AN INVESTIGATION INTO THE IMMUNE FUNCTIONAL, MORPHOLOGICAL, AND HISTOPATHOLOGICAL ALTERATIONS DURING THE COURSE OF GRAFT-VERSUS-HOST REACTIONS.

by

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ABSTRACT

The relationship between various immune functions, tissue damage, and splenomegaly was investigated during the course of Graft-versus-host (GVH) reactions.

The GVH-associated tissue lesions appeared at the time when both T- and B-cell function were severely suppressed and NK cell activity was at its peak and/or highly augmented. The development of tissue lesions correlated with donor NK cell activity. On the other hand, splenomegaly developed independently of both NK cell activity as well as tissue lesions.

Prolonged immunosuppression was associated with thymic dysplasia, but not splenomegaly. The immune functions recovered gradually, following severe suppression, and coincided with the gradual recovery of the thymus. The immune functional recovery was observed in the following order: LPS responsive B-cell function; Con A responsive T-cell function; PHA responsive T-cell function; and finally, T-cell dependent B-cell response T-cell function of immunodeficient GVH mice could be restored only when thymic medullary regeneration had occured. In contrast, the restoration of NK cell activity of NK-depressed mice was independent of thymic dysplasia.

These studies provide information regarding the possible mechanisms involved in inducing immunosuppression, tissue damage, and splenomegaly following GVH induction.

RESUME

Afin de mieux définir les mécanismes physiopathologiques impliqués dans la pathogénèse de la réaction du greffon contre le porte-greffe, nous avons établi dans un modèle murin une corrélation temporelle entre l'apparition des lésions tissulaires, la splénomégalie, et les diverses épreuves fonctionnelles immunologiques. L'apparition des lésions tissulaires coincide, d'une part, avec une forte augmentation de l'activité cytotoxique des cellulles K naturelles provenant du greffon, ainsi que, d'autre part, avec l'installation d'un état d'immunosuppression à la fois humoral et cellulaire. Par contre, la splénomégalie se développe indépendamment des lésions tissulaires et de l'augmentation de l'activité de cellulles K naturelles.

Nous avons observé une concordance étroite entre la sévérité de l'atteinte thymique, qui caractérise la réaction du greffon contre le porte-greffe, et la persistance de l'immunosuppression. L'amélioration de la dysplasie thymique s'accompagne d'un retour progressif vers la normale des diverses épreuves fonctionnelles immunologiques. Du plus précoce au plus tardif, on assiste à une regénération séquentielle et progressive des réponses suivantes: effet mitogénique du LPS pour les lymphocytes B, épreuves de transformation blastique des lymphocytes T par les lectines (ConA et PHA), production d'anticorps contre les antigènes thymo-dépendant. La récupération, naturelle ou induite, de la plupart des fonctions des lymphocytes T ne survient qu'au terme d'un processus de regénération au niveau de la région médullaire du thymus. Par contre, la récupération, naturelle ou induite de la fonction des cellules K naturelles est indépendante de la dysplasie thymique.

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To

my brother, Khalid Ghayur

and

my niece, Mira Mohsini

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CHAPTER SEVEN

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ABBREVIATIONS USED

AsGM1	Anti-asialo GM1
BM	Bone marrow
B6	C57BL/6
B6 bg/bg	C57BL/6 beige/beige
B6AF1	(C57BL/6xA)F1 hybrid
bg/bg F1	(C57BL/6 beige/beige X C3H/Hej beige/beige)F1 hybrid
+/bgF1	(C57BL/6 +/beige X C3H/Hej +/beige)F1 hybrid
C3H bg/bg	C3H/Hej beige/beige
C3H +/bg	C3H/Hej +/beige
Con A	Concanavalin A
cpm	counts per minute
CTL-L	Cytotoxic T lymphocyte-Line
FCS	Fetal Calf Serum
GVH	Graft-versus-Host
HBSS	Hank's Balanced Salt Solution
IFN	Interferon
IL-2	Interleukin-2
ITF	Insoluble thymic factor
LPS	E. coli Lipopolysaccharide
M	Molar
ml	milliliter
NK	Natural Killer
PCV	Packed cell volume
PFC	Plaque-forming cell
PG	Prostaglandin
PHA	Phytohemagglutinin
Poly I:C	Polyinosinic:polycytidilic acid
rpm	Revolutions per minute
S.E	Standard Error
SRBC	Sheep red blood cell
STF	Soluble thymic factor
TES	Thymic epithelial cell supernatant
ug/ml	Microgram per milliliter
/	per
E:T	Effector:Target
C'	Complement

PREFACE

Graft-versus-host (GVH) disease has become a clinical reality since the advent of bone marrow transplantation as a treatment for a variety hematopoietic and immune functional disorders. The various experimental models of GVH disease have provided information to understand and alleviate the complications observed in the clinical setting.

In the murine model, the classical features of the GVH reaction induction are: immunosuppression, tissue damage, and splenomegaly. Immunosuppression and tissue damage are also observed in clinical situations and both are the major complications limiting the success of bone marrow transplantation. In experimental models, it has been shown that the activation of donor T cells is essential for the initiation of the GVH reaction; whether donor T cells are responsible for inducing all the GVH-associated features or that different mechanisms are involved in affecting the different features is not yet fully understood.

To understand the relationship between and the mechanisms responsible for inducing immunosuppression of various immune functions, tissue damage, and splenomegaly we have performed comparative studies of these parameters in two experimental models: (i) GVH reactions in B6AF1 hybrids were induced by injecting different doses of either A or B6 PLC. Such a protocol induces GVH reactions of various intensities. (ii)

Parent into F1 hybrid GVH combinations of beige/beige (deficient NK but normal T-cell function) and +/beige (normal NK and T-cell function) mice were employed. Such a protocol enabled us to investigate the role of T-cells and NK cells in inducing various GVH-associated parameters.

By these experimental manipulations of the GVH reaction, we show that splenomegaly and tissue damage are two distinct features of the GVH reaction mediated by separate mechanisms and that tissue damage determines the duration of immuno suppression. We also show a dissociation between the suppression of various immune functions and between the suppression of various immune functions and splenomegaly early after GVH induction.

Our results demonstrate the importance of performing comparative studies between various GVH-associated parameters in providing a better understanding of the mechanisms responsible for affecting each parameter. Such studies may be of help in our understanding of clinical GVH disease.

CHAPTER ONE

THE GRAFT-VERSUS-HOST REACTION: A REVIEW

1.1 HISTORY OF GVH REACTIONS

The concept of a graft-versus-host (GVH) reaction originated as an error in the interpretation of the origin of cells involved in renal allograft rejection. While studying renal allograft rejection, Simonsen (1953) and Dempster (1953) suggested that, after several days residence in a foreign host, the cells which infiltrated the renal cortex were of local graft origin. It was suggested (Simonsen, 1953; Dempster, 1953) that these cells of renal graft origin were involved in an immunological reaction against the infiltrating host cells, i.e. A GVH reaction.

The validity of the GVH concept was later demonstrated independently by Simonsen (1957) and Billingham and Brent (1957). Simonsen (1957) showed that the grafting of immunologically competent cells (eg. spleen cells) into immature animals (chicks and mice) which were unable to defend themselves resulted in a disease which could only be explained on the basis of a GVH reaction. On the other hand, Billingham and Brent (1957) observed a devastating disease while trying to induce immunological tolerance in new-born mice by injecting immunologically competent adult spleen cells. They also concluded that a GVH reaction was the most straight forward interpretation of this disease.

Although the concept of GVH reactions became an experimental reality in the 1950's, the actual phenomenon of a GVH reaction was first observed, but not conceptualized, by Murphy in 1916. He observed that the grafting of chicken embryos with fragments of certain tissues (notably spleen and

bone marrow) from adult chicken donors resulted in a marked enlargement of the embryonic spleens (splenomegaly). Danchakoff in 1918 confirmed Murphy's observations. She further showed that the capacity to induce splenomegaly of the embryo was confined to cells of only certain organs (namely, spleen, liver, and bone marrow) belonging to a donor of the same species Splenomegaly has become one of the most commonly used parameters to measure the induction of a GVH reaction.

Simonsen (1962) defined three criteria which should be fulfilled in order to qualify a disease/syndrome as GVH. These criteria are: (1) the graft must contain immunologically competent cells. (2) The host must possess transplantation antigens that are lacking in the graft donor, so that the host appears foreign to it and is, therefore, capable of stimulating it antigenically. (3) The host itself must be incapable of mounting an effective immunological reaction against the graft (Simonsen, 1962). These three criteria are valid to this day.

1.2 FORMS OF GVH REACTIONS:

Several situations have been described which qualify as GVH reactions and, depending upon the situation, different names have been ascribed to each of these syndromes (GVH reaction): For example, Runt disease, Secondary disease, Parabiosis intoxication, and F1 hybrid disease.

In an attempt to induce tolerance, Billingham and Brent (1957), inoculated newborn mice with allogeneic adult splenic lymphoid cells. They noticed that in some strains of new born

mice a syndrome characterized by diarrhea, severe wasting, and high mortality, was observed which they termed "runt disease". This syndrome occured as a consequence of the induction of a GVH reaction in the newborn mice.

In an attempt to reconstitute the lymphoid organs of an adult lethally X-irradiated animal with lymphoid cells, Barnes and Loutit (1956), Trentin (1956), and Uphoff and Law (1958) discovered that animals reconstituted with syngeneic bone marrow cells recovered from the effects of irradiation and survived for a long time. However, animals reconstituted with allogeneic cells developed a wasting syndrome and died within a few months. This mortality rate was higher when parental spleen cells were employed to reconstitute X-irradiated animals or when the donor cells were presensitized to the host antigens. Trentin (1956) postulated that the wasting syndrome was the result of a GVH reaction. This disease was called "secondary disease".

The concept that GVH reactions are induced by lymphocytes was strengthened by experiments involving chronic cross-circulation (of peripheral blood) between parent and F1 hybrids. When F1 hybrids and one of the parents were parabiotically united, a lethal wasting syndrome developed in the F1 hybrids (Hilgard et al,1964; Cornelius and Martinez,1965; and Cauchi,1966). This syndrome was termed "parabiosis intoxication".

Finally, the injection of parental lymphoid cells into adult non-X-irradiated F1 hybrids induced a syndrome termed F1

hybrid disease (Billingham, 1968; Elkins, 1971). It was suggested that F1 hybrid disease was also due to a GVH reaction. It was postulated that parental lymphoid cells would recognize the H-2 antigens of the other parent on F1 cells as foreign and react against them. However, the F1 hybrid lymphoid cells would perceive the parental H-2 antigens on the grafted cells as self and would not react against the graft.

The different experimental procedures resulting in GVH reactions (as described above) provide insight into various clinical situations and an understanding of immune regulatory mechanisms. GVH reactions induced in either new- born or sensitized pregnant animals by adult, immunocompetent lymphocytes may provide insight into the role of maternal lymphocytes which may gain access to the fetus across placental barriers. It has been suggested that in humans transfer of maternal lymphocytes into the fetus may incite a GVH reaction and result in immunodeficiency diseases (Seemayer, 1979; Pollack et al, 1982). GVH reactions induced in X-irradiated adult animals provide a relevant model of clinical GVH disease in man, which may arise after therapeutic bone marrow engraftment of a patient who received a lethal dose of X-irradiation (Grebe and Streilein, 1976; Bortin and Rimm, 1977; Graze and Gale, 1979). The F1 hybrid disease in which the host (F1 hybrid) is believed to be specifically deficient in rejecting the parental grafts, inspite of a normal immune system, provides a tool to investigate the mechanism(s) of immune regulation and the effects of GVH reaction per se (Grebe and Streilein, 1976). In this situation, the effects of X-

irradiation and other therapeutic agents are avoided. F1 hybrid disease model can also be used to study histopathological alterations of the lymphoid and non-lymphoid organs and severe, prolonged immunosuppression. These symptoms are commonly observed in human recipients of bone marrow transplants who develop GVH disease (Bortin and Rimm, 1977; Graze and Gale, 1979). Both histopathological alterations and immunosuppression are consistently observed in the F1 hybrid GVH model (Seemayer, 1977, 1978; Lapp et al, 1985).

1.3 FACTORS DETERMING THE INTENSITY OF A GVH REACTION:

Several factors determine the intensity of a GVH reaction: (i) the degree of histoincompatibility between the donor graft and the host; (ii) the number of donor cells injected; (iii) the age of the host; (iv) presensitization of the donor cells; and, (v) the phenomenon of hybrid resistance. Since the degree of histoincompatibility (genetic disparity) between donor and host plays a central role in determining the intensity of GVH reactions, the effects of various degrees of genetic disparaties in inducing and determining the intensity of GVH reactions shall be presented in the following section.

1.3.1 HISTOINCOMPATIBILITY AND THE INTENSITY OF GVH REACTIONS:

1.3.1.1 H-2 ANTIGENS AND GVH INTENSITY

Genetic differences between host and the donor are major factors leading to the induction of a GVH reaction

(Bach, 1973). Of the different regions of the H-2 complex, it was originally thought that the K-end was more effective than the D-end in inducing GVH reactions (Demant, 1970). However, using congenic strains of mice which share the K and the D ends of the H-2, it was later discovered that it was not the H-2K locus by itself which was responsible for the stronger intensity of GVH reactions, but rather, the closely-linked cluster of immune response (Ir) genes are present within the I-region of the H-2 (Klein, 1973, Klein and Park, 1973; Livnat et al, 1973, and Widmar etal, 1973) In these studies splenomegaly was employed to assess the intensity of GVH induction. It was further demonstrated that an H-2K region differences without an H-2I region differences does not elicit any stronger GVH reaction than H-2D differences alone (Livnat et al, 1973; Klein and Park, 1973). It was suggested that perhaps a combination of various H-2 regions (I and K/D region of the H-2) would result in greater GVH intensity Klein and Park (1973) also demonstrated that H-2S plus H-2D region disparity had no additional effects on the intensity of GVH reactions as compared to that observed with an H-2D disparity alone. These workers suggested that the effects of the H-2S region on GVH reactivity is minimal and in many cases non-existent (Klein and Park, 1973). GVH reactions (as assessed by mortality) induced across T-region incompatibilities have also been reported (Elkins,1981; Eastcott et al 1981).

Further studies (Clark and Hildemann 1977a,b) showed that disparities across the entire I-region result in vigorous splenomegaly. The entire I-region splenomegaly was due to a

combined effect of I-A and I-C sub-region disparities However, Clarke and Hildeman (1977) showed that H-2I region differences alone required 40X more cells to produce comparable splenomegaly than that observed in combinations with disparities at multiple loci within the H-2 complex

Studies carried-out to determine the role of various sub-regions of the H-2I region on the induction of GVH reactions demonstrated that with I-A sub-region disparity, considerably higher numbers of donor cells were required to induce splenomegaly, however no mortality was observed (Clark and Hildeman 1977a,b) I-C sub-region disparate GVH combinations pre-immunization of donor cells with host antigens resulted in 50% mortality of the hosts (Clark and Hildeman, 1977a). These workers (Clark and Hideman, 1977a) also demonstrated that the severity of GVH reactions varied as a function of interallelic strain combination and were particulary influenced by host I-C region determinants (Clark and Hildeman, 1977b) Disparities of I-B or I-J sub-regions did not evoke detectable GVH reactions as assessed by splenomegaly (Clark and Hildeman, 1977a,b, Klein and Chiang, 1977).

Thus, these studies collectively demonstrate that I-A differences induce stronger GVH reactions, whereas I-C disparities can induce GVH reactions, but either higher donor cell dose or prior sensitization of donor cells with host antigens is required. In contrast, I-B and I-J sub-regions do not evoke detectable GVH reactions.

Wolters et al. (1981) studied the effects of different

regions of the H-2 complex on the activation of GVH effector cells using the delayed-type hypersensitivity (DTH) reaction as an assay system. In this assay system, spleen cells from donor mice were injected into an irradiated host (primary host). Spleen and lymph node cells from this primary allogeneic host were removed and transferred i.v. into a normal secondary recipient. These secondary recipients were then challenged in the hind foot with spleen cells syngeneic with the primary irradiated recipient. The DTH reactivity in such a system was shown to be directed exclusively to the I-region of the H-2 complex, whereas the H-2K/D regions did not induce anti-host DTH effector cells (Wolters et al. 1981). These workers (Wolters et al. 1981) confirmed the importance of the I-region in GVH induction by using an assay system different from the splenomegaly assay (Klein and Park, 1973; Livnat et al. 1973) It should be noted that both the DTH and splenomegaly assays measure the proliferative phase of the GVH reaction (proliferative phase; for details, see section 1.4.1).

In contrast, when GVH-induced weight loss and mortality were used as the assay system it was discovered that no significant differences were observed when the disparity between the host and donor was at the H-2K, H-2I, or H-2D regions alone (Klein and Chiang, 1976). It was concluded that there was a lack of correlation between the proliferative (splenomegaly) and effector (mortality/weight loss) phases of GVH the reaction induced across different regions of the H-2 complex (Klein and Chiang, 1976). Thus, these studies show that the H-2I region of the H-2 complex alone is more effective in

inducing splenomegaly and DTH response than H-2K/D region incompatibilities. However, using mortality as a measure of GVH reaction, H-2K, H-2D, or H-2I region incompatibility was equally effective.

1.3.1.2 MINOR OR NON-H-2 LOCI AND GVH INTENSITY:

Several studies have reported the induction of GVH reactions across minor or non-H-2 loci disparities when the donor cells were preimmunized with host antigens and/or injected in higher numbers (Cantrell and Hildeman, 1972, 1973, Cosgrave and Davis, 1971).

The non-H-2 loci have been selected for strong, moderate, and weak incompatibilities on the basis of mortality and splenomegaly by the use of congenic strain combinations of donor and host (Cantrell and Hildeman, 1972, 1973). It was demonstrated that, with decreasing immunogenetic incompatibility, increasing numbers of donor cells had to be injected to induce GVH reactions. However, with non-H-2 genetic disparaties, preimmunization of the donor cells with host antigens enhanced the GVH inducing potential; the weaker the histoincompatibility barrier, the greater was the potential efficacy of preimmunization in evoking GVH reactions (Cantrell and Hildeman, 1972). However, in certain strain combinations which differed only at multiple non-H-2 loci, severe GVH reactions (as assessed by donor cell proliferation and mortality) have been reported without prior sensitization of

donor cells (Korngold and Sprent, 1978; Eastcott et al., 1981). Wolters et al. (1980b), employing the DTH assay system (as described above), have also demonstrated GVH reactions in H-2 compatible donor-host combinations.

It has further been reported that non-H-2 and H-2 differences may interact with each other to increase the intensity of the GVH reaction (Abuaf et al., 1977; Motta et al., 1981; Pritchard and Halle-Pannenko, 1981). Pritchard and Halle-Pannenko (1981), however, concluded that the detection of the synergistic effect of H-2 and non-H-2 disparities depended upon the choice of an appropriate assay system, i e , it was suggested that splenomegaly results cannot be used to predict the outcome of a systemic GVH reaction as assessed by mortality and/or weight loss. Further studies on the effects of several different non-H-2 markers revealed that these non-H-2 markers can interact with each other in a variety of ways and affect the severity of GVH reactions (Motta et al 1981). Thus, the combined effects of two different non-H-2 genes may exert cummulative, synergistic, or suppressive effects on the intensity of GVH reactions (Motta et al, 1981).

It has also been demonstrated that in certain H-2 incompatible GVH combinations mortality may also be influenced by sex of the mice employed (Abuaf, 1977). In certain strains, especially C57BL/6, GVH reactions can be induced in mice by injecting lymphoid cells which have been previously sensitized (females sensitized to males). Thus, in this situation, GVH reactions can be induced across an H-Y disparity. Synergistic interactions between H-2 and H-Y disparities have been reported

to play a role in determining the GVH reaction intensity (Elkins and Silvers, 1982; Abuaf et al, 1977).

Thus, the studies cited above suggest that non-H-2 genetic disparities are able to induce GVH reactions by themself. These non-H-2 differences can also increase or decrease the intensity of GVH reactions by interacting amongst themselves and by interacting with H-2 differences.

The intensity of GVH reactions can also be determined by the degree of histopathological alterations observed in lymphoid and non-lymphoid organs (Billingham, 1968, Grebe and Strelien, 1976; Seemayer 1979; Seemayer et al., 1977,1978, Lapp et al., 1985; Becshorner et al , 1982a,b,1983). Bains and Dienner (1972) developed the focal assay to determine the lymphocytic infiltration and associated tissue damage in the liver following GVH induction. They suggested that the intensity of liver infiltrates was related to the dose of donor cells injected. Rappaport et al., 1979, conducted a detailed study on the kinetics of development of histopathological lesions following GVH induction across H-2 or non-H-2 loci. These workers demonstrated that when GVH reactions were induced across H-2 barriers lesions developed earlier and were more severe than when GVH reactions were induced across non-H-2 barriers.

The GVH-associated histopathological alterations in the lymphoid and non-lymphoid organs are a consistent and perhaps a more reliable criterion than mortality assay to assess GVH intensity (Santos et al, 1985). However, not much data are

available on the effects of H-2 and non-H-2 disparities in affecting histopathological alterations. Moreover, no data are available on the relationship either between the degree of histopathological alterations and the degree of splenomegaly or between the histopathological lesions and the DTH response during the course of a GVH reaction.

In most of the studies cited above GVH reactions were studied by examining only one of the parameters/symptoms associated with GVH reaction. When two or more parameters were used to assess GVH induction under similar conditions, it was clearly evident that the intensity of the proliferative phase (splenomegaly and DTH assay) did not correlate with the intensity of the effector phase (weight loss or mortality). In the following section a brief review of the two phases of GVH reaction shall be presented.

1.4 THE TWO PHASES OF THE GVH REACTION AND THEIR ASSESSMENT

The intensity of the GVH reaction is determined by different assay systems. However, it should be remembered that the introduction of immunocompetent cells into an immunoincompetent host triggers a complex series of events leading to various morphological, histopathological, and immune functional changes. Each of these changes may be caused by different mechanisms acting either independently of, or in combination with each other.

GVH reactions are broadly divided into two main phases: (1) An early proliferative phase which leads to morphological

changes, i.e., splenomegaly, lymph node enlargement or swelling of the foot pad as in the DTH assay ,and (2) A later effector phase responsible for histopathological changes in the lymphoid and non-lymphopid organs which lead to weight loss and mortality. However, the immune functional changes observed during GVH reactions may be due to a combination of both proliferative and effector phases of the GVH reaction.

The various assay systems employed to investigate the induction and intensity of GVH reactions provide information regarding a particular phase of a GVH reaction rather than the GVH reaction as a whole. For example, in the studies in which the proliferative phase (splenomegaly) and the effector phase (weight loss/mortality) of the GVH reaction were studied under similar conditions, a lack of correlation between the two phases was observed (Cantor et al., 1970a; Klein and Chiang, 1976; Livnat et al., 1973; Pritchard and Halle-Pannenko, 1981). Also, the potency of lymphoid cells from different lymphoid organs in inducing the symptoms of the two phases was found to be different (Gleichmann et al., 1976; Cantor and Asofsky, 1970; Cantor et al., 1970b; Cantor and Asofsky, 1972; Tigelar and Asofsky, 1972). This suggests that different effector cells may be involved during different phases of the GVH reaction

In the following section, a brief review of the assays employed to determine the induction/intensity of GVH reaction, the possible cell types, the mechanisms involved in affecting the proliferative phase, effector phase, and immune functional changes during GVH reactions shall be presented.

1.4.1 THE PROLIFERATIVE PHASE OF THE GVH REACTION

Following the induction of GVH reactions, the proliferation of different lymphoid cell types and the contribution of the donor and host cells have been assessed by various assays. For example: (1) Incorporation of DNA specific radio-isotopes such as, 3H-thymidine (Romano et al., 1976; Ford et al., 1981) and IUDR incorporation (Bennett,1971,1972,1973, Elkins, 1970). (2) Auto-radiography (Gowan,1962; Rolstad and Fossum, 1977). (3) Chromosomal markers (Fox, 1962) (4) Allo-antisera (Rolstad, 1976) (5) DTH response (Wolters et al , 1979a,b,;1980,1981). (6) Reconstitution of GVH mice with different sub-populations of either parental or F1 hybrid lymphoid cells (Romano et al., 1976; Ford et al., 1981). (7) Induction of GVH reactions with different lymphoid cell populations (Cantor and Askofsky, 1970; Cantor et al., 1970a,b; Cantor and Askofsky, 1972; Tigelar and Askofsky, 1972; Zollinger and Potworowski, 1979, Yamashita et al, 1982).

The proliferation of lymphoid cells early after the induction of GVH reactions results in characteristic morphological changes in the host such as splenomegaly (Simonsen, 1957; Billingham, 1968,), lymphnode enlargement (Levine, 1968; Haskova and Gansova, 1970; Ford et al., 1970) and swelling of the foot pads in the DTH assay (Wolters et al , 1979a,b;1980a,b;1981). During the proliferative phase of the GVH reaction, both donor as well as host cells proliferate. However, detectable morphological changes result as a consequence of donor cells' induced recruitment and

proliferation of the host cells. A brief summary of the donor cell type(s) involved in the initiation of the proliferative phase and the contribution of both the donor and host cells in affecting the morphological changes associated with the proliferative phase are presented.

1.4.1.1 DONOR CELL TYPE INVOLVED IN THE INITIATION OF THE PROLIFERATIVE PHASE:

The demonstration that peripheral blood lymphocytes from chickens (Simonsen, 1957), mice (Billingham and Brent, 1959), and rats (Billingham et al, 1962) were capable of producing GVH reactions indicated that certain cells within the circulating population of lymphoid tissue were fully capable of immunological reactivity. Further studies with lymphoid cells that were classified on morphological basis as "large" and "small" lymphocytes, demonstrated that the GVH-inducing capacity resided in the "small" lymphocyte population (Gowans, 1962; Gowans et, 1962; Gowans and McGregor, 1965, Billingham et al, 1962).

In the early seventies, Cantor and co-workers demonstrated in a series of articles that two donor lymphocyte populations act synergistically to induce morphological changes associated with the GVH reaction. Cantor et al. (1970a,b) and Cantor and Asofsky (1970) confirmed the earlier studies of Gowans (1962) and Gowans et al. (1962) and showed that indeed, the circulating lymphocytes are important in initiating GVH reactions. They showed that the splenomegaly producing capacity

of lymphocytes from different lymphoid organs was as follows: peripheral blood and thoracic duct lymphocytes, lymphnode cells, spleen cells, and, finally, bone marrow cells (BM) having the least capacity, if any at all. The synergistic interactions between the two lymphocyte populations were discovered when Cantor et al (1970a,b) and Cantor and Asofsky (1970) showed that the GVH-inducing capacity of splenocytes from aged NZB mice could be restored by adding a small number of splenocytes from young NZB mice. They (Cantor et al, 1970a,b; Cantor and Asofsky, 1970) suggested that the spleens of older mice were deficient in a cell population necessary for inciting GVH reactions. Cantor and Asofsky (1970) also showed that lymphoid cells derived from certain lymphoid organs (thymus or spleen) of thymectomized (normal Balb/c) animals were not able to induce splenomegaly by themselves. However, when the cells from thymectomized mice were mixed with cells from spleen or lymph nodes of non-thymectomized mice they were able to initiate significant splenomegaly. These workers (Cantor and Asofsky, 1970) suggested that at least two cell types are required to mediate GVH reactions. The ratio between these two sub-populations in different lymphoid tissues determines the degree of reactivity of that tissue.

A characterization of the two synergistically acting cell populations showed that both were thymus dependent (Cantor and Asofsky, 1972). One cell population was extremely sensitive to anti-thymocyte serum (ATS). It belonged to the rapidly recirculating T-lymphocyte pool i.e. present in the peripheral blood, thoracic duct, and lymph-nodes. This cell population was

termed as T2 (Cantor and Asofsky, 1972). The other T-cell population, sensitive to thymectomy but relatively resistant to ATS, was present in greatest concentration in the thymus and spleen. It recirculated slowly, if at all, and served as a precursor of the cell which inflicts splenomegaly This T-cell population was termed T1 (Cantor and Askofsky, 1972). The T1 cell, or the precursor cell activity, was amplified by the T2 or the amplifier cell (Cantor and Asofsky, 1972) Thus, it appears that the first cell to be activated following the induction of the GVH reaction is the T2 cell Cantor and Asofsky (1972) proposed two hypotheses regarding the relationship between T2 and T1 cell: (i) Both the T-cell populations (T1 and T2) belong to a single differentiated line but differ in their degree of maturation and (ii) there are at least two separate differentiated lines of T-cells in the peripheral tissues and thymus (Cantor and Askofsky, 1972).

Zollinger and Potworowski (1979) investigated the effects of two thymic stromal fractions: the insoluble thymic fraction (ITF) and the soluble thymic fraction (STF) for their capacity to induce distinct T-cell subsets. They found that ITF elicited a splenic cortisone-sensitive T-cell subset endowed with enhanced GVH-inducing capacity (splenomegaly). The STF increased the number of cortisone-resistant T-cells in the lymph nodes, but these cells were less active than ITF treated cells in inducing GVH reactivity. Treatment of ITF treated donor cells with STF resulted in cortisone-resistance and a decreased capacity to induce splenomegaly. Zollinger and

Potworowski (1979) suggested that the cortisone-sensitive and resistant subsets corresponded to the T1 and T2 populations, respectively, both of which cooperate in the induction of the GVH reaction. Zollinger and Potworowski (1979) suggested that the two synergistically acting T-cell populations represented two consecutive differentiation stages. Further studies in rats, using the lymph node enlargment assay (Yamashita et al., 1982), showed that the greater GVH-inducing capacity indeed resided in short-lived, cortisone-sensitive cells that had recently immigrated from the thymus.

The question regarding the histoincomptability requirements for T1 and T2 cell populations in the initiation of the proliferative phase of GVH reactions was investigated by Wolters and Benner (1979a,b,1980a,1981). These workers employed the DTH assay sytem and demonstrated that the ATS sensitive T2 cell population recognizes I-A determinants whereas the thymectomy sensitive T1 cell population recognizes K/D determinants. These H-2 recognition requirements are analogous to T-helper and T-cytotoxic/suppressor cell requirements (Zinkernagal et al, 1978a,b; Benacerraff and Germain, 1978; Feldman et al, 1977; Doherty et al, 1976). Wolters and Benner (1980) and Wolters et al. (1981) further demonstrated that T2 and T1 cells act synergistically only during the induction phase of the anti-host proliferative response, i.e., when T2 and T1 cells were separately activated by H-2 antigens, then combined and simultaneously transferred into a secondary recipient, no synergistic activity was observed. However, when T2 and T1 cells were activated by H-2 antigens together,

greatly enhanced anti-host proliferative responses were observed. These workers (Wolters and Benner, 1980a) suggested that the consequence of the differential specificity of receptors of T2 (I-A determinants) and T1 (K/D determinants) cells is that both sub-populations belong to different, yet parallel lines of T-cell differentiation. The role for T-helper cells in the initiation of the proliferative phase of the GVH reaction in rats was later confirmed by the use of monoclonal antibodies directed against T-helper cells (Brideau et al, 1980).

Miyazaki et al. (1982) showed that depending upon the duration of sensitization of donor cells with host cells in complete Freund's adjuvant, the capacity of donor cells to induce splenomegaly can either be increased or decreased and the capacity to induce cytotoxicity varied inversely to that of splenomegaly. These workers (Miyazaki et al., 1982) postulated that the differentiation of T-helper cells (T2 cell population) may have a common precursor in a certain time period after antigenic stimulation. After such a period of transitional differentiation the T-helper cells may differentiate irreversibly either into effector cells of DTH or effector cells of cytotoxicity. These workers (Miyazaki et al., 1982) suggested that a common precursor may exist for T-helper and T-cytotoxic/suppressor cells after antigenic stimulation.

The studies cited above collectively suggest that the proliferative phase of the GVH reaction is initiated by a mature, ATS sensitive, cortisone resistant, rapidly

recirculating, I-A recognizing cell, termed as T2 or T-helper cell (lyt 1+, L3T4+ cells). The response of the T2 cell is amplified by an immature, cortisone sensitive, sessile, K/D recognizing cell, termed as T1 or T-suppressor/cytotoxic cell (lyt 2+) However, it is not yet clear whether the T2 and T1 cells belong to the same or different lines of differentiation.

1.4.1.2 CONTRIBUTION OF DONOR AND HOST CELLS IN AFFECTING THE MORPHOLOGICAL CHANGES ASSOCIATED WITH THE PROLIFERATIVE PHASE:

Both the donor and the host cells proliferate and contribute to the morphological changes observed during the proliferative phase of GVH reactions However, the kinetics of the proliferative responses of the donor and the host cells vary and their actual contributions to morphological changes differ.

Gowans (1962), by employing autoradiographic techniques, showed that 3H-thymidine labelled donor cells were observed in the lymph nodes, Peyer's patches and splenic white pulp three hours after their injection. After 24 hours small lymphocytes transformed into large cells in the spleen (Gowans,1962). Fox (1962) studied donor cell proliferation by examining chromosomally marked (CBA T6) spleen cells after their injection into (CBAxC57BL/6)F1 mice. Fox (1962) noted that a burst of donor cell proliferation was observed by day 2-3 after GVH induction. At this time donor cells constituted up to 60% of the dividing cells in the host spleens, however no splenomegaly was observed. By day 4, donor cell contribution to

the dividing splenic cell was less than 10% whereas host cells (which were not chromosomally marked) contributed about 90% of the dividing cells. Furthermore, at the time when maximum splenomegaly was observed (between days 10-20), donor cell proliferation constituted approximately 1-2% of the dividing cells (Fox,1962). These results suggested that splenomegaly was almost entirely of host origin and that the majority of donor cell proliferation precedes morphological changes (Fox, 1962) Howard et al (1961) also showed that the host component constituted about 80-90% of the cells during peak splenomegaly

In the lymph node enlargement assay, Rolstad and Fossum,(1977), employing the autoradiography technique, also observed significant proliferation of donor cells by day 3, before lymph node enlargement was apparent. They (Rolstad and Fossum, 1977) showed that donor cell proliferation occurs before host cell proliferation commences and host cell proliferation correlates best with lymph node enlargement Ninety percent of the cells present in the enlarged lymph nodes were of host origin as assessed by the use of anti-sera (Rolstad, 1976). The advantage of the anti-sera technique in determining the host and donor component is that it measures both the dividing as well as non-dividing cells (Rolstad, 1976). The importance of host cell proliferation in affecting morphological changes was further strenghtened by the observation that X-irradiation (Howard et al., 1961; Ford et al., 1981) or treatment of F1 cells with mitomycin (Romano et al., 1976) depressed or abolished the morphological changes.

Furthermore, injections of normal F1 cells (but not parental cells) into X-irradiated mice restored the appearance of morphological changes (Ford et al., 1981).

The early proliferative burst of the donor cells that was observed after their injection into a semi-allogeneic or allogeneic host is stimulated by the histocompatibility antigens of the host. Ford and Atkins (1971) removed thoracic duct lymphocytes from the F1 hybrids 12-36 hours after they were injected with parental lymphoid cells. These thoracic duct lymphocytes were then injected into either F1 mice that were syngeneic with the primary recipient or F1 mice that were semi-allogeneic with the parental mice, i e., bearing different alloantigens from the primary recipient. The results from such experiments indicated that the recovered thoracic duct lymphocytes ('filtered' lymphocytes) were unable to cause lymph node enlargement in recipients syngeneic with the primary host, however, significant lymph node enlargement was observed in third party hosts (Ford and Atkins, 1971; Atkins and Ford, 1975; Ford et al., 1974). Further studies showed that only a portion of donor cells recognize and react specifically to the foreign antigens (Ford et al, 1974; Bennett, 1971; Sprent and Miller 1972a,b).

Specific activation (but not necessarily proliferation) of donor cells after recognition of host histocompatibility antigens is essential for the induction of maximum splenic enlargement associated with the proliferative phase of the GVH reaction. When parental cells were treated with mitomycin proliferation of F1 cells was 35-51% of parental cells not

treated with mitomycin (Romano et al , 1976) These results suggest that activation of donor cells (even in the absense of proliferation) can exert mitogenic effect on the F1 cells (Romano et al., 1976; Ford et al., 1981) Since the maximum morphological changes are observed at the time when the donor cell proliferation is minimum, it suggests that the recruitment and proliferation of F1 cells may be mediated by lymphokines released by T-cells following specific antigenic stimulation (for review of possible lymphokines involved, see Grebe and Streilien, 1976).

The recruited F1 cells which constitute the majority of cells in the enlarged spleens belong to cells of either macrophage and/or erythrocyte lineage (Howard, 1961; Elie and Lapp, 1976,1977; for review see, Billingham, 1968; Grebe and Streilein, 1976, Simonsen, 1962) In contrast, the enlargement of the lymph nodes was mediated by the recruitment of both T and B-cells of the host origin. However, the B-cells are the major cell type responsible for the enlargement of lymph nodes (Rolstad, 1976; Rolstad and Fossum, 1977; Ford et al., 1981)

Thus, the studies relating to the proliferative phase (morphological changes) of the GVH reaction show that : (1) The cell which initiates the GVH reaction is a T-cell. (2) Two T-cell populations, T-helper and T-cytotoxic/suppressor (T2 and T1), respectively interact to induce GVH reactions. (3) The activation of donor cells is specific and their proliferation is observed only early after GVH induction. (4) The donor cell that initiates the GVH reaction in H-2 diparate combinations is

a T-helper cell which is activated by I-A determinants of the host and its activity is amplified by T-cytotoxic/suppressor cells activated by H-2K/D determinants. (5) The morphological changes associated with the proliferative phase (splenomegaly, lymph node enlargement, and the DTH response) are due to donor cell induced recruitment and proliferation of host cells.

1.4.2 EFFECTOR PHASE OF THE GVH REACTION:

The effector phase of GVH reactions has been characterized by different parameters, such as histopathological lesions in the lymphoid and non-lymphoid organs; weight loss and runting; and, finally, mortality Mortality, after the induction of GVH reaction, is the most commonly used assay for the effector phase. Thus far the cells responsible for the effector phase have not been characterized and there are no parameters that can be used to accurately predict the intensity of the effector phase (Elkins, 1978).

It is believed that the weight loss, runting, and mortality of GVH mice results as a consequence of histopathological lesions in the intestines and gut-associated non-lymphoid organs. This tissue injury results in diarrhea, malabsorption, malnutrition, weight loss, and finally death (Hedberg et al., 1968; van Bekkum et al., 1974; vanBekkum and Knnan, 1977). Damage to the lymphoid organs results in severe immunosuppression and death may also be associated with anemia and infection (McBride, 1966). Thus, it appears that the initial or the earliest features of the effector phase may be the GVH- associated histopathological alterations in the

lymphoid and non-lymphoid organs.

The primary target of GVH reactions are lymphoid organs and cells (Lafferty et al, 1972 ; McBride, 1966, Billingham, 1968). Furthermore, the specificity and the reproducibility with which the epithelial cells are damaged in the lymphoid and non-lymphoid organs during the GVH reactions strongly suggest that epithelial cells are also primary targets of GVH reactions (Lapp et al., 1985). Lafferty et al , (1972) and Snover (1984) have suggested that organs and tissues (either lymphoid or non lymphoid) which possess a stem cell pool i e., rapidly proliferating cells with a capacity for self renewal (for example, the primary lymphoid organs and the epithelial cells lining the GI tract) are the primary targets of GVH reactions

The histopathological lesions resulting in significant injury to the epithelial thymus have profound implications on the immune system. The destruction of epithelial cells lining the GI tract are believed to be responsible for weight loss , diarrhea, and mortality. Two basic mechanisms have been proposed to account for the tissue damage observed during the effector phase of a GVH reaction. The first suggests that the epithelial cell damage is non-specific, mediated by the release of lymphokines from activated lymphocytes, i.e., the "innocent bystander" phenomenon. The second suggests that epithelial cell damage is mediated by donor lymphocytes which are specifically activated following their stimulation by the host alloantigens, i.e., specific cell-mediated mechanism. A brief review of the mechanisms and the cell types that may be responsible (or

assumed to play a role) for tissue damage during the effector phase of the GVH reaction shall be presented.

1.4.2.1 THE INNOCENT BYSTANDER PHENOMENON

The "innocent bystander" phenomenon states that the epithelial cell damage observed during the course of a GVH reaction results from the release of lymphokines or other cytotoxic agents during an interaction between host and donor lymphoid cells, leading to damage to the nearby epithelial cells.

The major support for this mechanism of tissue damage comes from experimental data showing that: (i) In F1 mice that received a parental intestinal graft followed by an injection of parental lymphoid cells, intestinal epithelial cell damage is observed both in the F1 intestines as well as transplanted parental intestinal tissues syngeneic with the parental donor cells (Elson et al., 1977; van Bekkum and Knnan, 1977; Mowat and Furgusson, 1981). (ii) In F1 rats tissue damage to transplanted parental kidney graft (in local GVH reactions) is observed when parental lymphoid cells are injected under the kidney capsule of the transplanted parental kidney (Elkins, 1966; Elkins and Guttmann, 1969). Damage to the parental kidney graft is observed only when the kidney is transplanted in a non-X-irradiated F1, so that F1 rats have a normal lymphoid element (Elkins, 1966). (iii) In F1 syrian hamsters, parental skin grafts are destroyed when parental lymphoid cells are injected subcutaneously (Billingham and Streilein, 1968). Thus, the main argument for the role of innocent bystander phenomenon

in tissue damage is that epithelial cell damage is observed in both the F1 organs as well as the parental grafts which are syngeneic to the parental lymphoid cells The host lymphoid cells play a major role in this phenomenon of tissue damage since irradiation of the host either blocks or decreases the intensity of tissue damage (Elkins, 1966).

However, several experimental observations cannot be either incorporated into the hypothses of "innocent bystander" phenomenon or contradict the non-specific mechanism for epithelial cell damage. It has been reported that F1 cells may enter the transplanted parental intestinal grafts (Ferguson and Parrott, 1972a,b). This observation is important as it has been reported that graft versus host reactions and host-versus-graft reactions occur side by side (McBride, 1966). Although it is believed that the F1 hybrid does not react immunologically towards the parental lymphoid cells, recent studies have demonstrated that the F1 hybrid can recognize and react against determinants on the parental lymphoid cells. Specific F1-anti-parent CTL responses have been observed (Shearer et al, 1976, Shearer, 1981; Shearer and Pollison, 1980; Warner and Cudkowicz, 1979; Ishikawa and Dutton, 1980; Nakano et al , 1981). Moreover, Shearer (1981) has demonstrated that the intensity of the F1-anti-parent CTL response is proportional to the parent-anti-F1 CTL response. Recent reports have also suggested that NK cells may be involved in tissue damage during the effector phase. Several studies have suggested that, as for the generation of CTLs, NK cells of both host and donor origin

are activated after GVH induction (Ghayur et al.; 1984; Lapp et al.; 1985; Mowat et al., 1985). Moreover, Suzuki et al. (1985) have demonstrated that NK cells appear earlier than the specific CTLs following allogeneic stimulation and that the early appearance of NK cells is essential for the generation of a specific CTL response. Mowat and Fergusson (1981), in support of the involvement of the "innocent bystander" phenomenon, have suggested that "the Ia+ F1 cells which infiltrate the transplanted parental intestinal grafts are activated to provide the signal for proliferation and recruitment by releasing lymphokines". The explanation suggests that the Ia+ F1 cells which infiltrate transplanted parental intestinal grafts recognize a "foreign" determinant on parental cells.

The experimental observations described above regarding the generation of F1-anti-parent CTL and the activation of host and donor NK cells suggest the possibility that parental grafts transplanted into a non-X-irradiated F1 host may be attacked by F1 lymphoid cells (cytotoxic/effector cells) and F1 grafts may be damaged by parental lymphoid cells. Thus, the possibility that tissue damage may be cell- mediated must be considered.

There is a second set of experimental observations which do not support a role for the "innocent bystander" phenomenon in tissue damage. These observations are the presense of lymphocytes in close contact with the damaged cells, a feature more in keeping with cell-mediated tissue damage (Seemayer et al., 1977, 1978; Slavin and Woodruff, 1974; Gallucci and sale, 1982; Weisdorf et al., 1983; Kaye et al., 1983).

1.4.2.2 ROLE OF MACROPHAGES IN TISSUE DAMAGE

GVH reactions have been reported to result in the induction of cytotoxic macrophages. Several workers (Fung and Sabbidini, 1976; Ptak et al., 1975) have demonstrated that macrophages derived from the spleens and peritoneal exudate cells of GVH mice can kill a variety of tumor target cells non-specifically. It has also been reported that the macrophage bactericidal capacity is also enhanced after GVH reaction induction (Cooper and Howard, 1961; Blanden, 1969, Anthony et al., 1984). Nestel et al. (1985) have recently reported that following the induction of GVH reactions there is massive production of H_2O_2 radicals by splenic and peritoneal exudate macrophages. It is possible that the macrophage cytotoxic activity as well as the production of H_2O_2 radicals may contribute to the tissue damage associated with the GVH reaction.

As mentioned in section 1.4.1.2, splenomegaly observed early after the induction of the GVH reaction is a consequence of donor cell induced recruitment, mainly of host macrophages. Elie and Lapp (1977) and Treiber and Lapp (1976) have proposed that early after GVH reaction induction the immune regulatory system may be working at supra-optimal levels to cope with the intense immune reaction. This over amplification of normal immune regulatory mechanisms may be involved in the induction of tissue damage [(like its involvement in immunosuppression as proposed by Elie and Lapp (1977) and Treiber and Lapp (1976)]

On the other hand experimental observations are reported which might argue against, but not exclude, the role of

macrophages in the initiation of tissue damage. Firstly, the maximum degree of splenomegaly, which constitute activated and recruited macrophages, does not correlate with the severity of tissue damage (Ghayur et al., 1985b; Lapp et al., 1985). Secondly, histological studies show that it is the lymphocytes (large granular lymphocytes) that are attached to the epithelial cells, whereas macrophages are filled with debris (Seemayer et al, 1977, 1978). Finally, the tissue damage precedes the accumulation of macrophages at the injured site, particularly in the thymus (Seemayer et al, 1977, 1978). These observations suggest that the initiation of the lesions may be mediated by lymphocytes and that macrophages are recruited at the site of tissue damage due to an inflamatory response.

However, several studies in which sterile intestinal grafts as well as grafts from conventional mice were used showed that the presence of bacteria increases the severity of tissue damage (Abrams et al., 1963; van Bekkum et al., 1974; van Bekkum and Knnan, 1977; Mowat and Furgusson, 1981). It was proposed that bacteria and their products intensify the inflammatory response resulting in more severe lesions (van Bekkum et al., 1974; van Bekkum and Knnan, 1977; Mowat and Furgusson, 1981). It is known that bacterial products, especially lipopolysacharide (LPS), in the presense of macrophage activating factor (MAF) or interferon (IFN) can induce cytotoxic activity in macrophages. A significant amount of IFN is produced *in vitro* cultures by spleen cells derived from GVH mice (Zwatsky et al., 1979). IFN activates macrophages

to kill tumors (Murray et al., 1985; Krammer et al., 1985; Celada et al., 1984; Keller, 1973) and increases H202 production and release from macrophages (Nakagawara et al., 1982).

The observations cited above collectively suggest that, although the initial tissue damage may be mediated by a lymphocyte, the presense of activated macrophages in the vicinity of the damaged site may play an important role in the progression and perhaps in maintenance of the tissue damage Since no kinetic study on the relationship between M0 cytotoxic activity, H202 production and the appearance and progression of histopathological lesions is available, it is difficult to assign a definitive role to M0 in GVH-associated tissue damage. Moreover, the question of the host and donor (specific or non-specific) nature of M0 in GVH-associated tissue damage is difficult to answer, since depletion of M0s and/or their precursors is technically difficult.

1.4.2.3 ROLE OF T-CELLS IN TISSUE DAMAGE.

It is generally believed that the GVH-associated tissue damage is caused by donor anti-host cytotoxic T-lymphocytes (CTL) which are activated by the histocompatibility antigens of the host (for reveiw see, Grebe and Streilien, 1976; McBride, 1966; Elkins, 1971,1978). The assumption that anti-recipient CTLs of donor origin are the effector cells in the GVH reaction is based upon earlier findings showing that parental strain mice tolerant to F1 hybrid antigens by neonatal inoculation with F1 hybrid bone marrow are incapable of

producing histologic alterations or clinical disease in F1 hybrids (Gowans and McGregor, 1965; Gowans et al., 1962). It was suggested that the antigens which induce tolerance to skin allografts were the same ones with which lymphocytes interact in GVH reactions (Gowans and McGregor, 1965; Gowans et al., 1962). However, in these studies, the possibility that such bone marrow reconstituted mice become chimeras was not considered (Gowans and McGregor, 1965; Gowans et al., 1962). It has been extensively reported that X-irradiated bone marrow reconstituted animals do become lymphoid chimeras (Gengozian et al., 1960, 1961; Doria et al., 1962; Rayfield and Brent, 1983). In addition, the intensity of local GVH reactions in guinea pigs correlated with the rapidity with which the donors rejected skin grafts (Brent and Medawar, 1963). However, several studies have reported a lack of correlation between skin graft rejection and GVH induction (Livnat et al., 1973; Kadish and Basch, 1876; Lapp et al., 1985; Ghayur et al., 1986).

The assumption that CTLs play a role in tissue damage was further strenghtened by the finding that lymphoid cells from immunized animals or lymphoid cells challenged in vitro were specifically cytotoxic for the immunizing cells in vitro (Moller, 1965a,b; Rosenau and Moon, 1961; Cerrotini et al., 1971; Cerrotini and Brunner, 1974). Specific cytotoxicity of target monolayers in vitro (Berke et al., 1969a,b) was later shown to be mediated by T-cells and was dependent upon cell contatct (Perlmann and Holm, 1969; Wilson and Billingham,

1967). The T-cell nature of cells capable of killing allogeneic target cells was confirmed by Golstein et al. (1972) and Wagner (1972).

Thus, the role for CTLs in GVH associated tissue damage is primarily based upon in vitro studies. However, Elkins (1971) has suggested that it is probable that the progeny of activated donor cells possess similar cytocidal properties for neighbouring cells in vivo during the course of a GVH reaction, but that the artificial nature of the target monolayers in vitro should be taken into consideration. Elkins (1971) further pointed to the fact that many tissues and cells in vivo might be shielded from this type of direct attack by connective tissue barriers and basement membranes.

The strongest evidence for the role of CTLs comes from experimental data showing that specific CTL's are generated both after skin graft rejection and in vivo sensitization with tumor allografts (Canty and Wunderlich, 1971; Rouse and Wagner, 1972; Cerrottini and Brunner, 1974). However, these associations are not universal, since data are also available which demonstrate a lack of correlation between skin graft rejection and GVH induction (Kadish and Bacsh, 1976; Livnat et al., 1973; Lapp et al., 1985; Ghayur et al., 1986) and between the generation of CTLs in MLC and the intensity of GVH reactions (van Elven et al., 1981).

Recent studies, in which the generation of specific CTLs and the development of effector phase of GVH reactions were studied, showed a lack of correlation between these two parameters. These studies demonstrated that the effector phase

of the GVH reaction, as assessed by mortality, is not observed even though donor-anti-host CTLs are generated *in vivo* (van Elven et al , 1981; Hamilton, 1984). In addition, injection of IL-2, a T-cell lymphokine that augments the CTL activity of normal mice, increases the incidence of mortality without increasing CTL activity of GVH mice (Judas and Peck, 1983). Finally, splenic CTL induction (Shearer and Pollison, 1980, Ishikawa et al , 1982)) as well as T-cell proliferative function and T-helper cell function (Ghayur et al., 1984, 1985a) became severely supressed as early as day 4 after GVH induction, whereas the GVH effector phase (as assessed by the development of histopathological lesions) appeared by day 12-16 after GVH induction.

The studies cited above do not negate the role of CTLs in the effector phase of GVH reactions since it is possible that CTLs may have simply migrated out of the lymphoid organs. It could also be that, due to the accumulation of host cells in the spleens of GVH mice, the donor anti-host CTLs are diluted out and, therefore, cannot be detected. If specific donor anti-host histocompatibility antigen-reactive CTLs are involved in the effector phase of the GVH reaction, then histopathological alterations should be observed in all organs and tissues due to the presense of foreign histocompatibility antigens. However, tissue damage during the GVH reaction is observed in specific tissues, i.e., those tissues with a rapidly proliferating (self renewing) stem cell population, eg,. lymphoid organs (Lafferty et al., 1972) and epithelial cells (Snover, 1984). Futhermore,

the type of lesions and the organs and tissues affected during the GVH reactions across the major or minor H-2 barriers cannot be distinguished (Seemayer et al., 1977, 1978, Rappaport et al , 1979; Lapp et al , 1985).

The fact that GVH associated mortality can be prevented by treating the donor inoculum with anti-thy antiserum or anti-thymocyte globulin (Muller-Ruchholtz et al , 1978, Kolb et al., 1979, Vallera et al , 1982; Korngold and Sprent, 1978, 1982; Hamilton et al., 1981) does not provide direct evidence for a role of CTLs in the effector phase It merely suggests that T-cells may be essential for the initiation of GVH reactions. Once the GVH reaction is initiated some other cell population may be activated which may be the effector during the effector phase of the GVH reaction. Synergy between two T-cell populations during the GVH reaction has been demonstrated (see section 1.4.1.1.). Recruitment of other cell population(s) following the initiation of GVH reactions by T-cells has also been suggested (Mowat and and Fergusson, 1981, Mowat et al., 1985). Several workers have, therefore, recently suggested that CTLs may not be essential in affecting the effector phase of the GVH reaction (van Elvan et al., 1981, Mason et al., 1981; Judas and Peck, 1983; Hamilton, 1984).

Recent studies employing lymphoid cells of nude mice, which lack mature T-cells, have shown that these cells are capable of inducing features of the effector phase of the GVH reaction, as assessed by weight loss/mortality and runting (Okunewick et al., 1981, 1985). These workers have suggested that some immature T-cell, probably the precursor of CTL (p-

CTL), is involved in the effector phase of the GVH reaction (Okunewick et al., 1981,1985). Other workers have also suggested that p-CTL, or a T-cell at a certain stage of its differentiation/maturation following allogeneic antigen induced activation may be the cell that inflicts tissue damage during the effector phase of the GVH reaction (Dokhelar et al., 1981; Clancy et al., 1983; Herberman et al., 1982).

A recently described cell type, natural killer (NK) cell,is believed to belong to the pre-T-cell (immature T-cell) (Herberman and Holden, 1978; Herberman et al., 1979; Kaplan, 1985) or the p-CTL pool (Nieminin and Seksela, 1984). The possible role of NK cells in the effector phase of the GVH reaction is presented in the following section.

1.4.2.4 NK CELLS AND TISSUE DAMAGE

Only recently has attention been directed towards the possible role of NK cells during the course of GVH reaction. Thus far, only a limited number of studies are available. Moreover, as is commonly the drawback with other studies in GVH research, detailed correlative studies on NK cell activity and the development of clinical features of the effector phase of the GVH reaction are not available. Therefore, in this section NK cell activity during both the proliferative and the effector phases of GVH reaction will be presented. A part of this thesis (chapters 3-6) shall deal with the kinetics of NK cell activity and the development of various features of GVH reactions.

Kiessling et al. (1977) reported that splenic NK cell

activity becomes severely depressed 30 days after the induction of a GVH reaction. However, kinetic studies on NK cell activity during the course of a GVH reaction showed that NK cell activity is augmented in the spleen, thymus, lymph nodes, and bone marrow early after GVH induction and then declines to undetectable levels (Ghayur et al., 1980; Roy et al , 1982, Clancy et al., 1983). The degree of early augmentation of NK cell activity was dependent upon the number and genotype of parental lymphoid cells injected (Pattengale et al , 1983, Varkila and Hurme 1985a; Ghayur et al , 1984)

Borland et al (1983) presented studies showing a correlation between the early augmentation of NK cell activity and the development of splenomegaly. These workers induced GVH reactions by injecting parental lymphoid cells intraperitoneally. However, in studies in which GVH reactions were induced by injecting parental lymphoid cells by the intravenous route, a lack of correlation was observed between NK cell activity and splenomegaly (Ghayur et al., 1984, 1985a; Varkila and Hurme 1985a, Lapp et al., 1985). Pattengale et al. (1983) demonstrated that both the degree and duration of early augmented splenic NK cell activity depended upon the number and strain of parental lymphoid cells injected to induce GVH reactions. This dose and strain dependance of augmented NK cell activity was later confirmed (Varkila and Hurme 1985a; Ghayur et al., 1984, 1985a; Lapp et al., 1985).

Borland et al. (1983) showed that the kinetics of early augmented NK cell activity in the intra-epithelial lymphocytes

was observed at the time when gut-associated changes are observed during GVH reactions (Mowat and Fergusson, 1981). Pattengale et al. (1983) reported that GVH-associated severe lesions (splenic dysplasia) were only observed in GVH mice in which early suppression of NK cell activity was observed. Furthermore, augmented NK cell activity after GVH reaction induction did not always correlate with the development of severe GVH reactions. Studies from our laboratory have shown that the GVH-associated lesions in the lymphoid and non-lymphoid organs appeared at the time when NK cell activity was at its peak. In addition, the severeity of the histopathological lesions correlated with the earlier appearance of peak NK cell activity, rather than with the overall augmented NK cell activity. (Lapp et al., 1985; Ghayur et al., 1984, 1985a). These studies constitute a part of this thesis and are presented in chapters 4 and 5.

The involvement of NK cells in the effector phase of the GVH reactions is supported by data showing that <u>in vivo</u> administration of anti-asialo-GM1 (AsGM1) antibodies directed against NK cells prevented GVH-associated mortality (Charley et al., 1984) and severe histopathological alterations in the skin. These authors (Charley et al., 1984) addressed the question of the origin (either host or donor) of NK cell activity during GVH reactions and its role in the effector phase of GVH reactions. They suggested that host NK cells may be involved in the effector phase of the GVH reaction (Charley et al., 1984). The argument that host NK cells are involved in GVH-associated tissue damage was based upon the findings that

administration of AsGM1 antibodies to the hosts prior to or at the time of GVH induction prevented GVH-induced mortality Elimination of endogenous NK cells from the donor inoculum had no effect (Charley et al., 1984). Varkila and Hurme (1985b) demonstrated that in vivo administration of AsGM1 antibodies to recipients prior to GVH induction abolished both the early augmentation of NK cell activity as well as GVH-associated mortality. Adminstration of the same antibodies to the donors prior to GVH induction had no effect on GVH-associated mortality (Varkila and Hurme, 1985b). These workers also suggested that NK cells of host origin may play an important role during the effector phase of the GVH reaction (Varkila and Hurme, 1985b). However, it is possible that alloantigen activated donor NK cells, rather than the endogenous donor NK cells, may also be important during the effector phase of the GVH reaction along with host NK cells. If this possiblity is true then the administration of AsGM1 antibodies to the host (at the time or prior to GVH induction) would eliminate NK cells of both the host as well as alloantigen activated NK cells of donor origin. Thus, the possibility that donor NK cells may also be important during the effector phase of the GVH reaction connot be completely ruled out.

We have also investigated the role of host and donor NK cells in inducing tissue damage duing the effector phase of the GVH reaction. These studies are presented in chapter six of this thesis.

In humans, Livnat et al. (1980) reported that patients

with leukemia or aplastic anemia showed normal or elevated NK cell activity by day 30 after bone marrow transplantation. This augmented NK cell activity remained elevated up to day 100 post- bone marrow transplantation. However, no correlation was observed between the augmented NK cell activity against K562 tumor targets and the development of GVH disease (Livnat et al., 1980). Lopez et al. (1979) provided data showing that high pretransplantation levels of NK cell activity against Herpes simplex virus-1 infected cells (NK-HSV-1) correlated with the devlopment of GVH disease. However, no correlation between high levels of donor NK-HSV-1 activity and development of GVH disease was observed (Lopez et al, 1979). In contrast, Dokhelar et al. (1981) reported a correlation between the early augmented NK cell activity against K-562 tumor targets (NK-K-562) and the development of GVH disease after bone marrow transplatation. Dokhelar et al. (1981) suggested that the early appearance of NK-K-562 cell activity which correlated with the development of GVH disease was of donor origin.

In humans, the data available for the origin of NK cell activity and the development of GVH disease are still controversial. The controversy exists due to the fact that different workers have employed different targets to determine NK cell activity. Recent studies in mice have, in fact, shown that NK cells that lyse YAC-1 targets and NK cells that lyse HSV-1 infected cells belong to distinct subpopulations of NK cells (Tang et al., 1985).

Thus, the studies cited above for the role of different mechanisms that are believed to play an important role during

the effector phase of the GVH reaction show that although different effector mechanisms may be involved in producing severe lesions, the mechanism that initiates the tissue damage is still being debated. Donor-anti-host specific CTLs are assumed to be the effector cells inflicting tissue damage, however, the assumption is mainly based upon in vitro data The in vivo data show a lack of correlation between specific CTL generation and the severity of the GVH effector phase. More recent evidence suggests that NK cells may play an important role in inflicting tissue damage, however their origin (either host of donor) is not clear. And finally no systematic kinetic study is available to show the kinetics of various immune functions and the initiation and progression of tissue damage

1.5 IMMUNOSUPPRESSION

A consistent feature of the GVH reaction is generalized immunosuppression. Both the proliferative phase (up to approximately 2 weeks after GVH induction) and the effector phase (beyond 2 weeks after GVH induction) of GVH reactions contribute to the state of severe immunosuppression. It appears that, at least, three different mechanisms come into play during the course of a GVH reaction and each contributes to the persistence of immune-suppression of the host at different times during the GVH reaction (Lapp et al., 1985). There appears to be sufficient overlap between the different

suppressive mechanisms to maintain the animal in a severely immunosuppressed state (Lapp et al., 1985). In the following section a brief review will be presented of the various events and mechanisms that play a role in the induction and maintainence of the immuno-deficient state of an animal undergoing a GVH reaction.

1.5.1 EARLY IMMUNOSUPPRESSION

Lapp and Moller (1969) demonstrated that as early as day 7 after GVH induction, GVH mice were deficient in their capacity to reject third party skin grafts. They proposed that GVH reactions alter the environment within the host, resulting in the prolongation of skin graft survival time. Sjoberg (1971, 1972) suggested three posibilities for the GVH-induced immunosuppression. These were : (i) antigenic competition between two cross-reacting antigens competing for the same antigen-reactive cell; (ii) lack of some essential factor in the host, and (iii) induction of some inhibitory factor.

Grushka and Lapp (1971, 1974) showed that when a normal thymus was transplanted into an immunosuppressed F1 hybrid undergoing a GVH reaction, GVH intensity increased as assessed by weight loss, and mortality. These workers suggested that the GVH reaction has a generalized immunosuppressive effect which suppresses not only the immune response to other antigens, but also the GVH reaction itself. They proposed that GVH-induced immunosuppression is caused by depletion of a thymic factor

which plays an important role at some critical stage in the normal immune response (Grushka and lapp, 1971, 1974). Further studies supporting the concept of thymic factor depletion as a cause of immunosuppression were presented by Lapp et al (1974). They demonstrated that the PFC response to SRBC of immunosuppressed mice could be partially restored by injecting either syngeneic thymocytes or thymic extracts. Similarly, *in vitro* T-helper cell function of GVH immunosuppressed mice could be restored by T-cell derived factors (Elie et al , 1974) These results strongly suggest that the GVH-associated immunosuppression could be at least partially due to depletion/lack of thymic and/or T-cell derived factors resulting in depressed T-helper cell function.

Besides the depletion of thymic and/or T-cell derived factors, release of suppressive factors and the generation of suppressor cells have also been reported to play a role in early GVH-induced immunosuppression. Studies by Sjoberg (1971, 1972) and Moller (1971) suggested a role for suppressor factors in GVH induced immunosuppression. Sjoberg (1972) demonstrated that a cell with macrophage-like properties was responsible for initiating early immunosuppression. Other workers also indicated that the macrophage could be the cell producing such suppressive factors (Byfield et al., 1973; Elie et al., 1974)

Elie and Lapp (1976, 1977) showed that during the early phase of GVH reactions there was a massive increase in the number of macrophages in the spleen (30% above normal), which suppressed T-helper cell function. It was further demonstrated

that these macrophages could perform normal functions, i.e., provide help in antibody production, if their numbers were reduced to normal levels (3-5% of the splenic cellularity). These results suggested that the suppressive effect of macrophages was quantitative rather than qualitative (Elie and Lapp, 1976,1977) and raised the possibility that the early macrophage-mediated suppression represented an amplified normal immune regulatory function (Elie and Lapp, 1977). Recent studies (Lapp et al., 1980) have shown that the accumulated macrophages in the spleens of GVH mice release massive amounts of prostaglandin (PG) E which suppress T-helper cell function. Removal of PGE-producing macrophages delayed the onset of severe immunosuppression. Furthermore, in vivo administration of Indomethacin (a PG synthetase inhibitor) partly reversed early immunosuppression (Lapp et al., 1980). A regulatory role for macrophages and their products has also been suggested by several other workers (Treiber and Lapp, 1976; Hoffman and Dutton, 1971; Waldman and Gottlieb, 1973; Calderon et al., 1975; Optiz et al., 1976).

Following the activation of T-cells by mitogens or antigens, interluekin-2 (IL-2), a T-cell lymphokine, is released by T-cells (Smith, 1980; Gillis, 1983). IL-2 is an essential requirement in the proliferation and maturation of cells involved in T-cell mediated immune responses and perhaps in T-helper cell dependent B-cell responses. The proliferative responses of GVH cells to mitogens (Ghayur et al., 1984, 1985a, 1986a;) and CTL generation (Shearer et al., 1980) become

severely suppressed as early as day 4 after GVH induction. IL-2 production by (Niven and Lapp, 1984; Nestel et al., 1986; Joseph et al., 1985), and the expression of IL-2 receptors on (Joseph et al., 1985) GVH spleen cells become severely suppressed by day 8-15 after GVH induction. It is not clear at the moment whether excess production of PGE by macrophages present in the GVH spleen may play a role in the suppression of IL-2 production and thereby cell proliferation. However, Prud'homme et al. (1984), using spontaneously diabetic rats, have shown that PGE production by "activated" macrophages suppresses both IL-2 production as well as cell proliferation Furthermore, IL-2 production and proliferative responses could be partially restored by Indomethacin (Prud'homme et al., 1984). Chouaib et al. (1984) have reported that PGE2 activates T-suppressor cells which inhibit IL-2 production. Another suppressor cell type in the spleens of GVH mice which suppresses mitogen responses of normal F1 splenocytes has recently been described (Holda et al., 1985a,b; Maier et al., 1985). This suppressor cell does not express thy-1, asialo GM1, surface Ig and is non-adherent. The cell is termed a natural suppressor cell and its activity can be demonstrated 7-10 days after GVH induction. A suppressor cell with neither T-cell nor macrophage properties has also been reported by Parthenais and Lapp (1978). However, the suppressive potential of this cell was only apparent when parental T-cells were eliminated from GVH spleens (Parthenais and Lapp, 1978).

Several workers have also demonstrated suppressor cells

that express T -cell surface markers in the spleen of GVH mice. The two assay systems extensively employed to study the suppressive effects of suppressor T-cells on normal cells are the PFC assay that measures the T-helper cell dependent B-cell responses (Shand, 1976,1977; Pickel and Hoffman, 1977a,b; van Elven et al., 1981; Rolink et al., 1983); and the in vitro generation of CTLs (Pollison and Shearer,1980; Shearer and Pollison, 1980; Ishikawa et al., 1982; Shearer and Levy, 1983; Hurtenbach and Shearer, 1983; Hurtenbach et al., 1984; Argyris, 1984). Suppressor T-cells may be either H-2 specific, i.e , directed specifically against H-2 antigens of the other parent on F1 cells (Pickel and Hoffman, 1977) or non-specific (Argyris, 1984; Shearer and Levy, 1983). Different groups of workers have reported various surface markers on T-suppressor cells, suggesting that perhaps different subpopulations of suppressor T-cells are activated at different times after GVH induction. The various surface markers reported on the suppressor T-cells are : Thy 1; lyt 1+2-; lyt 1+2+; lyt 1-2+; lyt 1+2+3+ (Argyris, 1984; Ishikawa et al., 1982; Hurtenbach and Shearer, 1983; Hurtenbach et al., 1984; Pickel and Hoffman, 1977; Shand, 1977).

Hurtenbach and Shearer (1983) and Hurtenbach et al. (1984) studied the surface markers on suppressor T-cells early after induction of the GVH reaction. These workers showed that between 5-7 days post-GVH induction the GVH suppressor activity could be abrogated by anti-lyt 1, anti-lyt 2, and anti-thy 1 antibody and complement treatment. The suppressor activity

became insensitive to anti-lyt 1, but not anti-lyt 2 and anti-thy 1 antibodies, by day 8 post GVH induction. From day 14 after GVH-induction, these workers reported that, for a brief period of time, the suppressor activity was insensitive to any of the antibodies directed against T-cell surface markers However, sensitivity to anti-thy 1, but not to anti-lyt 1 and lyt 2 antibodies, reappeared again (Hurtenbach and Shearer, 1983; Hurtenbach et al., 1984). Hurtenbach and Shearer (1983) and Hurtenbach et al. (1984) demontrated that the T-suppressor cells were of donor origin.

In the studies cited above the suppressor cell activity was measured in vitro by mixing GVH spleen cells with normal F1 cells. It has been reported that although thy-1 positive cells in the GVH spleen inhibited the reponse of normal F1 cells, they failed to inhibit the response of normal cells of the same genotype as those used to induce the GVH reaction (Parthenais and Lapp, 1978). It was, therefore, suggested that the increased suppressor activity by thy-1+ cells in the GVH spleens may simply reflect an increase in cytotoxic or cytostatic cells of donor origin (Parthenais and Lapp, 1978). Pollison and Shearer (1980) also suggested that suppressive activity of GVH spleen cells could be accounted for, at least in part, by parent anti-F1 cytotoxic cells.

The direct suppressive effects of GVH spleen cells on normal B-cell function have not been studied as extensively as those of T-cells. However, suppression of B-cell proliferative

responses to LPS by factors (PGE) (Lapp et al., 1980) and suppressor cells (Maier et al., 1985; Holda et al., 1985a,b) during GVH reactions have been reported. Furthermore, Abbuzro and Rowley (1983) have suggested that the augmented NK cell activity during the early phase of GVH reactions may suppress B-cell function. Regulation of B-cell responses by NK cells has recently been reported by several workers (Abruzzo and Rowley,1983; Storkus and Dawson,1986; Brieva et al., 1984; Nabel et al., 1982).

The studies cited in this section suggest that several mechanisms may be involved in inducing immunosuppression early after the induction of GVH reactions. These mechanisms are (i) depletion/lack of thymic and/or T-cell derived factors; (ii) A quantitative change in the splenic macrophage population; (iii) PGE production; and (iv) activation/generation of different types of suppressor cells.

Active suppressor cells disappear and the presence of PGE can not be detected between 20-30 days after GVH induction, yet animals remain suppressed (Parthenais et al., 1974; Parthenais and Lapp, 1978; Shand 1976,1977; Lapp et al., 1985). The fact that immune suppression persists after the disappearance of active suppressor mechanisms suggests that prolonged suppression may be due to the depletion of a cell or factor that is essential for initiating the induction and/or effector function of the immune response. In the next section a brief review of the mechanisms involved in persistant immunosuppression are presented.

1.5.2 PERSISTANT IMMUNOSUPPRESSION

As mentioned in the preceding section, one of the mechanism of GVH associated immunosuppression is the depletion/lack of thymic and/or T-cell derived factor(s) The GVH reaction induces a functional thymic deficiency which can be corrected by the transplant of a normal thymus (Grushka and Lapp, 1971; 1974). Atrophy or involution of the thymus has been described as a characteristic feature in murine GVH reactions (Simonsen, 1962; Heim et al., 1967). Heim et al (1967), described GVH-associated thymic changes as a morphological disorganization of the thymus accompanied by destruction and depletion of thymocytes. The specific effects of GVH reaction on the thymic medulla were not described in these earliar studies (Heim et al., 1967). It was proposed that the thymic involution/atrophy observed after the induction of GVH reactions was a hormone-mediated phenomenon dependent upon an intact pituitary-adrenal axis (stress related) and not an immunological phenomenon (Heim et al., 1967).

Studies from this laboratory have extensively investigated the thymic architecture in mice undergoing either chronic or acute GVH reactions (Seemayer et al., 1977, 1978; Seddik et al., 1980; Lapp et al., 1985). Seemayer et al. (1977, 1978) showed that induction of both chronic (across multiple-minor loci) and acute (across H-2 differences) GVH reactions in F1 hybrids resulted in marked thymic atrophy as early as day 6

after GVH induction. The thymic involution was largely due to depletion of thymocytes in the thymic cortex. In addition to thymic atrophy, light and electron microscopic studies revealed profound thymic dysplasia as characterized by an effacement of the cortico-medullary demarcation, injury of medullary epithelial cells and loss of Hassall's corpuscles. These medullary changes started to appear by day 14 and were maximal by day 21-28 post GVH induction (Seemayer et al., 1977).

In order to differentiate between stress- related and GVH specific thymic alterations adrenalectomized mice were used as recipients. When GVH reactions were induced in adrenalectomized mice, thymic atrophy, i.e., cortical thymocyte depletion, was not observed. However, pathological changes were observed in the thymic medulla including cortico-medullary demarcation effacement, broadening and disorganization of the medulla, emperipolesis of epithelial cells by lymphocytes, dissappearance of Hassall corpuscles, epithelial cell injury and the progressive accumulation of macrophages laden with debris (Seemayer et al., 1978, Seddik et al., 1980). It was therefore suggested that the GVH associated thymic cortical changes were hormone mediated (stress-related), whereas the GVH associated thymic medullary changes were related to the GVH reaction per se and thus due to an immunological phenomenon (Seemayer et al., 1978; Seddik et al., 1980, Lapp et al., 1985). Utilizing floresence labelled antibody specific for a soluble thymic factor (Potworowski et al., 1977), it was further demonstrated that thymic medullary epithelial cells,

which are normally positive for soluble thymic factor (STF), could not be detected in the GVH-induced dysplastic thymus (Potworowski et al., 1979). Studies have also demonstrated that the intensity of thymic histopathological lesions depended upon both the donor-host genetic combinations (Rappaport et al., 1979; Seemayer et al., 1977, 1978) and the number of parental lymphoid cells injected to induce GVH reactions (Ghayur et al., 1984, 1985a). The studies on the effect of donor cell dose and genotype on the intensity of thymic dysplasia are presented in chapters 5 and 6 of this thesis.

Seddik et al. (1979, 1980) examined the relationship between the thymic dysplasia and T-cell immunodeficiency. GVH reactive mice were sacrificed at different days after GVH induction and their thymocytes were used to recontitue T-cell function in adult thymectomized, X-irradiated, bone marrow reconstituted (ATxBM) mice (Seddik et al, 1979,1980).The ATxBM mice were then tested for their ability to reject third party H-2 incompatible skin grafts, T-helper cell function, and mitogen responsiveness (Seddik et al., 1979, 1980). The results from these studies demonstrated a close association between the progression of thymic medullary injury and the decline in T-cell functions (Seddik et al., 1979, 1980, 1984a, 1984b). It was suggested that the injury to the thymic epithelium resulted in a T-helper cell maturational defect.

Mendes et al. (1985a, 1985b) investigated the nature of the T-cell functional defect associated with the thymic

dysplasia. Using interleukin-1 (IL-1) and IL-2 as probes to study the nature of the T-cell functional defect and its association with thymic medullary injury, these workers (Mendes et al., 1985a, 1985b) demonstrated that GVH thymocytes (obtained from dysplastic thymuses) failed to respond to IL-1. When GVH thymocytes were stimulated with IL-2 plus mitogen (PHA) up to 60% of the normal response was observed (Mendes et al., 1985a, 1985b). These results suggested that the defect in the GVH T-cells may not be in the IL-2 responsive cell population, but rather in the T-helper cell population responsible for IL-2 production. Studies on IL-2 production by GVH spleen cells demonstrated that, indeed, these cells were severely deficient in IL-2 production (Mendes et al., 1985a, 1985b). Chapters 7 and 8 of this thesis shall deal with the role of GVH-associated thymic dysplasia in determining the duration of T-cell immunodeficiency.

Thus, the experimental data cited above concerning the association between T-cell immunodeficiency and thymic injury in GVH mice, suggest that thymic epithelial cell damage results in a maturational defect in the T-helper, IL-2 producing cell population. This defect may play an important role in GVH-associated prolonged T-cell immunodeficiecy (Lapp et al., 1985).

1.5.3 B-CELL IMMUNODEFICIENCY

Earlier studies on B-cell function during the GVH

reaction suggested that B-cell function and/or production may be directly affected by the GVH reaction. Moller (1971) and Treiber and Lapp (1978) demonstrated that the PFC response to the thymic independent antigen ,LPS, was depressed following GVH induction. T-cell responses, but not B-cell responses, could be stimulated in GVH mice by repeated antigenic challenge (Treiber and Lapp, 1978). When GVH bone marrow was used to reconstitute adult thymectomized, X-irradiated mice it was shown that by day 7-10 after GVH induction GVH bone marrow was ineffective in reconstituting B-cell function (Seddik et al., 1985b). These results collectively suggested that B-cell function and/or production in the bone marrow is affected by the GVH reaction (Seddik et al., 1985b).

Recent studies employing immunofluorescent labelling of cytoplasmic and surface μ chains, which identify pre-B and B-cells respectively (Osmond, 1980), have demonstrated alterations in the number of pre-B and B-cells in the bone marrow and spleen at different days after the induction of GVH reactions (Xenocostas et al., 1986). By this approach it was demonstrated that the number of B-cells in the spleen declined 10-25 fold within 14 days after GVH-induction. In the bone marrow, both the pre-B and B-cells numbers declined to 1-10% of normal levels and preceded the marked reduction in the spleen (Xenocostas et al., 1986). These results suggest that B-cell suppression is due to a defect in B-cell genesis in the bone marrow, the primary site of B-cell production (Osmond, 1980). Although, histopathological changes in the bone marrow of GVH

mice have been described (Githens et al., 1968; Hirabayashi, 1981; Ishihara and Shimanina, 1980) no correlative study on the influence/effects of these structural changes on B-cell function is available.

1.6 CONCLUDING REMARKS

The literature survey of the GVH field presented in this chapter shows that the GVH reaction results in morphological, histopathological, and immune functional changes. The morphological changes associated with the GVH reaction are affected by the donor cell induced host cell recruitment. The histopathological changes appear to be cell-mediated, however the cell type responsible for initiating these pathological changes is still controversial. Moreover, the origin (either host or donor) of the effector cell which initiates the histopathological changes is also not clear. However, the progression of histopathological lesions may be due to the inflammatory response at the site of tissue damage. Both the morphological as well as the histopathological changes may be involved in bringing about the immune functional changes associated with the GVH reaction.

The literature survey also reveals that most of the workers have employed one parameter or another that measures either proliferative (morphological changes) or effector (histopatho-

logical changes) phases of the GVH reaction at one particular period of time. Only a few workers have employed two or more parameters which measure either the proliferative (splenomegaly) or the effector (mortality) phase of the GVH reaction and have observed a lack of correlation between these two phases. Thus, the use of one GVH associated parameter at one particular time after GVH induction only provides information on the GVH reaction at that time and is not necessarily useful for predicting later events. The available data suggest that following the induction of a GVH reaction a series of events /mechanisms are triggered and that during each phase of the GVH reaction a particular mechanism or combination of mechanisms may be involved. The GVH reaction, thus appears to be a dynamic ongoing process.

No systematic kinetic studies are available on the relationship between the morphological, histopathological, and immune functional changes. Lack of such studies raises certain crucial questions that may be essential to our understanding of the rather complex GVH reaction. The questions to be addressed in this thesis are the following:

(1) What is the relationship between the various immune functions, i.e., T-cell, B-cell, and NK cell activity, following GVH induction?.

(2) What is the relationship between the various immune functions, splenomegaly, and histopathological alterations after GVH induction?.

(3) Which immnue cell function correlates best with the development of the histopathologiacl lesions?.

(4) What is the origin of the cell type that correlates with the development of histopathological lesions ,i.e., host or donor?

(5) What is the relationship between the degree of splenomegaly and the degree of histopathological alterations?.

(6) What is the contribution of splenomegaly and thymi dysplasia in determing the duration of immunosuppression?.

(7) Do the animals that survive the initial GVH reaction recover from thymic dysplasia and immunosuppression?. If so, what are the kinetics of the regeneration of the thymus and the immune function?.

And finally, (8) Can the immune functions (T-cell and NK cell) of GVH immunosuppressed mice be restored?.

These questions are intriging not only as an experimental curiosity, but are also important for understanding clinical GVH disease.

CHAPTER TWO

MATERIALS AND METHODS

2.1 ANIMALS

Mice of inbred strains A (H-2a), C57BL/6 (H-2b) and their F1 hybrids (AxC57BL/6)F1 (B6AF1) were used in most of the studies presented in this thesis. For the studies presented in chapter six, mice of inbred strains C57BL/6 beige/beige J (B6 bg/bg) (H-2b) and littermates B6 +/bg; C3H/Hej bg/bg 2J (C3H bg/bg) (H-2k) and littermates C3H +/bg were purchased from Jackson Laboratories, Bar Harbor, Maine. Appropriate breeding pairs, (B6bg/bg x C3H+/bg) and (B6+/bg x C3Hbg/bg) were established to produce B6xC3Hbg/bgF1 (bg/bgF1) and +/bgF1 mice which served as recipients. B6bg/bg and B6+/bg mice produced in the same litter by appropriate breeding served as donors.

All mice used in this thesis were between the ages of 12-20 weeks at the start of the experiments. The mice were bred and maintained in our animal colony at the McIntyre Medical Sciences Building, McGill University, Montreal, Canada. Syngeneity between animals of a given strain was assured by strict brother-sister mating and the animals chosen as breeders were never more than 4 generations removed from a common brother-sister mating.

2.2 GRAFT-versus-HOST (GVH) REACTION INDUCTION

Animals of parental strains A and B6 were sacrificed under ether euthanasia. Their spleens, brachial, axillary, inguinal, and cervical lymph nodes were removed. Spleen and lymph nodes of parental strains A and B6 were pooled

seperately. A single lymphoid cell suspension was prepared by gently pressing the pooled spleens and lymph nodes through a #50 mesh stainless steel screen placed over a petri dish containing Hank's balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, N.Y.). Small clumps of cells that passed through the screen were broken up by gently pipetting with a pasteur pipette. The cells thus obtained were washed three times by centrifugation at 1,000 rpm (500g) for 6-8 minutes in HBSS. After the last wash the cells were passed through a cotton gauze to remove any clumps.

Cell counts were done by the trypan blue dye exclusion method. F1 recipients were warmed under an infra-red lamp in order to dilate the tail veins. Different doses ; $10x10^6$, $20x10^6$, or $30x10^6$ of lymphoid cells of either parental strain A or B6, in a volume of 0.3ml, were injected intravenously (i.v) into F1 recipients via the tail veins using a tuberculin syringe and a 26 gauge needle.

For the studies presented in chapter 6, single cell suspensions of pooled spleens and lymph nodes from B6bg/bgJ and B6+/bgJ littermates were prepared separately and $50x10^6$ lymphoid cells, in a volume of 0.3ml., of either B6bg/bg or B6+/bg origin were injected i.v into either bg/bgF1 or +/bgF1 mice, thereby producing four experimental groups of GVH-reactive mice:

(i) B6 bg/bg parental cells injected into bg/bgF1 mice.

(ii) B6 bg/bg parental cell injected into +/bgF1 mice.

(iii) B6 +/bg parental cells injected into bg/bgF1 mice.

(iv) B6 +/bg parental cells injected into +/bgF1 mice

2.3 SKIN GRAFTING

Full thickness skin grafts were applied according to the technique described by Bliss (1965). Donor C3H/HeJ +/bg mice were sacrificed. Pinch grafts were taken from their mid-dorsal area, using fine curved scissors, after the skin was shaved and washed with a 70% ethanol solution. The grafts were then placed in a petri dish containing a sterile filter paper soaked with physiological saline. The grafts were placed with the epidermal side down. The fat and connective tissue were removed using curved Metzenbaum scissors.

Recipient mice of either B6 bg/bg or B6 +/bg genotype were anesthesized and then placed on a clean surgical cork board. A small piece of cotton soaked with ether was placed in front of the animal's nose to ensure a proper level of anesthesia. The lateral chest wall of the recipients was shaved and cleaned with 70% ethanol. Graft beds of the same size as the donor grafts were prepared by removing recipient skin, taking care not to injure the chest wall or the mammary and thoracic blood vessels. Donor grafts were then applied on the graft beds on the lateral chest wall, dusted with sulphadiazine powder and secured in place by "scotch" tape.

Graft dressings were removed on the 10th post-operative day and grafts were visually inspected daily. The grafts were scored from 0 to 100 based on the amount of healthy

epithelium. Grafts with 10% or less healthy epithelium were considered rejected.

Since C3H/HeJ served as donor strain for all skin grafting and B6 bg/bg or B6 +/bg served as recipients, the B6 bg/bg or B6 +/bg effector cells responsible for allograft rejection were directed against the C3H H-2k haplotype It should be noted that the same H-2k haplotype of C3H strain served as the stimulus/target for the donor B6 lymphoid cells that were injected into B6C3H bg/bgF1 or B6C3H +/bgF1 mice to induce a GVH reaction (see chapter 6)

2.4 PREPARATION OF LYMPHOID CELL SUSPENSIONS FOR NATURAL THE KILLER (NK) CELL ASSAY

2.4.1 THYMUS CELL SUSPENSION

Normal control and GVH-reactive mice were sacrificed by ether euthanasia. To remove the thymus, the mouse was placed in a supine position and the thoracic cavity was opened by a longitudinal, mid-ventral incision from the xiphisternum to the neck. Using fine curved forceps, each thymic lobe was removed and any contaminating lymph nodes were removed. A single cell suspension of thymocytes was prepared by gently pressing the thymic lobes through a #50 mesh stainless screen into a petri dish containing HBSS. Thymus cells obtained from normal and each group of GVH-reactive mice were pooled seperately.

2.4.2 BONE MARROW (BM) CELL SUSPENSION

Normal and GVH-reactive mice were sacrificed as described above. The mice were placed in a supine position, and an incision was made in the skin over the femurs and tibiae. The skin was peeled in order to expose the muscle. The muscles were removed and the femurs and tibiae exposed. The head of each bone was cut-off and the marrow was aspirated with a #26 gauge needle in a 1ml. syringe containing a small amount of HBSS. BM cells obtained from animals in the same experimental group were pooled.

2.4.3 LYMPH NODE CELL SUSPENSION

Normal and GVH-reactive animals were sacrificed under ether euthanasia. The lymph nodes, namely, brachial, axillary, inguinal, and cervical were removed and placed in HBSS. Single cell suspensions of lymph nodes were prepared. Lymph node cells obtained from normal and different groups of GVH mice were pooled seperately.

2.4.4 SPLEEN CELL SUSPENSION

Spleens were removed from normal and GVH mice under ether euthanasia. Splenic single cell suspensions were made by gently pressing the spleens through a #50 mesh stainless steel screen placed over a petri dish containing HBSS. Spleen cells obtained from each group of mice were pooled seperately.

2.5 PARTIAL PURIFICATION OF NK CELLS FROM SPLENOCYTES, THYMOCYTES, LYMPH NODE CELLS, AND BM CELLS.

Single cell suspensions from normal and GVH reactive spleens, thymuses, lymph nodes, and BM were prepared separately in HBSS, as described in section 2.4.
The cells from each of the above organs were washed twice in HBSS. The cells were resuspended in HBSS supplemented with 2% fetal calf serum (FCS) and incubated in disposable plastic petri dishes for a period of 60-90 minutes at 37oC in 5% CO2 This procedure, at least partially, removed macrophages (MO)/monocytes, since such cells are plastic adherent

After the period of incubation, the non-adherent cells were removed with a pasteur pipette, layered onto ficol hypaque (density 1.09), and spun at 1,000 rpm (500g) for 15-20 minutes. Ficol hypaque was prepared by adding 112 ml. of hypaque M 60% (Winthrop laboratories, Aurora, Ontario, Canada) to 400 ml. of 9% Ficol 400 (Pharmacia Chemicals, Uppsala, Sweden) solution. This mixture of ficol and hypaque gave a density of 1.09. Layering of non-adherent cells onto ficol hypaque removed red blood cells and some granulocytes which settled to the bottom of the tube, whereas the dead cells floated to the surface of the medium.

Following ficol hypaque layering and centrifugation, the cells at the interface, between ficol hypaque and HBSS, were gently removed with a pasteur pipette and washed three times with HBSS. The cells were then resuspended in RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 5% FCS, counted and adjusted to a desired concentration to

achieve different effector:target (E:T) cell ratios.

The cells thus obtained (partially purified cells) after plastic adherence and ficol layering, were used as effectors in the NK cell assay , as described in section 2.7. These cells will be referred to as partially purified NK cells or NK cells in the text.

2.6 TREATMENT OF NK CELLS WITH ANTI-THY-1 SERUM PLUS COMPLEMENT (C')

Anti-Thy-1.2 serum was raised in the laboratory according to the methods of Reif and Allen (1966) and Raff (1969). The anti-serum was employed at a dilution that achieved maximum (> 90%) cytotoxicity when tested on thymocytes. Splenocytes which were partially purified by plastic adherence and ficol hypaque layering, as described in section 2.5, were adjusted to a desired concentration and were then incubated with anti-Thy-1.2 antiserum for 30 minutes at 37oC, followed by 45 minutes incubation with absorbed guinea pig C' (GIBCO, Burlington, Ontario, Canada). Splenocytes treated with medium (HBSS) and C' served as controls. The cells were washed three times in HBSS and resuspended in RPMI-1640 supplemented with 5% FCS. These cells served as effectors in the NK cytotoxicity assays, as described in section 2.7. Cytotoxicity of anti-Thy-1.2 serum was determined by trypan blue dye exclusion.

2.7 NK CELL ASSAY

Single cell supensions of spleen, lymph nodes, thymus and BM were obtained as described in section 2.4. The cells from these lymphoid organs were then partially purified by plastic adherence and ficol hypaque layering as described in section 2.5. These partially purified cells were resuspended in RPMI-1640 supplemented in 5% FCS, adjusted to a concentration of $10x10^6$ cells/ ml, and served as NK effector cells in the NK cell cytotoxicity assay. In some experiments, serial dilutions of effector cells were performed to achieve different E:T cell ratios.

YAC-1, a Maloney virus-induced lymphoma of strain A/Sn (H-2a); P-815 , a methylcholentherene induced mastcytoma of strain DBA/2 (H-2d), and Eb, a lymphoma of strain DBA/2 (H-2d) origin, were used as targets in the NK cell cytotoxicity assay. The tumor target cells were labelled with 51Cr. For 51Cr labelling, approximately $3x10^6$ tumor cells were suspended in 1 ml. of RPMI-1640 supplemented with 5% FCS and incubated with 0.1 ml of Na2Cr04 (Specific activity 1UCi/ml) (NEN, Chicago) for 1 hour at 37oC. Following this incubation, tumor target cells were washed three times, and resuspended in RPMI-1640 supplemented with 5% FCS at a final concentration of $1x10^5$ cells/ ml.

Different effector cell numbers in a volume of 50ul/well were plated in quadruplicate or sextuplicate cultures in V bottom microtiter plates (Dynatech Laboratories Inc.). 51Cr labelled target cells/well in a volume of 100ul were added to the effector cells. This resulted in a final volume of

150ul/well. The plates containing NK effector cells and 51Cr labelled target cells were incubated for 4 hours at 37oC in a humidified atmosphere containing 5% CO2. The plates were then centrifuged at 1,000 rpm (500g) for 5 minutes and 100ul aliquots of supernatant was asseyed for 51Cr release in a Nuclear Chicago gamma counter. Percent cytoxicity was calculated by the formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm - spontaneous cpm}}{\text{Maximum cpm - spontaneous cpm}}$$

Experimental cpm were derived from aliquots (100ul) of supernatants taken from wells in which 51Cr labelled targets were incubated for 4 hours with effector cells. Spontaneous cpm were derived from aliquots (100ul) of supernatants taken from wells in which 51Cr labelled targets were incubated for 4 hours in medium alone. Maximum cpm were obtained by counting aliquots (100ul) of 51Cr labelled targets incubated for 4 hours in medium alone. The spontaneous release was always less than 10% of the maximum 51Cr release.

The % cytotoxicity data is presented as the mean of quadruplicate or sextuplicate cultures. The variability (mean $\pm$ S.E.) of 51 Cr release in individual wells at a given E:T cell ratio of individual spleen as well as spleens pooled from three animals was also determined in some experiments (chapter 3).

2.8 COLLECTING SUPERNATANTS FROM CELLS OF NORMAL AND GVH REACTIVE LYMPHOID ORGANS

B6AF1 mice that were injected with $30x10^6$ B6 parental lymphoid cells (PLC) and age-and-sex matched normal B6AF1 mice were sacrificed under ether euthanasia. Their thymus, spleen, lymph nodes, and BM cells were removed and pooled separately. Single cell suspensions of pooled thymuses, spleens, lymph nodes, and BM of normal and GVH-reactive mice were prepared in HBSS, as described in section 2.4.

The thymic, splenic, lymph node, and BM cell concentration was adjusted to $30x10^6$ cells / ml, and kept at room temperatures for 2 hours. The cells were then centrifuged at 1,000 rpm (500g) for 10 minutes and supernatants collected The supernatants were then spun at 1,500 rpm for 15 minutes ; approximately 0.5 ml. of the supernatants at the top of the tubes were discarded and supernatants were collected with the help of a pasteur pipette, leaving approximately 1 ml at the bottom of the centrifuged tube. The supernatants were stored at 4oC till further use (never more than 24 hours).

2.9 TREATMENT OF NORMAL B6AF1 THYMOCYTES, LYMPH NODE, AND BM NK CELLS WITH SUPERNATANTS DERIVED FROM NORMAL OR GVH-REACTIVE LYMPHOID CELLS

Five to six normal B6AF1 mice between the ages of 10-12 weeks were sacrificed under ether euthanasia. Their thymuses, lymph nodes, and BM cells were removed, and single cell suspensions were made separately in HBSS + 2% FCS as described in section 2.4.

Partially purified NK cells from each of the above mentioned lymphoid organs were obtained, as described in section 2.5. The partially purified NK cells were aliquoted, 15-20x10^6 cells/ml. Aliquots from each of the lymphoid organs were washed twice and resuspended in a volume of 1 ml in either : (i) medium alone (HBSS+2% FCS), (ii) supernatants derived from cells of different lyphoid organs of normal B6AF1 mice, and (iii) supernatants derived from cells of lymphoid organs of GVH-reactive B6AF1 mice. The cells were incubated for a period of 60-90 minutes at 37oC in humidified atmosphere containing 5% CO2.

Following this incubation the cells were washed twice and resuspended in RPMI-1640 + 5% FCS, 10x10^6 cells/ml., and tested for their spontaneous cytotoxicity against 51Cr labelled YAC-1 tumor targets in the NK cell cytotoxicity assay which is described in section 2.7.

2.10 PREPARATION OF PROSTAGLANDINS (PG) E1, E2, AND F2

PG E1 (Lot number 447HY), PG E2 (Lot number 522KA), and PG F2 (Lot number 143JT), were a generous gift from Dr. J. Pike, Upjohn, Kalamazoo. Stock solutions of PGs E1, E2, and F2 were prepared in 95% ethanol and stored at -30oC till further use. Desired concentrations of PGs were obtained by diluting them in RPMI-1640+5% FCS. Fresh dilutions of each PG were made each time just prior to their use.

2.11 PREPARATION OT POLYISINIC-POLYCYTIDILIC ACID (POLY I:C)

Poly I:C (Sigma Chemicals, St.Louis, MO.) was weighed on an Mettler balance and dissolved in HBSS. The concentration of Poly I:C in HBSS was adjusted either to 100ug/0.3ml when used in vivo or to 200ul/ml when used in vitro treatment of normal and GVH-reactive splenocytes. A fresh solution of Poly I:C was prepared each time just prior to its use.

2.12 ADMINISTRATION OF POLY I:C IN VIVO

Poly I:C was dissolved in HBSS. 100ug of Poly I:C in a volume of 0.3ml was administration intra-peritoneally to normal and GVH-reactive mice 18-24 hours prior to the collection of their sera for interferon (IFN) assay and removal of their spleens for NK cell cytotoxicity.

2.13 IN VITRO TREATMENT OF SPLENIC NK CELLS WITH PGs AND POLY I:C

Single cell suspensions of pooled spleens from 4-6 normal B6AF1 mice and from 4-6 B6AF1 injected with $30x10^6$ B6 parental lymphoid cells were prepared separately in HBSS. Splenocytes were partially purified to obtain NK cells as described in section 2.5. NK cells from normal and GVH-reactive mice were aliquoted, $20x10^6$ cells/ml. Aliquots were washed twice and resuspended in a volume of 1 ml , in either : (i) medium alone (RPMI-1640+5% FCS), (ii) various concentrations of different PGs alone, (iii) Poly I:C (100ug/ml) alone, and (iv) various concentrations of different

PGs in combination with Poly I:C. These aliquots were incubated for a period of 60-90 minutes at 37oC in a humidified atmosphere containing 5% CO2. Following these treatments cells were washed twice, resuspended in RPMI-1640+5% FCS at a concentration of $10x10^6$ cells/ml, and tested for their spontaneous cytotoxicity against 51Cr labelled YAC tumor targets in NK cell cytotoxicity assay as described in section 2.7.

2.14 SERUM COLLECTION FOR INTERFERON (IFN) ASSAY

Normal and GVH reactive B6AF1 mice were anesthesized with ether and their blood was collected aseptically in separate tubes by cardiac puncture. The animals were placed in a supine position, the thoracic cavity was opened and the heart exposed. Using #26 gauge needle and a 1 ml. tuberculin syringe, blood was collected by cardiac puncture under sterile conditions. The blood was allowed to clot for 60 minutes at room temperature, and then centrifuged for 10 minutes at 1,500 rpm (500g). The serum was removed with a sterile pasteur pipette and was stored at -30oC until assayed for IFN titers. Serum from the following groups of animals was collected:

(1) Normal B6AF1 mice

(2) Normal B6AF1 mice injected with Poly I:C

(3) GVH-reactive B6AF1 mice

(4) GVH-reactive B6AF1 mice injected with Poly I:C

In each group, serum from 4 animals/group/day was pooled.

2.15 IFN ASSAY

The IFN assay was performed in Dr.Jean-Marie Dupus' laboratory, Institut Armand-Frappier, Laval. A brief description of the assay follows:

The test serum samples were obtained from normal mice, normal mice injected with Poly I:C, GVH mice, and GVH mice injected with Poly I:C as described in section 2.14. The IFN standard was obtained from The National Institutes of Health, Bethesda, Maryland (4000 units of IFN/ml). Both the IFN standard and the test samples were serially diluted in MEM.E 1x without FCS, and each dilution was plated in triplicate wells in a volume of 250ul/well. The highest dilution of the test sample (serum containing IFN) and the standard was also added to extra wells (3 wells/sample) to test the cytotoxicity of the sample. The plates were then exposed to ultraviolet light for 15 minutes to inactivate possible virus which might exist in the samples.

L2 cells (fibroblasts) which were grown as monolayers were trypsanized to inactivate IFN that may have been produced by the L2 cells and were then washed 1x, counted, and adjusted to a concentration of $25x10^5$ L2 cells/ml in MEM.E 1x with 10% FCS. These L2 cells were then plated in a volume of 0.2ml/well ($5x10^6$ L2 cells/well) containing different dilutions of the standard IFN and various test samples. The cells were incubated for 24 hours at 37oC in 5% CO2. After 24 hours of incubation, the plates were examined microscopically to confirm the formation of monolayers, then the IFN containing

samples were removed by reversing the plates onto sterile gauze. The virus (Sindbis) was then added to the L2 cell monolayers.

After addition of the virus to L2 cells, the plates were further incubated for a period of 72 hours at 37oC in 5% CO2. Following this incubation the plates were read under the microscope and the L2 cells in each well were graded on a scale of 0 to 4+ (the wells in which all the L2 cells were destroyed by the virus were graded as 4+). In the absence of IFN the virus would destroy all the L2 cells, whereas the presence of IFN would protect the L2 cells from viral destruction.

2.16 ERYTHROCYTE ANTIGEN

Sheep red blood cells (SRBC) were obtained from a solution containing a 1:1 ratio of sheep blood and Alserver's solution (Institut Armand Frappier, Laval, Canada). The sheep blood was diluted 1 in 10 with isotonic saline, centrifuged at 1,500 rpm for 10 minutes and the supernatant was discarded. For in vivo immunization, the SRBC were resuspended in isotonic saline at 30x the packed cell volume (PVC). Aliquots of 0.3ml, containing approximately $5x10^8$ SRBC, were injected i.v. via the tail vein of the animal.

2.17 THE IN VIVO DIRECT PLAQUE FORMING CELL (PFC) ASSAY

Spleen cells were assayed for the total number of direct PFC's to SRBC using the technique of Cunninigham and

Szenberg (1968) as modified in this laboratory (Elie and Lapp, 1977; Treiber and Lapp, 1978; Parthenais and Lapp, 1978). The animals were injected with 5 x 10^8 SRBC, sacrificed four days later and their spleens were prepared for the PFC assay. Splenic single cell suspensions were made in a final volume of 15 mls of HBSS. 0.05 ml of spleen cells was mixed with 0.07 ml of a 10% SRBC solution, and 0.5 ml of guinea pig C' diluted 1:20 in HBSS. The mixture was distributed into 4-5 chambers made from two precleaned microscope slides (Fisher Scientific Co., N.J.) held together by double sided "scotch" brand tape. A blank solution, containg SRBC and C' only, was used to fill the last slide when necessary. The chambers were sealed with warm paraffin wax and incubated at 37oC for one hour. The plaques were counted by macroscopic examination and confirmed, if necessary, microscopically.

2.18 MITOGEN ASSAY

Splenic mitogen responses were tested according to the technique described by Kirchner et al. (1975). Mice were sacrificed by ether euthanasia and their spleens removed aseptically. Splenic single cell suspensions were made as described in section 2.4. For the mitogen assay, spleen cells resuspended in RPMI-1640 supplemented with 1% Gentamycin, 2mM L glutamine (Grand Island Biological Co., Grand Island, N.Y.) and 5% FCS (Flow Laboratories, Mississauga, Ontario, Canada). Concanavalin A (ConA) (Pharmacia Fine Chemicals, Uppsala, Sweden),

Phytohemagglutinin (PHA) (Wellcome Research Reagents Ltd., Beckman, England), and E.Coli Lippopolysaccharide (LPS) (Sigma Chemical Co., St. Louis, Mo,) were weighed, resuspended in HBSS, and then diluted into various concentrations (100ul/ml; 50ul/ml; 25ul/ml; 12.5ul/ml; and 6.25ul/ml) ConA, PHA, and LPS, in a volume of 0.1ml was added to different tubes each containing $3x10^6$ splenocytes in a volume of 0 9ml. A volume of 200ul ($6x10^5$ cells/well) was plated in triplicate into flat bottom microtiter plates (Flow Laboratories Inc , Mississauga, Ontario, Canada) and incubated at 37oC in an atmosphere of 5% CO2 and 95% air for 48 hours. After this incubation period, the plates were pulsed with thymidine methyl 3H (TdRH3 NEZ 027, New England Nuclear Co., Boston, MA.; specific activity 6.7 Ci/mmole); at a final concentration of 1uCi/well, for a period of 12-16 hours. The cultures were harvested, after a total incubation of 60-64 hours, using a multiple automated sample harvester onto glass fiber filters (Mandel Scientific, Rockwood, Ontario, Canada). The filters were air dried and placed in scintillation vials, each containing 6ml scintillation fluid (Scintiverse, Fisher Scientific Co , N.J.), and counted in a beta counter (LKB Rackbeta). The results are presented as either :

(1) mean counts per minute (cpm) $\pm$ S.E. of triplicate cultures.

(2) Net cpm, calculated as follows:

mean cpm in the presence of mitogen - mean cpm without mitogen

(3) % of nomal resonse, calculated as follows:

$$\frac{\text{Net cpm of experimental group}}{\text{Net cpm of normal splenocytes}} \times 100$$

2.19 TREATMENT OF SPLENOCYTES WITH PGs PRIOR TO MITOGEN ASSAY

Singles cell suspensions of normal and GVH-reactive spleens were made, washed twice, aliquoted, and resuspended in RPMI-1640+5% FCS. Tubes containing 25-30x10^6 splenocytes were treated with either medium alone or with different concentrations of either PG E1 or PG E2, for a peroid of 60 minutes at 37oC in a humidified atmosphere containing 5% CO2 Following these treatments , the cells washed twice and then resuspended in RPMI-1640+5% FCS and 1% Gentamycin at a concentration of 3.3x10^6 cells/ml, and tested for their ability to respond to T-cell mitogens (ConA and PHA) The protocol for mitogen assay is described in detail in section 2.18

2.20 INTERLEUKIN-2 (IL-2) PRODUCTION

On different days after GVH induction, spleens were removed from normal and GVH mice, under sterile conditions, and single cell suspensions made. The splenocytes were washed twice and resuspended in RPMI-1640+5% FCS at a final concentration of 3.3x10^6 splenocytes/ml, in a volume of 1 6 ml, in 15 ml conical tubes (Starstedt, Canada). The desired concentrations of T-cell mitogens (ConA and PHA) were then added to the cells. In some experiments, LPS a B-cell mitogen

which does not induce IL-2 production, was used as control. Splenocytes in the presence of mitogens, were incubated for 18 hours at 37oC in 5% CO2. Following this incubation, $6x10^5$ splenocytes/well were plated in triplicate for the mitogen assay (section 2.18) and the remaining cells were spun at 1,000 rpm (500g) for 10 minutes and supernatants containing IL-2 were collected. This protocol allowed us to assess mitogen responsiveness as well as mitogen production by the same cell population.

2.21. IL-2 ASSAY

Supernatants containing IL-2 were tested for their ability to support the growth of IL-2 dependent cytotoxic-T-lymphocyte line (CTLL), as assessed by 3H-thymidine incorporation.

The concentration of CTLL cells was adjusted to $20x10^4$ /ml in Dulbecco's modified eagles medium supplemented with 5% FCS. Two x 10^4 CTLL cells, in a volume of 100ul/well, were plated in round bottom 96 well plates. 100ul of supernatant, which was collected from normal and GVH-reactive splenocytes following stimulation with either ConA or PHA (and in some experiment with LPS) was added to triplicate wells containing CTLL cells. These CTLL cells were then incubated at 37oC in 5% CO2 for 40 hours. After this incubation period, the plates were pulsed with Thymidine methyl 3H at a final concentration of 1uCi/well, for a peroid of 8-12 hours. Following this incubation period, the cells were harvested onto glass fiber

filters. The filters were air dried, placed in scintillation vials containing 6ml of scintillation fluid. The results are expressed as mean net cpm $\pm$ S.E. of triplicate cultures or as % of normal response.

2.22 DETERMINATION OF SPLEEN INDEX (SPLENOMEGALY)

Animals were randomly selected from a pool of GVH-reactive mice and from a pool of age-and-sex matched normal B6AF1 mice on different days after GVH induction Each animal was weighed, numbered, and then sacrificed Spleens from each of the animals were removed asceptically and placed in a petri dish that had been previously numbered and weighed The petri dish containing the spleen was then weighed. The weight of spleen of an individual animal was determined as follows

- weight of petri dish+spleen - weight of petri dish alone

Spleen Index (which determines splenomegaly) was determined by the method of Simonsen (1962);

$$= \frac{\dfrac{\text{Spleen weight of GVH-reactive animal}}{\text{Total weight of GVH-reactive animal}}}{\dfrac{\text{Spleen weight of normal animal}}{\text{Total weight of normal animal}}}$$

2.23 HISTOLOGY

On different days after GVH induction, GVH-reactive mice were sacrificed by ether euthanasia and their thymuses, lymph nodes, salivary glands, liver, lung, and pancreas were removed and fixed in 5% formalin. Parrafin sections of each of these organs were stained with hematoxylin and eosin and

examined. The histopathological lesions were graded as normal, mild-slight, and moderate-severe. The photomicrographs and the descriptions of various degrees of histopathological lesions are presented in the result section of chapters 4-7.

CHAPTER THREE

STUDIES ON THE STATUS OF NK CELL ACTIVITY IN DIFFERENT LYMPHOID ORGANS DURING THE COURSE OF GVH REACTIONS.

3.1 INTRODUCTION

It is well-established that the GVH reaction results in severe prolonged immunosuppression of both the T-and B-cell function. However, the status of NK cell activity in different lymphoid organs after GVH induction is still poorly defined. Experiments were therefore, designed to investigate; (1) The effect of PLC genotype on the status of splenic NK cell activity following GVH induction in F1 mice; (2) The effect of PLC dose on the status of NK cell activity in different lymphoid organs of F1 mice; (3) The status of NK cell activity in different lymphoid organs of F1 mice undergoing a chronic GVH reaction.

3.2 EXPERIMENTAL DESIGN

The experimental design is shown in figure 2.1 a, b, c, d.

In the first series of experiments studies were undertaken to confirm the presence of the "classical" NK cell activity in the partially purified cells that we have used as NK cells (section 2.6). The following aspects of NK cell activity were assessed in the normal mice: (i) NK cell activity in the partially purified splenocytes, thymocytes and BM cells against YAC targets which are NK sensitive (i.e., organ distribution of NK cell activity); (ii) P-815 target cell (NK insensitive target) killing by splenocytes; and (iii) effect of anti-thy 1.2 serum plus C' treatment of normal splenocytes on YAC killing.

In the second series of experiments, studies were designed to investigate the effects of GVH reactions on splenic NK cell activity. In these studies GVH reactions were induced in B6AF1 mice by injecting $30x10^6$ lymphoid cells of either parental strain A or B6. Eight days later, each group of GVH-reactive animals and normal F1 mice was randomly divided into two groups. One group of animals were injected with SRBC on day 8 post-GVH induction, to confirm suppression of PFC response to SRBC, which is a hallmark of GVH reaction induction. The animals in the other group were used to determine NK cell activity on day 8 post-GVH induction. In these studies NK cell activity of individual spleens and pooled splenocytes (3 animals/group) within a group was also determined at different E:T cell ratios. Percent NK cell mediated killing of YAC-1 targets was also calculated for an individual well at a given E:T cell ratio to determine the variability in cytotoxicity within the wells.

In the third series of experiments kinetics of splenic NK cell activity during the course of GVH reactions was studied GVH reactions were induced in B6AF1 mice by injecting either $30x10^6$ A or $30x10^6$ B6 PLC. On different days post-GVH induction 3 -4 animals/group were sacrificed. Spleens from these animals within a group were pooled, and NK cell activity for each group of pooled splenocytes was determined.

In the fourth series of experiments, we studied : (1) the kinetics of NK cell activity in different lymphoid organ and

(ii) the effects of parental lymphoid cell dose on the kinetics and the degree of augmented NK cell activity. In these studies B6AF1 mice were injected with different doses, $30x10^6$, $20x10^6$, or $10x10^6$, of B6 PLC. On different days post-PLC injection animals were randomly selected from different groups of F1 mice and their spleens, thymuses, lymph nodes, and BM cells were pooled seperately. Single cell suspensions were made of each organ seperately and tested for their NK cell activity. Furthermore, on day 8 post-PLC injections animals in each group were also tested for their immune functional status in the PFC assay. Suppression of the PFC response to SRBC was used as a criterion for the GVH reaction induction. NK cell activity in different lymphoid organs (spleen, thymus, lymph nodes, and BM), of the group of B6AF1 mice in which suppression of PFC response was observed (B6AF1 mice injected with $30x10^6$ B6 PLC), was then followed for 3-4 months post-GVH induction.

Next, studies were designed to investigate whether supernatants derived from cells of various lymphoid organs (lymph nodes, spleen, and BM) of GVH-reactive animals were able to induce/augment NK cell activity (spontaneous killing of YAC-1 tumor targets) in cells of different lymphoid organs (lymph node, BM) of normal B6AF1 mice. GVH reactions were induced in B6AF1 mice by injecting $30x10^6$ B6 PLC, since this cell dose induced GVH reactions as assessed by suppression of PFC response to SRBC.

Spleen, lymph nodes, and BM cells were removed from 4-6

GVH-reactive animals on day 8 post-GVH reaction induction and single cell suspensions were made. Supernatants from cells of these lymphoid organs were collected (see section 2.9). Partially purified NK cells were obtained from lymph nodes and BM of normal B6AF1 mice. These NK cells from BM and lymph nodes of normal mice were tested for their NK cell activity after they had been treated with supernatants derived from cells of different lymphoid organs of GVH-reactive mice (see section 2.10).

3.3 RESULTS

3.3.1 ENDOGENOUS NK CELL ACTIVITY IN DIFFERENT LYMPHOID ORGANS OF PARENTAL AND F1 MICE

The endogenous splenic, thymic, and BM NK cell activity of parental strains A and B6 and their B6AF1 hybrids is shown in table 3.1. At all E:T cell ratios tested, splenic NK cells from parental strain A possessed lower cytolytic activity against YAC-1 targets, than compared with the endogenous splenic NK cell activity of parental strain B6 and B6AF1 mice. Futhermore, as can be seen in table 3.1, the splenic NK cell activity of B6AF1 mice is similar to that of parental strain B6 rather than of parental strain A. These results are consistent with data previously reported (Petranyi et al, 1975 ; Kiessling et al, 1975a). Furthermore, NK cell activity against YAC targets was low in the BM (as compared to spleen) and non-existent in the thymus (Table 3.1). These results are in accordance with the organ distribution pattern

of classical NK cells as reported earlier by Kiessling et al. (1975) and Herberman et al. (1975).

3.3.2 P-815 TARGET CELL KILLING BY PARENTAL AND B6AF1 SPLENOCYTES

Table 3.2 shows the killing of P-815 tumour target cells by splenocytes taken from parental strains A or B6, as well as their B6AF1 hybrids. As can be seen, the splenocytes from either the parental strain mice or the F1 hybrids did not lysis P-815 tumour targets effectively. It has previously been reported that P-815 tumours cells are resistant to NK cell mediated lysis (Keissling et al, 1975; Herberman et al, 1975).

3.3.3 EFFECT OF ANTI-THY-1.2 SERUM PLUS C' TREATMENT ON ENDOGENOUS SPLENIC NK ACTIVITY OF B6AF1 HYBRIDS

Figure 3.2 demonstrates the effect of anti-thy 1.2 serum plus C' treatment on splenic NK cell activity of B6AF1 mice. Such treatment did not reduce NK cell activity against YAC targets at any E:T cell ratio; rather slight enhancement of NK cell activity was observed in this study. Similar results showing no effect of anti-thy 1.2 serum plus C' treatment on NK cell activity have been previously reported (Keissling et al., 1975a,b; Shellam, 1977; Clark et al., 1979; Borland et al., 1983).

Collectively, these data (Table 3.1, Table 3.2, and Fig.3.2) show that the partially purified splenic cells we have used as NK effectors do contain cells which mediate the

"classical" NK cell type of killing, as assessed by their organ distribution, non-adherence to plastic, insensitivity to anti-thy 1.2 serum plus C' treatment, inability to kill P-815 (NK insensitive) tumour targets, and their ability to kill YAC-1 (NK sensitive) tumour targets.

3.3.4 NK CELL ACTIVITY FOLLOWING GVH REACTION INDUCTION

GVH reactions were induced in B6AF1 mice by injecting either $30x10^6$ A or $30x10^6$ B6 parental cells. In the first series of experiments, splenic NK cell activity was studied on day 8 after GVH-induction.

Table 3.3 demonstrates splenic NK cell activity of individual animals within a group and splenic NK cell activity of pooled splenocytes from 3 animals/group. The data demonstrate that on day 8 post-GVH reaction induction augmented NK cell activity is observed in all of the individual animals as well as the pooled splenocytes in both the groups of GVH-reactive mice (A-->B6AF1 & B6-->B6AF1) when compared with normal B6AF1 splenocytes. However, NK cell activity observed in the splenocytes of B6AF1 mice that were injected with $30x10^6$B6 PLC was higher than the NK cell activity observed in the splenocytes of B6AF1 mice that received $30x10^6$A parental lymphoid cells (table 3.3; 40% killing versus 29% killing at 50:1 E:T cell ratio, with the pooled splenocytes).

Table 3.3 also shows the mean $\pm$ of NK cell mediated killing of YAC-1 targets at different effector:target cell

ratios (usually 4 well/E:T cell ratio were tested). As can be seen, the variability (± S.E of the mean) between the wells at a given E:T cell ratio was always very small (< 10% of the mean killing observed at a given E:T cell ratio). Thus, these data show that augmented NK cell activity during GVH reactions is observed in individual animals as well as in pooled splenocytes.

Table 3.3 also shows that GVH reactions augment the cytotoxic activity of effector cells that can lyse P-815 target cells. The effect of the donor genotype in augmenting P-815 effector cell cytotoxicity is evident. Strain A PLC induce much greater P-815 effector cell cytotoxicity than B6 PLC. This augmentation of the P-815 effector cell activity by the GVH reaction shall be discussed in detail in chapters 4 and 6.

3.3.5 KINETICS OF SPLENIC NK CELL ACTIVITY FOLLOWING GVH INDUCTION

Figure 3.3 shows the kinetics of splenic NK cell activity of B6AF1 mice injected with either $30x10^6$ A or $30x10^6$ B6 PLC. As can be seen, augmented splenic NK cell activity was observed as early as day 4 post-GVH-induction, and by day 8 post-GVH-induction, NK cell activity reached its peak in both GVH-reactive groups (A-->B6AF1 and B6-->B6AF1). After day 8 post-GVH-induction, NK cell activity started to decline, eventually falling to below control levels. Thus, the splenic NK cell activity in the two GVH-reactive groups follows a

similar kinetics, namely, an augmentation followed by later depression. However, the peak splenic NK cell activity observed in the group of B6AF1 hybrids injected with $30x10^6$ B6 PLC was higher than that observed in B6AF1 mice injected with $30x10^6$A PLC (33% kill vs 24% kill). Moreover, the decline of NK cell activity in B6AF1 mice injected with $30x10^6$B6 PLC was slower than that observed in B6AF1 mice injected with $30x10^6$A PLC (Figure 3.3). The return to near-normal levels of splenic NK cell activity of B6AF1 mice injected with $30x10^6$B6 PLC was observed by day 17 post-GVH-induction, whereas, below normal splenic NK cell activity in B6AF1 mice injected with $30x10^6$A PLC was observed by day 14 post-GVH-induction (Figure 3.3).

3.3.6 ASSESSMENT OF GVH REACTION INDUCTION: SUPPRESSION OF PFC RESPONSE TO SRBC

Table 3.4 demonstrates the PFC response of B6AF1 injected with either $30x10^6$ A or $30x10^6$ B6 PLC. Table 3.4 shows that the B6AF1 mice which were injected with $30x10^6$ A PLC became totally immuno-suppressed. However, severe but incomplete suppression of the PFC response to SRBC was observed in B6AF1 mice injected with $30x10^6$ B6 PLC by day 8 post-PLC injection (9% of the normal response).

3.3.7 KINETICS OF NK CELL ACTIVITY IN DIFFERENT LYMPHOID ORGANS OF B6AF1 MICE FOLLOWING PLC INJECTION: EFFECT OF PLC DOSE

Different doses $30x10^6$, $20x10^6$, or $10x10^6$ of parental

strain B6 lymphoid cells were injected into groups of B6AF1 mice and the kinetics of NK cell activity in different lymphoid organs was followed.

3.3.7.1 KINETICS OF SPLENIC NK CELL ACTIVITY:

Figure 3.4 illustrates the splenic NK cell kinetics of B6AF1 mice injected with either 30, 20, or $10x10^6$ B6 PLC. NK cell activity started to increase by day 3 post-PLC injection in the groups of B6AF1 mice that were injected with $30x10^6$ B6 PLC and reached a peak by day 8 after GVH induction (three experiments). Augmented NK cell activity in the groups of B6AF1 mice injected with $10x10^6$ and $20x10^6$ B6 PLC reached a peak by day 12 post-PLC injection in two experiments and on day 15 in one experiment. The highest NK cell activity was observed in B6AF1 mice that were injected with $30x10^6$ B6 PLC (32% killing on day 8), followed by B6AF1 mice with $20x10^6$ B6 (26% killing on day 12), and $10x10^6$ B6 PLC (21% on day 12), respectively. Although, the peak NK cell activity in F1 mice injected with $20x10^6$ and $10x10^6$ B6 PLC fluctuated between days 12-15 after GVH induction, it was never observed on or earliar than day 8 post-GVH induction. However, the peak NK cell activity in F1 mice that received $30x10^6$ B6 PLC was consistently observed on day 8 after GVH induction.

In all groups of B6AF1 mice that recieved different doses of B6 PLC, NK cell activity after reaching a peak started to decline, and returned to control levels by day 17 post-PLC

injection and then became suppressed by day 20 post-PLC injection.

Figure 3.4 further shows the splenic NK cell activity of B6AF1 mice undergoing chronic GVH reactions. The induction of GVH reactions was determined by the severe suppression of the PFC response to SRBC (Table 3.5). As can be seen, the splenic NK cell activity of B6AF1 mice undergoing a chronic GVH reaction (F1 mice injected with $30x10^6$ B6 PLC), was severely suppressed up to day 150 post-GVH induction

3.3.7.2 KINETICS OF LYMPH-NODE NK CELL ACTIVITY

Figure 3 5 illustrates the NK cell kinetics of lymph node cells of B6AF1 mice injected with different doses of B6 PLC. Slightly augmented NK cell activity was exhibited on day 3 post-GVH induction by lymph node cells of B6AF1 mice injected with $30x10^6$ B6 PLC Slightly augmented NK cell activity by lymph node cells of B6AF1 mice injected with $20x10^6$ B6 PLC was observed on day 6 post-GVH induction, whereas no augmentation of NK cell activity of lymph node cells of B6AF1 mice injected with $10x10^6$ B6 PLC was observed up to day 8 post-GVH induction The peak NK cell activity in the lymph-node cells of B6AF1 mice injected with $30x10^6$ B6 and $20x10^6$ B6 PLC was observed on day 8 post-GVH induction, whereas peak NK activity in lymph node cells of B6AF1 mice injected with $10x10^6$ B6 PLC was observed on day 10 post-GVH induction. The highest peak of NK cell activity was observed in B6AF1 mice injected with $30x10^6$ B6 PLC (26% killing), whereas the peak NK

cell activity observed in the lymph node cells of B6AF1 mice injected with $20x10^6$ and $10x10^6$ B6 PLC was approximately the same, namely 14% and 13% respectively. In all groups of GVH-reactive B6AF1 mice, the NK cell activity returned to control levels by day 15-17 post-PLC injection.

Figure 3.5 further shows that the lymph node NK cell activity of B6AF1 mice undergoing a chronic GVH reaction (B6AF1 mice injected with $30x10^6$ B6 PLC) remained severely suppressed up to day 150 post-GVH induction.

3.3.7.3 KINETICS OF THYMIC NK CELL ACTIVITY

Figure 3.6 illustrates the thymic NK cell activity of B6AF1 mice injected with different dose of B6 PLC. The initial appearance of NK cell activity in the thymus of GVH- reactive B6AF1 mice was dependent upon the number of B6 PLC injected. Thymic NK cell activity was first observed on day 3 post-PLC injection in B6AF1 mice injected with $30x10^6$ B6 PLC, on day 6 in B6AF1 mice injected with $20x10^6$ B6 PLC and on day 8 in B6AF1 mice injected with $10x10^6$ B6 PLC. In all groups of GVH reactive B6AF1 mice the thymic NK cell activity remained elevated up to day 12 post-PLC injection and then returned to normal (undetectable) levels by day 15 post-PLC injection.

Figure 3.6 further shows that the thymic NK cell activity of GVH-reactive B6AF1 mice (i.e., F1 mice injected with $30x10^6$ B6 PLC) remained undetectable up to day 60 post-GVH induction but, in contrast to the spleen and lymph node NK cell activity,

a dramatic increase in thymic NK cell activity was observed beyond day 60 after GVH-induction.

3.3.7.4 KINETICS OF BM NK CELL ACTIVITY

Figure 3 7 illustrates the BM NK cell kinetics of B6AF1 mice injected with either $30x10^6$, $20x10^6$, or $10x10^6$ B6 PLC. Augmented BM NK cell activity was observed on day 3 post-PLC injection in B6AF1 mice which were injected with $30x10^6$ B6 and $20x10^6$ B6 PLC. On the other hand, in B6AF1 mice injected with $10x10^6$ B6 PLC augmented NK cell activity was observed on day 10 post-PLC injection. The highest degree of augmented NK cell activity was observed in the BM of B6AF1 mice that were injected with $30x10^6$ B6 PLC (22% killing on day 8 post-PLC injection). The BM NK cell activity started to fall, after reaching a peak level, in all groups of GVH-reactive F1 mice and, by day 20 post-PLC injection had declined to normal or below normal levels.

Figure 3.7 further shows that the BM NK cell activity of GVH-reactive B6AF1 mice remained suppressed up to day 50 post-GVH induction. However, like the thymus and in contrast to spleen and lymph nodes, dramatically augmented NK cell activity was observed in the BM beyond days 60 after GVH induction.

3.3.8 PFC RESPONSE TO SRBC OF B6AF1 MICE INJECTED WITH DIFFERENT DOSES OF B6 PLC

Table 3.5 shows that among the B6AF1 mice injected

with different doses of B6 PLC, only the group of F1 mice that received $30x10^6$ B6 PLC became severely immunosuppressed (11% of the normal response) by day 8 post-PLC injection. Whereas, only partial suppression of PFC response to SRBC was observed in F1 mice injected with $20x10^6$ B6 PLC (75% of the normal PFC response) and no suppression of PFC response was observed in the group of F1 mice injected with $10x10^6$ B6 PLC (100% of the normal response) on day 8 post-PLC injection. The studies regarding the duration of immunosuppression in different groups of GVH mice are presented in chapter seven of this thesis.

3.3.9 EFFECT OF SUPERNATANTS FROM GVH-REACTIVE CELLS ON NORMAL BM AND LYMPH NODE NK CELLS

GVH reactions were induced in B6AF1 mice by injecting $30x10^6$ B6 PLC. This PLC dose resulted in a chronic GVH reaction, as assessed by suppression of PFC response to SRBC (Table 3.4 & 3.5). Supernatants were derived from spleen cells, lymph node cells and BM cells of GVH-reactive B6AF1 mice (day 8 after GVH induction) and normal B6AF1 mice as described in detail in section 2.9.

3.3.9.1 EFFECT OF SUPERNATANTS FROM GVH REACTIVE CELLS ON NORMAL BM NK CELLS

Figure 3.8 demonstrates the effect of supernatants derived from GVH-reactive spleen and BM cells on normal BM NK cell activity. As can be seen, the supernatants derived from the GVH-reactive spleen cells and BM cells

induced/augmented NK cell activity of normal BM NK cells at all E:T cell ratios tested. However, the supernatants derived from day 8 GVH-reactive spleen cells were more effective in inducing/augmenting normal BM NK cell activity than supernatants derived from day 8 GVH-reactive BM cells. Supernatants derived from normal B6AF1 spleen and BM cells also induced/augmented NK cell activity of normal BM cells. However, the supernatants from normal cells were much less effective in inducing/augmenting NK cell activity in normal cells than the supernatants derived from GVH cells.

3.3.9.2 EFFECT OF SUPERNATANTS FROM GVH-REACTIVE CELLS ON NORMAL LYMPH NODE CELLS

Figure 3.9 demonstrates the effect of supernatants derived from GVH-reactive spleen and lymph node cells on NK cell activity of normal lymph node cells. As can be seen, the treatment of normal partially purified lymph node NK cells with supernatants derived from GVH-reactive spleen cells or lymph node cells dramatically induced/augmented NK cell activity in normal lymph node cells at all E:T cell ratios tested. The supernatants derived from day 8 GVH-reactive spleen cells induced /augmented normal lymph node NK cell activity to a greater degree at all E:T cell ratios than supernatants derived from day 8 GVH reactive lymph node cells. In contrast, supernatants derived from normal B6AF1 spleen cells, but not lymph node cells, only slightly augmented NK cell activity of normal lymph node cells.

3.4 DISCUSSION

In this study we have demonstrated that GVH reactions induce/augment NK cell in different lymphoid organs of F1 mice early after GVH reaction induction. Furthermore, regardless of the dose of B6 PLC injected into B6AF1 mice, an increase in NK cell activity is observed in splenocytes, lymph node cells, thymocytes, and BM cells between days 1-12 post -PLC injection. However, the initial appearance of NK cell activity in the thymus and the magnitude of peak of NK cell cytotoxic activity in the spleen are dependent upon the number of PLC injected. After reaching a peak, NK cell activity in all organs and in all groups of B6AF1 mice that were injected with different doses of PLC declined to normal and/or below normal levels.

We have also shown that a dose of $30x10^6$ B6 PLC resulted in a GVH reaction as assessed by immunosuppression of the PFC response to SRBC. The long-term effects of a chronic GVH reaction on NK cell activity in different lymphoid organs were also studied. The data showed that although NK cell activity in spleens and lymph nodes remained suppressed beyond day 50-60 post GVH induction, it intensified in the thymus and BM of GVH-reactive mice. Finally, the data presented in this chapter show that supernatants derived from GVH-reactive splenocytes, lymph node cells, and BM cells are much more effective in inducing/augmenting NK cell activity in normal lymph node and BM cells than supernatants derived from spleen, lymph nodes,

and BM cells of normal mice.

On the basis of the NK cell kinetic studies presented in this chapter, it is proposed that the GVH reaction can be divided into three separate phases: (1) Early phase: This phase is characterized by an increase in NK cell activity in the spleen, lymph nodes, thymus, and BM followed by a return to control levels; (2) Intermediate phase: This phase is characterized by suppressed NK cell activity in spleen, lymph nodes, thymus, and BM; (3) Late phase: This phase is characterized by continued suppression of NK cell activity in the spleen and lymph nodes and dramatic reappearance of NK cell activity in thymus and BM. The results presented in this chapter clearly demonstrate that both the time at which NK cell activity is assessed and the organ selected to determine NK cell activity are critical factors to be considered in the determination of NK cell status following GVH induction..

The initial report on NK cell status during GVH reactions demonstrated depressed splenic NK cell activity (Keissling et al., 1977) . However, recent studies from our laboratory (Roy et al., 1982; Ghayur et al., 1986), as well as reports by other workers (Borland et al., 1983; Pattengale et al., 1983; Mowat et al., 1985, Varkila and Hurme, 1985), have demonstrated an early augmentation followed by a decline in NK cell activity in several different parent into F1 hybrid GVH combinations. Furthermore, Clancy et al. (1983) has recently described an early augmentation (upto day 10-12) and a later decline (by day 20) of NK cell activity in the spleen, lymph nodes, thymus, and

BM of GVH rats. Thus, the studies in rats confirm our earlier studies (Roy et al., 1982; Ghayur et al., 1980) and those presented in this chapter. In humans, Dokhelar et al. (1981) have also reported early augmentation followed by severe depression in peripheral blood NK cell activity in bone marrow transplant patients who developed GVH disease. Thus, the first two phases of NK cell activity during the course of GVH reactions/GVH disease have been documented.

The mechanism of augmentation of NK cell activity early after GVH induction is not clear. However, this augmentation of NK cell activity could be due to the release of lymphokines from GVH-reactive cells. The data presented in this chapter (Figures 3.8 and 3.9) showing that the supernatants from GVH-reactive cells of different lymphoid organs can effectively induce/augment NK cell activity of cells derived from different lymphoid organs of normal mice support the above proposal. Moreover, we have previously reported production of interferon (IFN) (Zawatsky et al., 1979) and PGE (Lapp et al., 1980) by the GVH reactive cells of different lymphoid organs *in vitro*. IFN production has also been reported following initiation of mixed lymphocyte reactions (MLR) *in vitro* (Kirchner et al., 1979) (MLR is a *in vitro* correlate of GVH reactions). IFN is one of the most potent potentiators of NK cell activity (Gidlund et al., 1978; Djeu et al., 1979; Trinchieri and Santoli, 1978; Senik et al., 1979). IFN has been shown to increase the lytic efficiency of mature NK cells as well as recruit and provide a maturational signal for pre-NK cells. (

Saksela et al., 1979; Herberman et al., 1979; Minato et al , 1980; Senik et al., 1980). Although, PGE by itself inhibit NK cell cytotoxic activity (Roder and Klein, 1979; Brunda et al , 1980; Bankhurst, 1982) , a combination of IFN and PGE can at least in vitro, enhance the lytic ability of NK cells (Targen, 1981). Furthermore, it is also of interest to note that significant augmented NK cell activity is observed in BM of GVH-reactive mice. NK cells (Haller et al., 1977; Haller and Wigzell, 1977) as well as IFN producing cells (DeMaeyer et al , 1967, 1970; 1975; DeMaeyer-Guignard et al., 1969) originate in the BM Collectively, it would appear that the early augmented NK cell activity could be possibly due to a combination of different factors, namely :(1) Increased lytic efficiency of mature NK cells of both host and donor origin (donor NK cells present in the inoculum) and recruitment and maturation of pre-NK cells by lymphokines such as IFN released after the induction of GVH reactions. (2) Increased numbers of NK cells produced and probably released into the periphery by the BM in response to the intense immune reaction that occurs early after the induction of GVH reactions.

The reasons for the depressed NK cell activity during the intermediate phase are not clear at the moment However, several possibilities may exist, each of which may not be mutually exclucive : (1) depression of IFN and PGs production by GVH- reactive cells, since NK cell activity during this phase could be restored by in vitro treatment of spleen cells with PGs and poly I:C, an IFN inducer (Ghayur et al., 1981),

(2) depletion of nucleated cells in the spleen and bone marrow of GVH mice (Xenacostas et al., 1986) and (3) maturational defect in the splenic NK cells during this phase . These three possibilites for depressed NK cell activity are discussed in detail in chapter 9.

The late phase of GVH reactions, as characterized in this chapter by continued suppression of NK cell activity in the spleens and lymph nodes and a dramatic increase in NK cell activity in the thymus and BM, has not been reported thus far. This recovery of thymic and BM NK cell activity is of interest. In humans, it has been reported that following allogeneic bone marrow transplantation, NK cell activity in the peripheral blood recovers earliar than other immune functions (Livnat et al., 1980; Noel et al., 1978; Ringden et al., 1979). NK cells originate in the BM and are then exported to the periphery. Furthermore, NK cells are present in the normal thymus as a resident population (Zoller et al., 1981), and are believed to be of T-cell lineage (Herberman and Holden, 1978; Herberman et al, 1979). Thus, it is possible that the augmented BM NK cell activity may represent the regeneration of BM of GVH reactive mice following its depletion by the GVH reaction (Xenacostas et al., 1986; Ghayur et al., 1986a,b; also see chapter 9). The augmented thymic NK cell activity may be due to the exportation of pre-T-cells (possibly NK cells) to the thymus by the regenerating BM. It has been reported that cells with surface markers of NK cell (asialo GM1) as well as NK cells with cytotoxic activity are the earliest to appear in the thymus

during its ontogeny (Habu et al., 1980; Koo et al., 1982). A second possibility for the augmented NK cell activity may be related to the regeneration of the thymic microenvironment of the regerating thymus (Ghayur et al., 1985, 1986) following GVH- induced thymic injury. The studies regarding the regeneration of thymic architecture are described in chapter 7 of this thesis.

An analysis of data on NK cell activity (Table 3.3 & Fig.3.3) and the PFC response to SRBC (Tables 3.4 & 3.5) shows that by day 8 after GVH-induction, although complete to severe suppression of the PFC response to SRBC is observed, NK cell activity is highly augmented. This suggests a complete dissociation between T-cell dependent B-cell responses and NK cell activity early after GVH reaction induction.

Thus in this chapter, we have demonstrated that GVH reactions induce highly augmented NK cell activity in the spleen, lymph nodes, thymus, and BM early after GVH reaction induction. However, later during the course of GVH reactions NK cell activity remains depressed in the spleen and lymph nodes, but reappears in the thymus and BM. The early augmented NK cell activity appears to be due , at least in part, to substances released by cells in the different lymphoid organs of GVH-reactive mice. Finally, the peak NK cell activity after GVH induction appears at a time when the PFC response to SRBC is completely and/or severely suppressed.

In the following two chapters the relationship between NK

cell cytotoxicity, T and B-cell functions, and the development of GVH-associated tissue injury to the non-lymphoid and lymphoid organs, respectively, are presented.

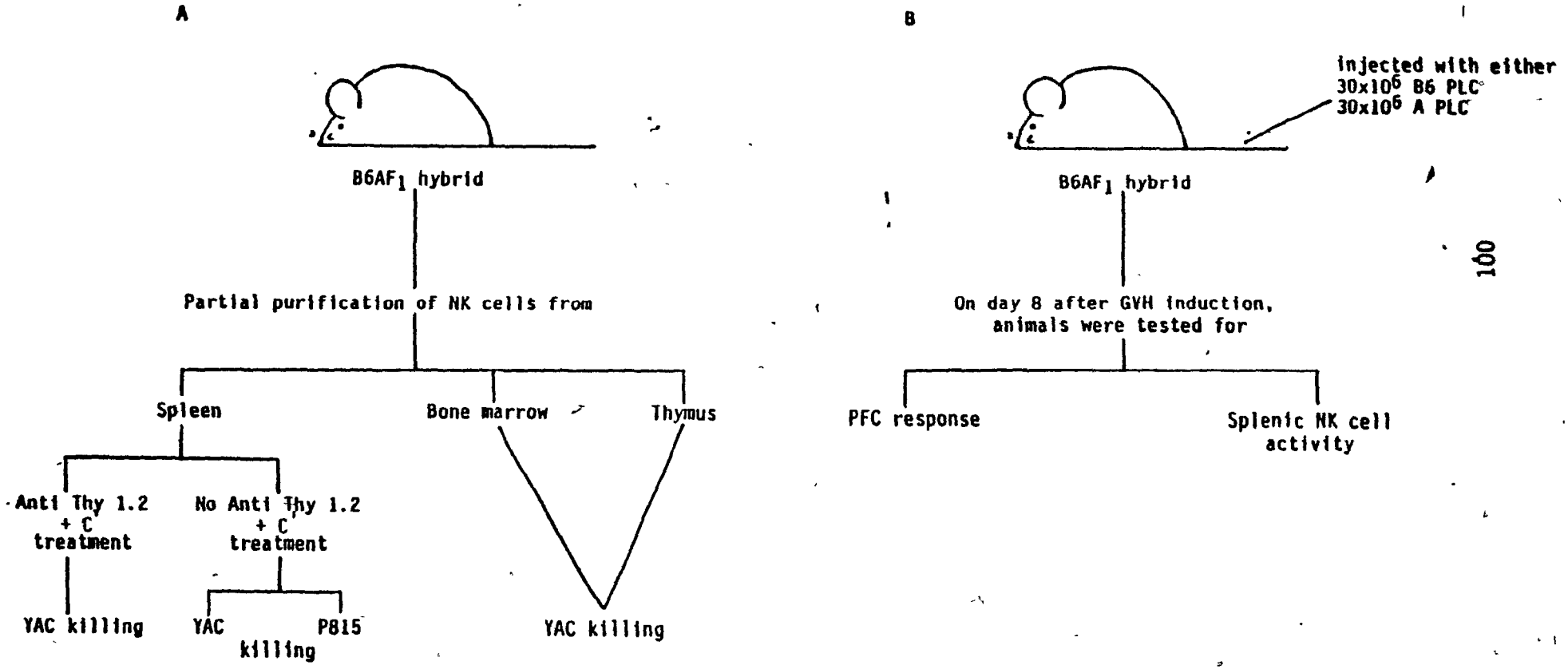

Figure 3.1 Experimental design used to investigate the status of NK cell activity (in different lymphoid organs) during the course of GVH reactions. (also see next page).

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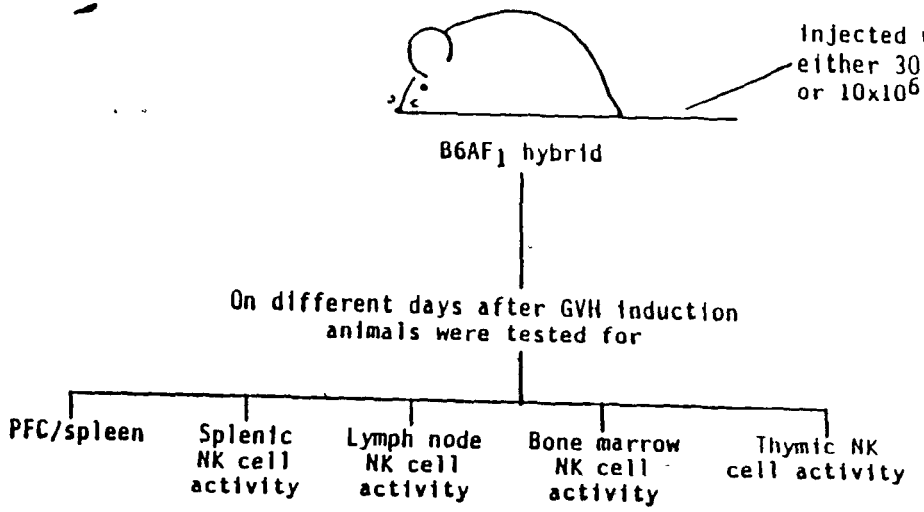

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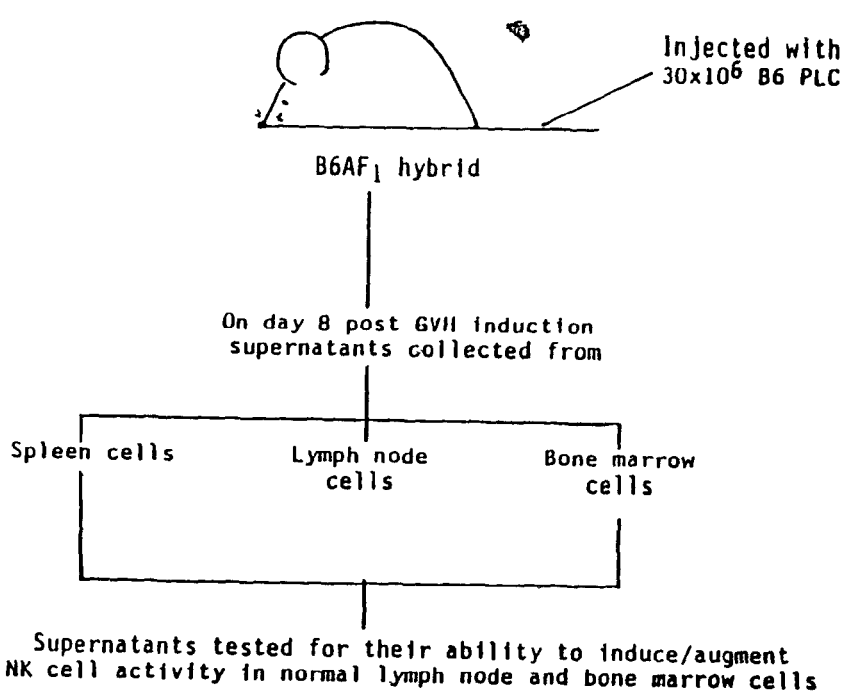

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Figure 3.1 Cont'd.

Table 3 1 · Endogenous splenic, thymic, and bone marrow NK cell activity of parental strains A and B6 and B6AF1 hybrids against YAC targets

Organ Assayed [a]	Animal Genotype	EXPT 1				EXPT.2				EXPT.3			
		EFFECTOR : TARGET CELL RATIO											
		50:1	25:1	12:1	6:1	50:1	25:1	12:1	6:1	50:1	25:1	12:1	6:1
SPLEEN	A	3.0	2.5	2.0	2.2	5 3	2.5	1.0	0 8	3.8	2.0	2.00	1.5
	B6	12.6	7.0	4.5	3.7	21.0	10.4	6.6	2.5	11.0	7.1	5.00	- *b
	B6AF1	14.0	10.4	5.3	3.1	20.2	12.3	7.2	3.0	13.3	10.5	5.00	-
THYMUS	A	1.2	0.5	-1.2	-0.8	0.5	-1.2	-0.8	-1.9	-2.4	-0.8	-1.2	-
	B6	-1.2	0 8	0.7	-1.2	1.8	-2.1	-1.0	0.8	1.5	-1.2	0.5	-
	B6AF1	-0.9	-2.4	-0.1	-1.3	0.6	0.8	-0.6	0.2	0.2	-0.7	-0.8	-1.2
BONE MARROW	A	2.0	1.5	0.6	-	1.8	1 2	-	-	2.9	1.3	-	-
	B6	3.8	2.5	-	-	4.0	2.5	-	-	4.9	3.5	-	-
	B6AF1	4.6	1.6	1.1	-	5.6	2.0	-	-	3.2	2.1	-	-

a Spleen cells, thymus cells and bone marrow cells from 3 animals/strain/experiment were pooled.

*b Not done.

Table 3.2 : P-815 target cell killing by splenocytes from parental strains A and B6 and B6AF1 hybrids

Animal Genotype[a]	EXPT.1		EXPT.2	
	EFFECTOR : TARGET CELL RATIO			
	50:1	25:1	50:1	25:1
A	0.9	0.7	0.6	-0.2
B6	1.5	0.9	1.4	1.0
B6AF1	1.9	0.4	2.4	-*[b]

a Spleen cells from 3 animals/strain/experiment were pooled

*[b] Not done

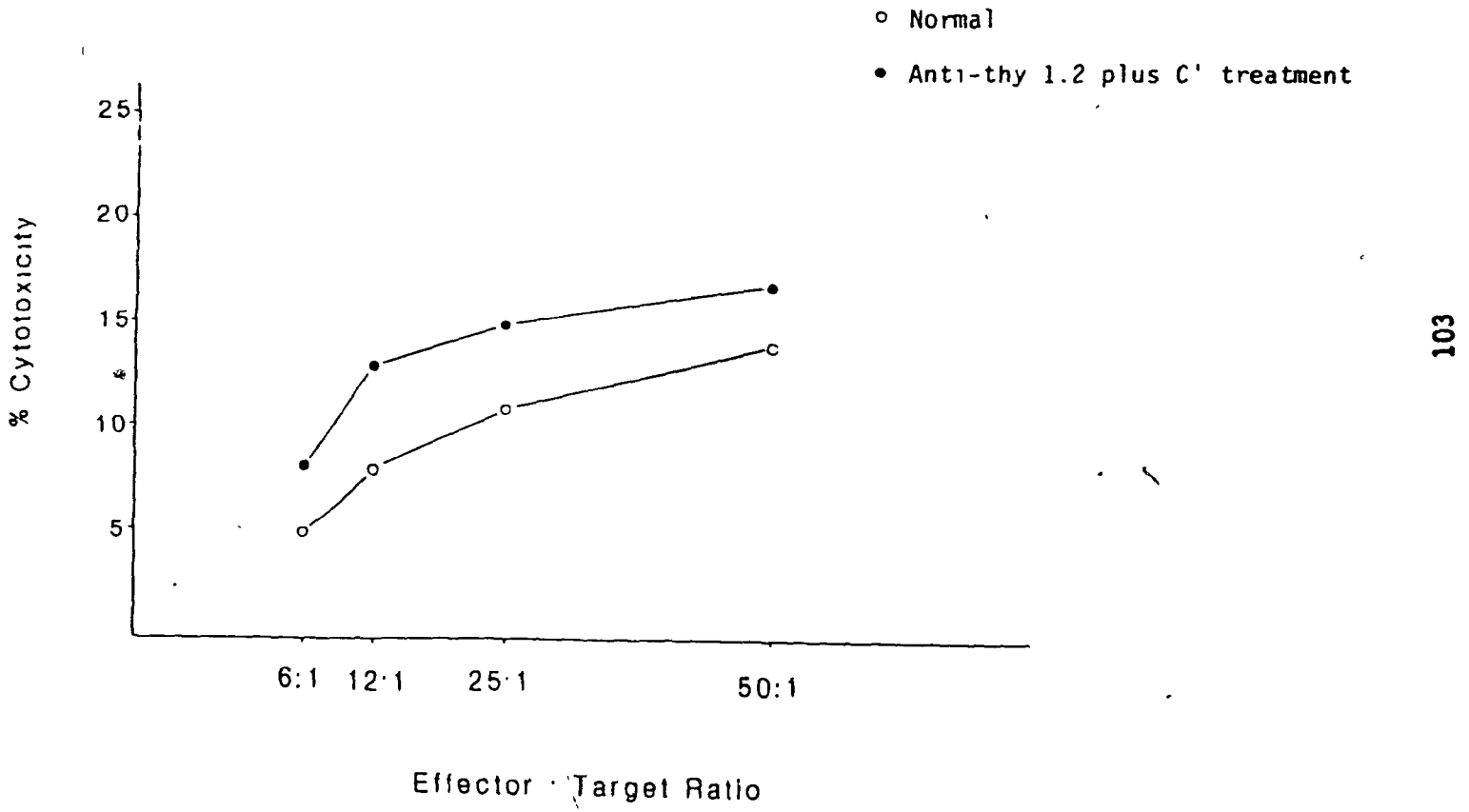


Figure 3.2 Effect of anti-thy 1.2 serum plus C' treatment on B6AF1 splenic NK cell activity against YAC targets

Table 3.3 : NK cell activity and P815 target cell killing of splenocytes obtained from normal F1 and GVH reactive mice.

Donor strain and number of cells injected to induce GVH reactions	Non-specific cytotoxicity against YAC and P815 at different E:T ratios					
	Splenocytes from individual animals				Pooled splenocytes	
	YAC		P815		YAC	P815
	50:1	25:1	50:1	25:1	50:1	25:1
-	10.0 ± 0.9	6.8 ± 0.6	-0.9 ± 1.7	0.7 ± 1.9		-
-	12.2 ± 0.7	6.3 ± 0.9	0.9 ± 1.4	-	10.9 ± 0.9	
-	12.5 ± 1.0	-*d	-	-		
B6 30x10^6	34.0 ± 0.9	22.2 ± 0.7	13.1 ± 1.3	10.0 ± 0.2		
B6 30x10^6	32.7 ± 0.8	20.9 ± 1.4	14.6 ± 2.5	9.1 ± 0.7	40.5 ± 1.7	11.5 ± 1.6
B6 30x10^6	36.1 ± 1.4	22.5 ± 0.9	-	-		
A 30x10^6	27.6 ± 1.8	21.9 ± 1.1	57.1 ± 2.4	55.2 ± 2.8		
A 30x10^6	22.2 ± 1.3	15.4 ± 1.6	58.6 ± 3.7	55.9 ± 2.6	29.8 ± 1.6	46.1 ± 2.1
A 30x10^6	24.1 ± 1.5	11.9 ± 1.2	-	-		

a % cytotoxicity of individual wells (4 wells) at a given E:T ratio was calculated. The data is presented as the Mean ± S E.
b Splenocytes were taken from B6AF1 mice 8 days after GVH induction.
c,d The experiment was repeated two times with similar results. Results of one experiment are shown.
* Not done.

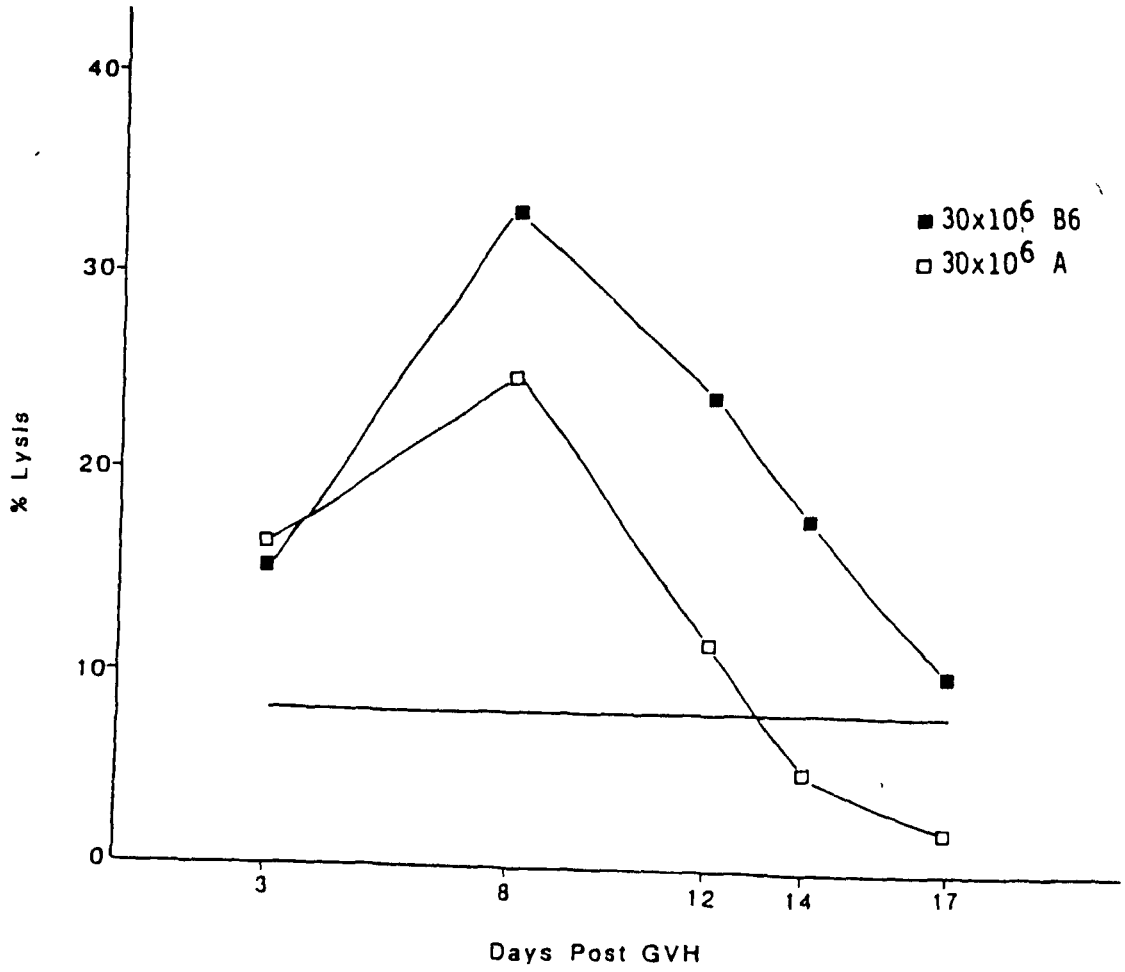


Figure 3.3 Kinetics of splenic NK cell activity against YAC targets of B6AF1 mice injected with either $30x10^6$A or $30x10^6$B6 PLC. The experiment was performed two times. Each experiment gave similar results. Results of one experiment are shown. At each point the normal and GVH splenocytes were tested in the same cytotoxicity assay. The effector:target cell ratio used was 50:1. The horizontal line is the mean splenic NK cell activity of normal B6AF1 mice.

Table 3.4 : PFC/spleen response to SRBC of B6AF1 mice injected with either $30x10^6$ B6 or $30x10^6$ A PLC.

Number and strain of parental lymphoid cells injected to induce GVH Reactions[a]	Number of animals / group	Mean PFC/spleen ± S.E. x 10^{-3}	% of normal PFC spleen response to SRBC
-	9	112.5 ± 11.5	100
$30x10^6$ B6	9	9.8 ± 2.1	8.6
$30x10^6$ A	9	0.2 ± 0.2	0.1

a B6AF1 mice were injected with SRBC on day 8 after PLC injection.

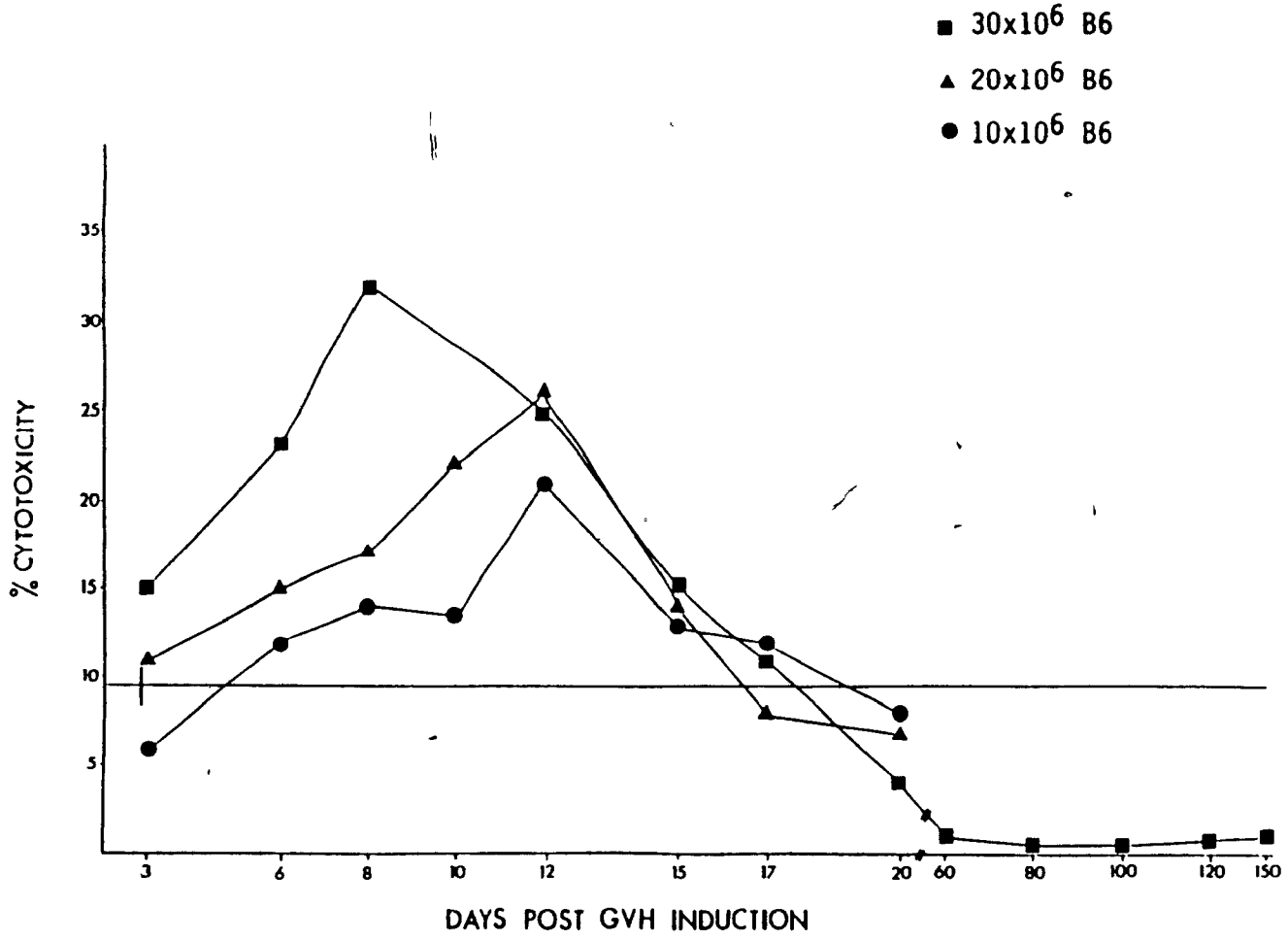


Figure 3.4. Kinetics of splenic NK cell activity of B6AF1 mice injected with different doses of B6 PLC against YAC targets. The experiment was repeated three times. Each experiment showed similar kinetics. Results of one experiment are shown. The horizontal line is the mean(±S.E) splenic NK cell activity of normal B6AF1 mice. The effector:target cell ratio used was 50:1.

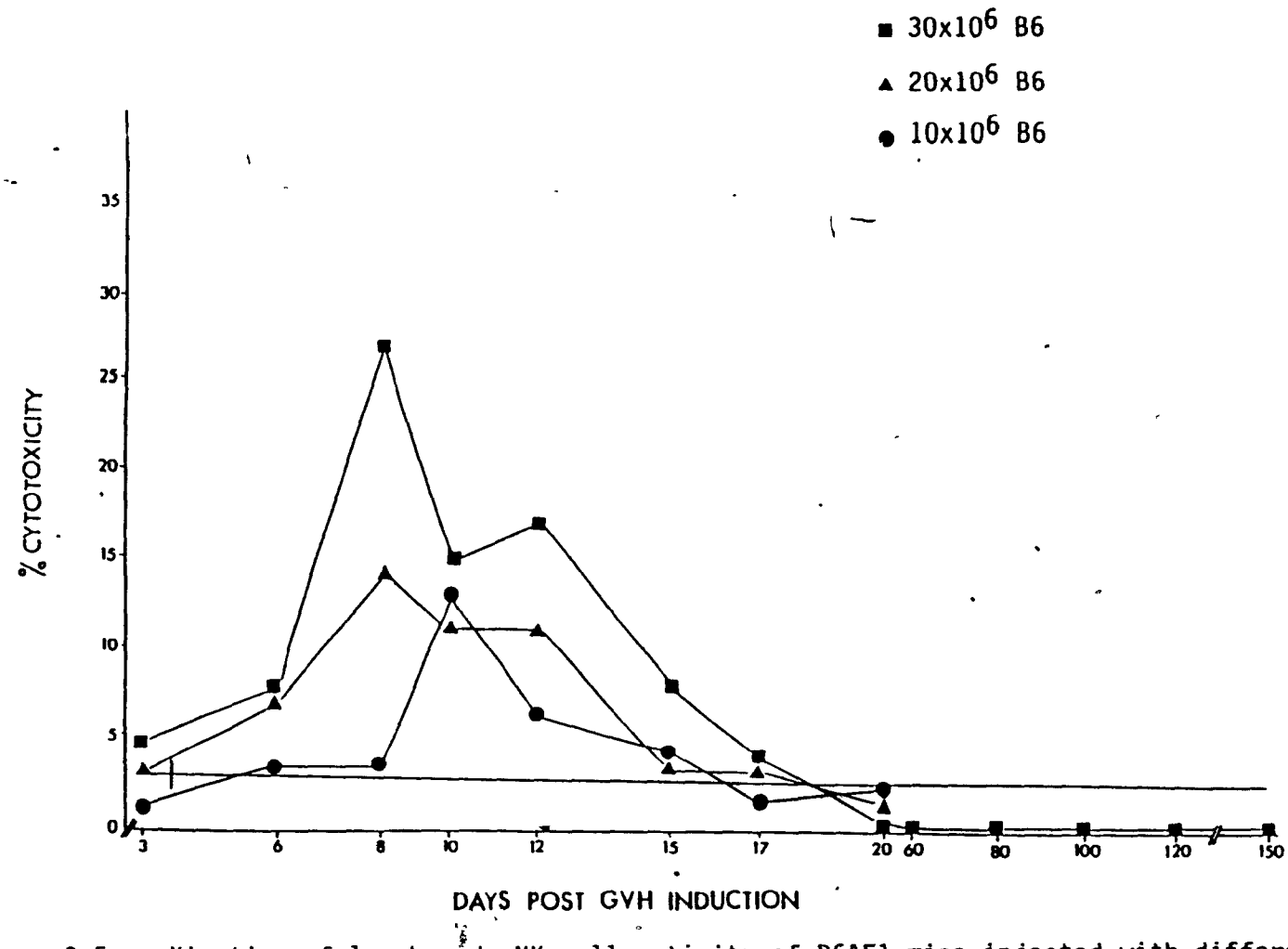


Figure 3.5. Kinetics of lymph node NK cell activity of B6AF1 mice injected with different doses of B6 PLC against YAC targets. The experiment was repeated three times. Each experiment showed similar kinetics. Results of one experiment are shown. The horizontal line is the mean(±S.E) lymph node NK cell activity of normal B6AF1 mice. The effector:target cell ratio used was 50:1.

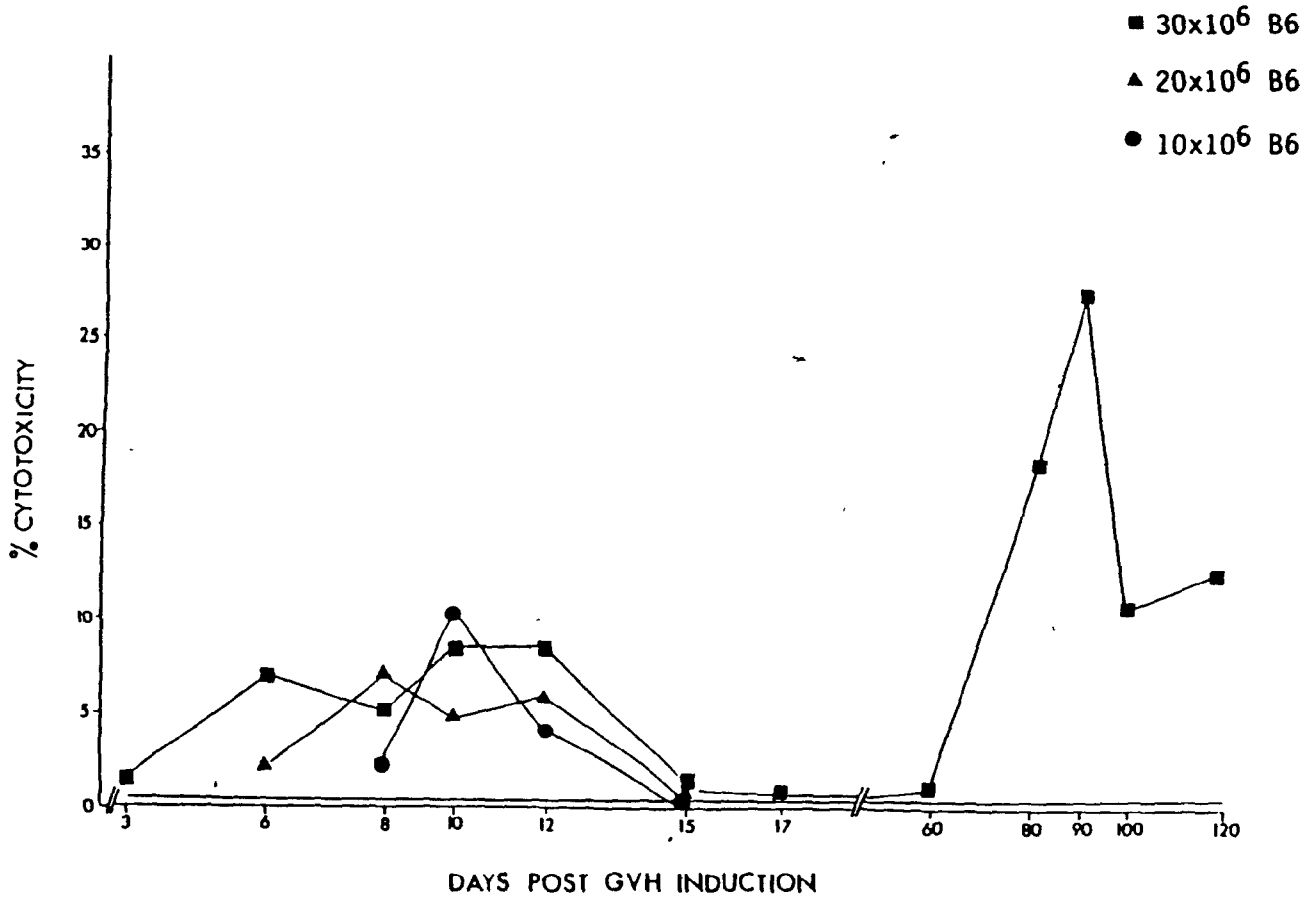


Figure 3.6. Kinetics of thymic NK cell activity of B6AF1 mice injected with different doses of B6 PLC against YAC targets. The experiment was repeated three times. Each experiment showed similar kinetics. Results of one experiment are shown. The effector:Target cell ratio used was 50:1. The horizontal line is the mean thymic NK cell activity of normal B6AF1 mice.

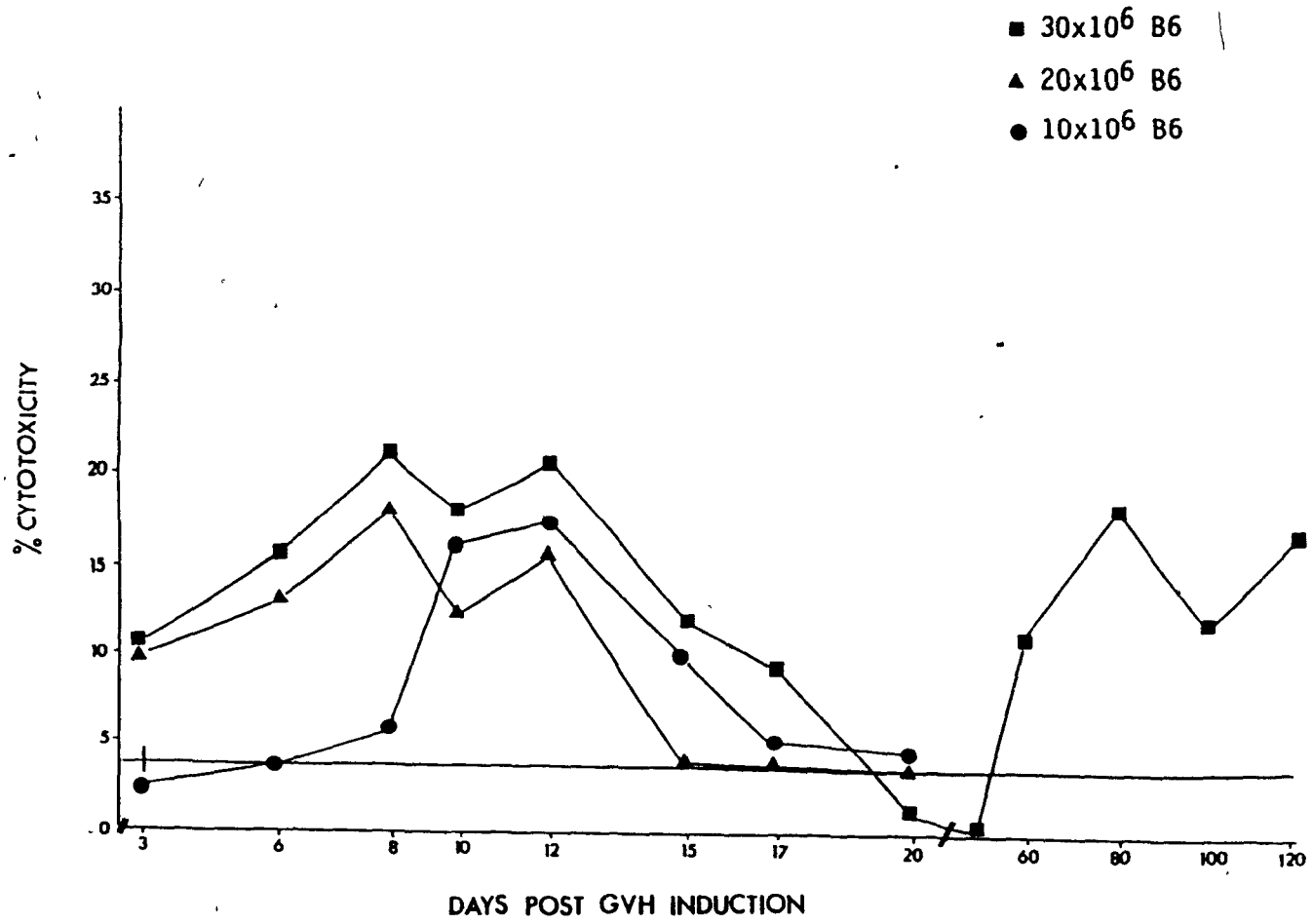


Figure 3.7. Kinetics of bone marrow NK cell activity of B6AF1 mice injected with different doses of B6 PLC against YAC targets. The experiment was repeated three times. Each experiment showed similar kinetics. Results of one experiment are shown. The horizontal line is the mean(±S.E) bone marrow NK cell activity of normal B6AF1 mice. The effector:target cell ratio used was 50:1.

Table 3.5 PFC/spleen response to SRBC of $B6AF_1$ mice injected with different doses of B6 PLC

Number and strain of parental cells injected to induce GVH reactions [a]	No. of mice /group	Mean no. of PFC/spleen $\pm$ S.E. $\times 10^{-3}$	% of normal PEC to SRBC
-	9	97.5 $\pm$ 4.5	100
30×10^6 (B6)	12	11.5 $\pm$ 1.6	11.7
20×10^6 (B6)	12	73.2 $\pm$ 2.6	75.0
10×10^6 (B6)	12	97.6 $\pm$ 5.8	100.1

(a): $B6AF_1$ mice were injected with SRBC on day 8 after PLC injection.

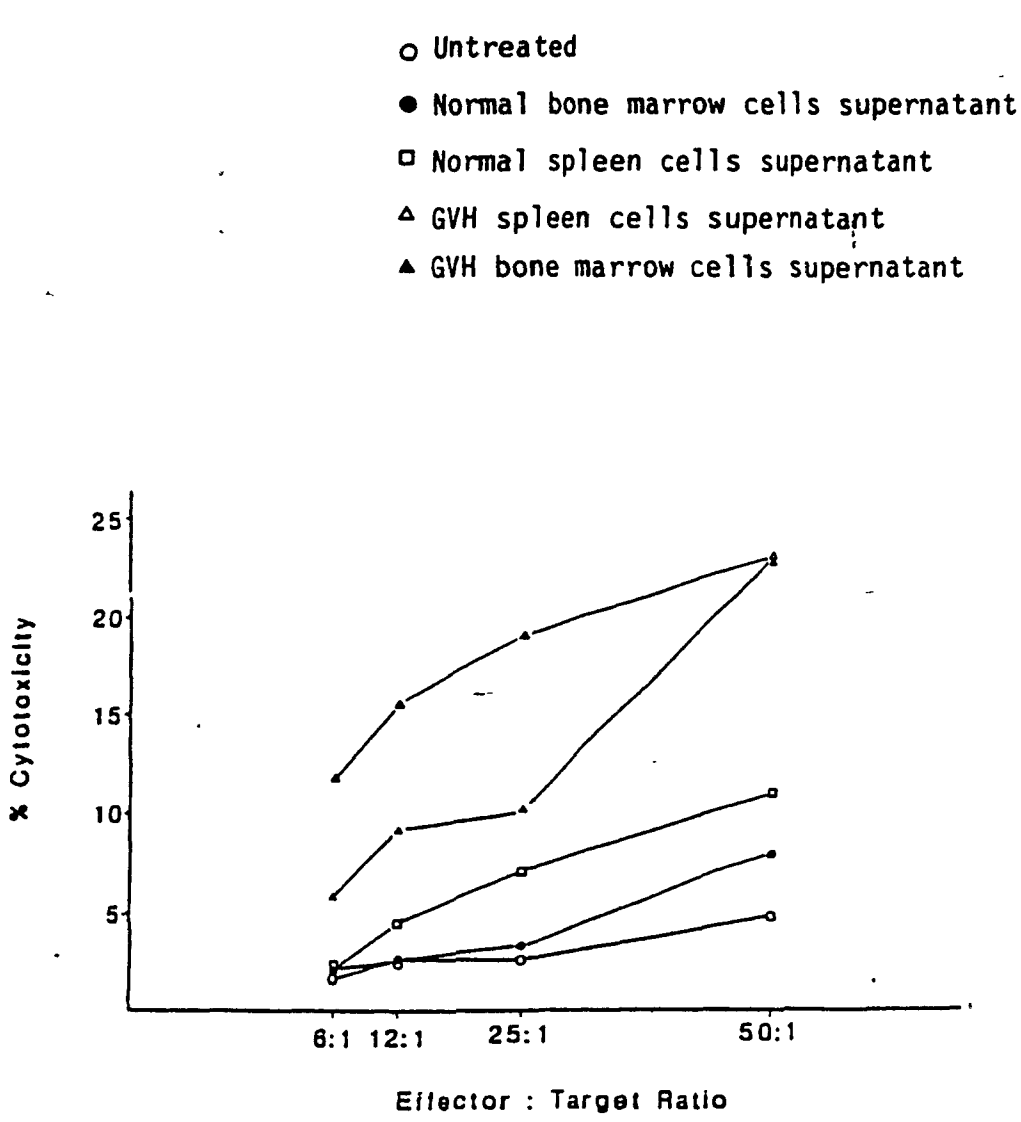


Figure 3.8. Effect of supernatants derived from normal and GVH reactive spleen and bone marrow cells on normal B6AF1 bone marrow NK cell activity against YAC targets. The supernatants were obtained from spleen and bone marrow cells of GVH-reactive B6AF1 mice 8 days after the injection of $30x10^6$B6 PLC. The experiments were performed two times. Each experiment gave similar results. Results of one experiment are shown.

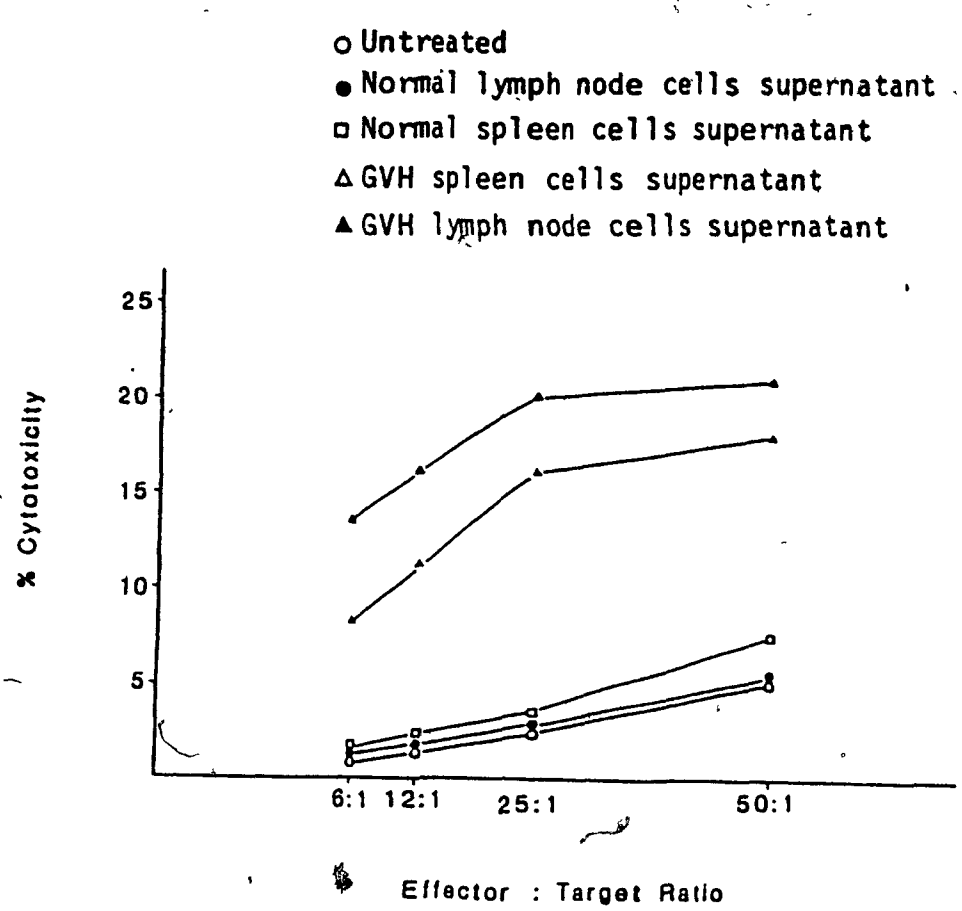


Figure 3.9. Effect of supernatants derived from normal and GVH-reactive spleen and lymph node cells on normal lymph node NK cell activity against YAC targets. The supernatants were obtained from spleen and lymph node cells of B6AF1 mice 8 days after the injection of 30×10^6 B6 PLC. The experiments were performed two times. Each experiment gave similar results. Results of one experiment are shown.

CHAPTER FOUR

THE RELATIONSHIP BETWEEN SPLENIC NK CELL ACTIVITY; T- AND B-CELL FUNCTIONS; AND THE DEVELOPMENT OF HISTOPATHOLOGICAL LESIONS IN THE NON-LYMPHOID ORGANS AFTER THE INDUCTION OF GVH REACTIONS.

4.1 INTRODUCTION

The studies presented in chapter 3 demonstrated that the magnitude of augmented splenic NK cell activity was dependent upon the number of PLC injected into B6AF1 hybrids However, GVH-associated suppression of the PFC response to SRBC was observed only in those F1 mice in which peak NK cell activity appeared earlier (F1 mice injected with $30x10^6$ B6 PLC) In this chapter we have further investigated (1) the relationship between the GVH induced augmented splenic NK cell activity and the GVH-induced suppression of splenic T-and B-cell function; (2) the relationship between the GVH-induced augmented splenic NK cell activity and the development and severity of GVH-induced histopathological lesions in non-lymphoid organs (liver, pancreas, and salivary glands).

4.2 EXPERIMENTAL DESIGN

The experimental protocol is outlined in figure 4.1 GVH reactions of different intensities were induced in B6AF1 hybrids by injecting different doses, 30, 20, , or $10x10^6$, of either parental strain A or B6 lymphoid cells. The relationship between splenic NK cell activity (YAC killing) and other "non-specific" cytotoxic activities, i.e , P-815 and Eb tumor target cell killing, splenic T- and B-cell function, and the development of histopathological alteration in the liver, pancreas, and salivary glands of these GVH-reactive mice was investigated. The rationale for studying the relationship

between splenic NK cell activity and the development of histopathological changes in the liver, pancreas, and salivary glands was three fold: (i) liver (Bain and Diener, 1972), pancreas (Seemayer et al., 1983), and salivary glands (Clancy et al., 1981) are characteristic non-lymphoid target organs for GVH reactions; (ii) the histopathological alterations in the liver, pancreas, and salivary glands appear concomitently with histopathological changes in lymphoid organs (Seemayer et al , 1977, 1978); (iii) recent studies have shown that generalized augmented NK cell activity correlates significantly with the development of GVH reactions in both man (Dokhlar et al , 1981) and mouse (Borland et al., 1983).

On different days after PLC injection, animals were randomly picked from a pool of B6AF1 mice that were injected with different doses of PLCs and from a pool of normal B6AF1 mice. These animals were sacrificed, their spleens and bone marrow (BM) cells were removed, and single cell suspensions were prepared. Splenocytes of individual spleen were divided into two aliquots. Cells in one aliquot were tested for the PFC response to SRBC and for mitogen response to Con A, PHA, and LPS. The cells in the other aliquot were pooled from several mice and were treated as described in chapter 2, (section 2.5) to obtain NK cells. The splenic NK cells obtained from each group of B6AF1 animals on day 16 post-PLC injection were further divided into two aliquots. Cells in one aliquot were treated with anti-thy 1.2 serum plus C', whereas the cells in the other aliquot were treated with C' and medium (RPMI-1640)

and served as controls. NK cells in both these aliquots were then tested for their ability to kill NK sensitive YAC-1 tumor targets and also NK insensitive P815 tumor targets Like the splenocytes, BM cells from each group were also pooled, partially purified, and tested for their ability to kill YAC targets in a 4 hour 51Cr release assay (section 2 7) The liver, pancreas, and salivary glands from each animal were removed and examined histologically.

4.3 RESULTS

4.3.1 DIRECT PFC RESPONSE TO SRBC

Tables 4 1a and 4.1b demonstrate the results of the PFC/spleen and PFC/10^6 spleen cell responses to SRBC, respectively, of B6AF1 mice injected with different doses of either strain A or B6 PLC. On day 4 after PLC injection, $30x10^6$ and $20x10^6$ strain A PLC induced much greater suppression than the eqvivalent cell dose of B6 strain donor cells On day 12 after PLC injections, the groups of B6AF1 mice that received either 30, 20, or $10x10^6$ A strain PLC, became totally suppressed. In contrast, total suppression of the PFC response to SRBC was observed only in the group of B6AF1 mice that received $30x10^6$ B6 PLC. The groups of B6AF1 mice that received $20x10^6$ B6 or $10x10^6$ B6 PLC demonstrated partial recovery and even enhancement, respectively, of the PFC response to SRBC on day 12 after PLC injection. These data are summarized in figures 4.2a and 4.2b.

4.3.2 CON A, PHA, AND LPS MITOGEN RESPONSIVENESS

Tables 4.2a and 4.2b demonstrate the Con A, PHA and LPS responses of B6AF1 mice injected with different doses of either strain A or B6 PLC. The mitogen data presented are from the same GVH-reactive mice for which the PFC data are presented in tables 4.1a and 4.1b. Table 4.2a demonstrates that on day 8 after PLC injections significant suppression of Con A, PHA, and LPS responses was observed in all groups of B6AF1 mice that received different doses of either strain A or B6 PLC. The degree of suppression of mitogen responses within a GVH combination was, however, dependent upon the number of PLC injected into B6AF1 mice. When equal numbers of strain A or B6 PLC were injected into B6AF1 mice, strain A lymphoid cells induced a greater degree of suppression of mitogen responses than did B6 parental strain lymphoid cells (Table 4.2a). Table 4.2b shows that on day 16 after PLC injection, B6AF1 mice that received either 30, 20, or $10x10^6$ A strain PLC were severely suppressed for Con A, PHA, and LPS responses. In contrast severe suppression of these mitogen responses was observed on day 16 in B6AF1 mice that received $30x10^6$ B6 PLC, while partial recovery of Con A, PHA, and LPS responses were observed in B6AF1 mice that received either $20x10^6$ B6 or $10x10^6$ B6 PLC. The mitogen data for Con A, PHA, and LPS are summarized in figures 4.3a, 4.3b, and 4.3c, respectively.

4.3.3 SPLENIC CELLULARITY

Table 4.3 shows the splenic mononuclear cell numbers of B6AF1 mice injected with different doses of either

B6 or A PLC. On day 8 after PLC injections, no significant differences were observed between the splenic mononuclear cell numbers of B6AF1 mice that received different doses of either B6 or A PLC and normal age-and sex-matched B6AF1 mice However, on day 16 after PLC injection, reduction in splenic mononuclear cell numbers was observed only in B6AF1 mice that received $30x10^6$ B6 PLC, but not in B6AF1 mice that received either $20x10^6$ B6 or $10x10^6$ B6 PLC. On the other hand, on day 16 after PLC injection, reduction in splenic cellularity was observed in the spleens of B6AF1 mice injected with $20x10^6$ A and $30x10^6$ A PLC, but not in the spleens of B6AF1 mice that received $10x10^6$ A PLC. These results are summarized in figure 4 4.

3.4 KINETICS OF SPLENIC NON-SPECIFIC CYTOTOXICITY DURING GVH REACTIONS: EFFECT OF DONOR CELL GENOTYPE

4.3.4.1 KINETICS OF SPLENIC NK CELL ACTIVITY

The kinetics of splenic NK cell activity in GVH reactive B6AF1 mice against YAC-1 (H-2a), a NK cell sensitive target, are shown in fig.4.5. Two distinct patterns of splenic NK cell activity are observed in B6AF1 mice injected with different doses of either strain A or B6 PLC. In animals that received either $30x10^6$ or $20x10^6$ A strain or $30x10^6$ B6 strain PLC, splenic NK cell activity peaked early, i.e., on day 8 after GVH induction, and then declined rapidly to normal levels by day 20-24 after GVH induction. In contrast, in mice that

received $10x10^6$ A and either $10x10^6$ or $20x10^6$ B6 strain PLC, peak splenic NK cell activity was delayed, i.e., peak NK activity was observed on day 16 after GVH induction, and then returned to normal levels by days 20-24 after GVH induction. Figure 4.5 also shows that in all groups of B6AF1 mice that received different doses of either A or B6 PLC the maximum increase in NK cell activity was dependent upon the number of cells injected. For example, 42%,31%, and 26% killing of YAC was observed in F1 mice injected with either 30, 20, or $10x10^6$ B6 PLC respectively. Similarly, 35% ,29%, and 22% killing of YAC targets was observed in F1 mice injected with either 30, 20, or $10x10^6$ A PLC, respectively. The return of splenic NK cell activity to normal and/or below normal levels was more rapid in B6AF1 mice that received different doses of A PLC. Moreover, B6 PLC induced greater overall splenic NK cell activity at each cell dose than the equivalent dose of A PLC (Figure 4.5). Inspite of the differences in NK cell activity observed between different groups of GVH-reactive mice, the kinetics of splenic NK cell activity in all experimental groups followed a similar pattern, namely, an augmentation followed by a decline to normal and/or near normal levels.

4.3.4.2 KINETICS OF BM NK CELL ACTIVITY

Figure 4.6 demonstrates the NK cell activity of BM cells of B6AF1 mice that were injected with different doses of either B6 or A PLC. As can be seen, in the groups of F1 mice that received different doses of B6 PLC , the bone marrow NK

cell activity reached a peak by day 8 after PLC injections, and then declined to near normal levels by day 16 after PLC injections. Moreover, in B6AF1 mice that received different numbers of B6 PLC the magnitude of peak NK cell activity in the BM was dependent upon the dose of PLC injected. NK cell activity in the BM of F1 mice that received different doses of A PLC was also found to be augmented on day 4 after GVH induction. However, unlike F1 mice that received B6 PLC , no further augmentation in this activity was observed (i e., no clear peak in NK cell activity was observed) beyond day 4 after GVH induction. Moreover, in B6AF1 mice that received different doses of A PLC all groups showed the same degree of augmentation of NK cell activity (i.e., no clear dose dependent effect was observed as was seen with B6 PLC). NK cell activity in these groups also declined to near normal levels by day 16 after PLC injections.

4.3.5 P-815 AND Eb TUMOR TARGET CELL KILLING BY GVH SPLENOCYTES

The splenic non-specific cytotoxicity data for P-815 (H-2d), an NK insensitive target, are presented in Figure 4.7. The influence of the genotype in activating effector mechanism(s) responsible for P-815 killing is evident. Marked P-815 cytolysis was observed in mice injected with different doses of A strain lymphoid cells, whereas negligible effects were observed in B6AF1 mice that received different numbers of B6 cells. Since YAC-1 is a lymphoma cell line of H-2a genotype

which expresses both H-2d and H-2k, and P-815 is a mastocytoma cell line of H-2d genotype (DBA/2 origin), we posited whether the differences observed might be related to target genotype To resolve this issue, Eb, a second H-2d genotype (DBA/2 origin) lymphoma cell line target, was employed under the same conditions. Figure 4.8 demonstrates the killing of Eb targets by day 8 GVH-reactive splenocytes at different E:T cell ratios. As can be seen, on day 8 after GVH induction, the killing of Eb targets by F1 mice treated with $30x10^6$ B6 PLC was greater than that of F1 mice injected with $30x10^6$ A strain PLC, at all E:T cell ratios tested. These results are similar to those presented in fig.4.5 for YAC-1 killing. These data suggest that, depending upon the donor cell genotype, either different cytotoxic mechanism(s) or different sub-populations of the same family of effector cells are activated during the course of GVH reactions.

4.3.6 EFFECT OF ANTI-THY 1.2 SERUM PLUS C'TREATMENT ON YAC AND P-815 TARGET CELL KILLING BY GVH SPLENOCYTES

To determine whether the cytolysis of YAC and P-815 targets could be attributable to cytotoxic T-lymphocytes (CTL), partially purified splenic cells from different groups of GVH-reactive B6AF1 mice were pretreated with anti thy 1.2 serum and C' prior to testing in a 4 hour 51Cr release cytotoxicity assay. Tables 4.4 and 4.5 show that pretreatment of partially purified spleen cells with anti-thy 1.2 serum plus C' failed to abrogate both YAC-1 and P-815 cytolysis respectively. These

data suggest that YAC (Table 4.4) and P-815 (Table 4.5) killing observed during the course of GVH reactions is not mediated by T-cells.

4.3.7 HISTOLOGY OF NON-LYMPHOID ORGANS DURING THE COURSE OF GVH REACTIONS

GVH-associated histopathological changes were studied in B6AF1 mice injected with different doses of either B6 or A PLC. Histopathological changes were graded as normal, mild or moderate-severe according to the degree of lymphocytic infiltrates and ductular pathalogical changes in the liver, pancreas, and salivary glands on days 8 and 16 after PLC injection. The degree of GVH-associated lesions were characterized as mild when the cellular infiltrates were observed only around the ducts. In tissues that were characterized as moderate-severe, the infiltrating cells spilled into the parenchyma of the organ.

On day 8 after PLC injections, no tissue alterations were recognized in the majority of B6AF1 mice receiving different doses of B6 PLC and $10x10^6$ A PLC (Table 4.6). However, in mice that received 20, or $30x10^6$ A PLC varying degrees of ductular injury associated with mononuclear cell infiltrates were present in the majority of mice at day 8 post-PLC injections (Table 4.6).

No lesions were noted on day 16 in animals receiving 10 or $20x10^6$ B6 PLC, whereas moderate-severe changes were appreciated

in those B6AF1 mice given $30x10^6$ B6 PLC. In contrast, moderate-severe ductular alterations associated with intense cellular infiltrates were manifest on day 16 in animals injected with either $20x10^6$ or $30x10^6$ A strain PLC. Only mild ductular injury associated with slight cellular infiltrates were noted in B6AF1 mice injected with $10x10^6$ A PLC (Table 4.6). Photomicrographs showing normal, mild, and moderate-severe lesions in liver, pancreas, and salivary gland are shown in Figures 4.9-4.17.

4.4 DISCUSSION

The data presented in this chapter demonstrate that GVH reactions induced in B6AF1 hybrids by three different doses (10, 20, and $30x10^6$) of A strain PLC, stimulate both NK cell cytotoxic activity and P-815 target cell killing, induce histopathological alterations in the liver, salivary glands, and pancreas, and cause profound immunosuppression of T- and B-cell function. In contrast, GVH reactions induced in F1 hybrids by similar numbers of B6 strain PLC resulted only in a marked increase in NK cell activity. However, slightly augmented P-815 target cell killing, histopathological changes, and immunosuppression of T- and B-cell function were observed only in F1 mice that received $30x10^6$ B6 PLC, whereas, 10 and $20x10^6$ B6 PLC were ineffective. The GVH-induced tissue lesions were apparent (day 8 after GVH induction) when both T- and B-cell function were severely suppressed and NK cell activity and P-815 target cell killing were at their peak or highly augmented.

The severity of histopathological lesions which developed later (day 16 post-GVH induction) correlated with an early temporal peak of NK cell activity and augmented P-815 target cell killing, but not with the overall NK cell activity

Depending upon the donor lymphoid cell genotype employed, different non-specific cytotoxic effector cell populations were activated to different degrees. The lysis of YAC-1 (H-2a) (Fig.4.5) and Eb (H-2d) (Fig 4.8) cell lines by splenocytes from mice injected with $30x10^6$ parental A or B6 strain lymphoid cells was similar, the greatest effect being observed in recipients of B6 PLC. In contrast, highly augmented P-815 (H-2d) cytolysis (Fig 4.7) was observed in mice which received $30x10^6$ A strain PLC. The effector cells mediating the lysis of YAC, P-815, and Eb targets appear to be highly efficient since lysis of these targets was observed in a 4 hour assay. These results suggest that the cells mediating the lysis of YAC, P-815, and Eb targets represent either separate effector populations (eg., NK, M0 etc.) with different specificities for target cell recognition or sub-populations (eg., NK1, NK2, etc.) of the same effector cell population. The activation of different non-specific effector cells (eg., NK cells and M0) during GVH reactions and differential susceptibilities of targets to lysis by cells derived from GVH animals have been previously described (Ptak et al., 1975; Fung and Sabbadini, 1976; Hansen et al., 1982). Similarly, heterogeneity within the NK cell population has also been reported (Tai et al., 1980; Lust et al., 1981).

The NK effector cells involved in the lysis of YAC targets, like the effector cells mediating the lysis of P-815 targets, were not eliminated by anti-thy 1.2 serum plus C' treatment (Tables 4.4 and 4.5), suggesting that these effector cells are not T-cells. Two recent reports showing NK cell cytotoxicity during the course of GVH reactions have produced conflicting data on P-815 killing. Pattengale et al. (1983) reported P-815 target cell killing in a GVH system (B6--->B6xDBA/2F1) in which severe GVH disease was induced. In their study, (Pattengale et al., 1983) cells affecting the P-815 target cell lysis expressed Thy-1 antigens. However, Borland et al. (1983) reported no spontaneous P-815 killing in a different GVH combination, that is, CBA into CBAxBalb/c. The basis for these discrepencies, in relation to our study, is not entirely clear. Conceivably the strains of animals employed contribute to these divergent findings. This interpretation appears plausible since, in our study, the activation of effectors for P-815 targets was observed only in recipients of A strain lymphoid cells. In one of the GVH combinations in which P-815 effector cells bore thy 1 antigens (B6-->B6xDBA/2) (Pattengale et al., 1983), the donor anti-host cytotoxic-T-lymphocyte response could have been directed against the H-2d haplotype (DBA/2 and P-815 are of identical H-2 haplotypes). In our GVH combination (A-->B6AF1) in which P-815 target cell lysis occured, the parent anti-F1 CTL response would have been directed against the B6 (H-2b) haplotype. However, the killing of Eb, which is of the same haplotype as P-815, was similar to

YAC killing rather than the P-815 killing in the two GVH combinations employed in the present study. Therefore, we propose that cytotoxic T-lymphoctes are not principally involved in this process.

The origin of effector cells for P-815 targets was not established in the present study. However, it is known that MO/monocytes are activated during the course of GVH reactions (Ptak et al., 1975; Fung and Sabbadini, 1976; Hansen et al., 1982). Although techniques were employed to remove adherent cells, MO/monocyte contamination of the effector population remains a possibility, especially since P-815 targets are sensitive to activated MO/monocyte (Roder et al., 1979).

Analysis of data relating to non-specific cytotoxic cell activity and the initial development and subsequent severity of tissue injury during the course of GVH reactions yields several interesting associations. Firstly, when $30x10^6$ A and $20x10^6$ A strain PLC were employed to induce GVH reactions, NK cell activity and P-815 target cell killing peaked or were highly augmented by day 8 post-GVH induction and histopathological alterations first appeared at the same time. These F1 hybrids subsequently developed severe GVH-associated pathological alterations. Secondly, when $10x10^6$ A strain PLC were employed to induce GVH reactions, the appearance of peak NK cell activity and P-815 target cell killing was delayed (day 16 after GVH induction); the initial appearance of histopathological lesions was also delayed and the tissue alterations were minimal (graded as mild). Thirdly, in F1

recepients of $30x10$ $B6^6$ PLC , peak NK cell activity and slightly augmented P-815 target cell killing (8-10%) were observed 8 days after GVH induction, yet tissue alterations that developed later during the course of GVH reactions (day 16 post-GVH induction) were of moderate-severe intensity. Fourthly, in F1 recipients of $20x10^6$ B6 and $10x10^6$ B6 PLC, a significant increase in NK cell activity was observed which peaked at day 16 after GVH induction; nevertheless, P-815 target cell lysis was not observed, nor did GVH-induced histopathological lesions develop.

The results described above show that moderate to severe lesions developed in mice that displayed an early peak in NK cell activity and an augmented P-815 target cell killing. In contrast, in those groups of F1 mice in which NK cell activity and/or P-815 killer cell activity peaked later, tissue alterations were either mild or not detectable. The findings suggest that the magnitude of tissue injury which develops during the course of GVH reactions may be related to the rapidity with which NK cell activity peaks, but not with the overall augmented NK cell activity, and, as well, may be related to an increase in activity of cytotoxic cells capable of killing P-815 target cells. The data presented in this chapter do not address the question of whether interactions between NK cells and P-815 target cell effectors are responsible for the more severe tissue lesions observed in some GVH combinations. The question regarding the role of NK cells and P-815 effector cells in GVH-associated tissue damage is

addressed in detail in chapter 6. However, a correlation between the early appearance of NK cell activity and the development of acute GVH disease in recipients of allogeneic bone marrow transplants has been described (Dokhelar et al., 1981). The data presented in this study support this observation and also suggest that the rate of augmentation of NK cell activity may predict the magnitude of tissue injury associated with GVH reactions.

The effect of donor genotype on the BM NK cell activity following GVH reaction induction has not been reported thus far The data presented in figure 4 6 demonstrate the effects of donor genotype on the kinetics of BM NK cell activity As can be seen, B6 PLC at all doses induce BM NK cell activity which peaks on day 8 after PLC injection. However, the magnitude of peak BM NK cell activity is dependent upon the number of PLC injected. Furthermore, the kinetics of BM NK cell activity is similar to that observed in the spleen, namely an augmentation followed by a decline in NK cell mediated killing of YAC targets. In contrast, in the A-->B6AF1 GVH combination, no PLC dose-dependent effect was observed that influenced the magnitude of BM peak NK cell activity, as was witnessed when different doses of B6 PLC were injected into B6AF1 mice. Moreover, no sharp peak was noted. Once NK cell activity in the BM was augmented (day 4) it remained slightly augmented and then started to decline slowly with time. In this chapter we also demonstrate that the magnitude as well as overall augmented splenic NK cell activity following GVH reaction

induction are dependent upon the strain and number of donor cells injected. A similar dependence of splenic NK cell activity on donor cell genotype and dose of donor cells injected has previously been reported (Pattengale et al., 1983; Ghayur et al., 1984; Varkila and Hurme, 1985a). It is of interest to note that NK cells originate in the BM and are then transported to the periphery (spleen) (Haller and wigzell, 1977; Haller et al., 1977). Thus, in light of the data presented on BM NK cell activity, it is plausible that the differences observed in the kinetics and overall augmentation of splenic NK cell activity, between animals receiving PLC of different genotypes may reflect the ability of donor cells to stimulate production and/or release of NK cells from the BM to the periphery. If this is the case then our data would suggest that the greater degree of overall NK cell activity observed in the spleens of B6AF1 mice injected with $30x10^6$ B6 and $20x10^6$ B6 PLC, as compared to 30 and $20x10^6$ A PLC, may reflect a substantial contribution from the host bone marrow.

A comparison of non-specific effector cell activity (Figures.4.5 and 4.7) and T and B-cell function (Table 4.1a,b and 4.2a,b) in B6AF1 hybrids which develop moderate-severe histopathological lesions shows that the NK cell and P-815 effector cell activity reach their peak and remain augmented for several days after T and B-cell activity have been severely suppressed. The data in this report also show that in groups of B6AF1 mice that display an early rapid augmented NK cell activity (and severe lesions) severe suppression of T and B-

cell function is observed early after GVH reaction induction Thus, a clear dissociation between NK cell activity and T and B-cell function is observed early after GVH reaction induction van Elvan et al. (1981) have demonstrated that the ability of donor cells to generate suppression following GVH induction correlates with their capacity to induce severe GVH reactions The data presented in this report support the work of van Elvan et al (1981) and furthermore demonstrate that an early rapid augmentation of NK cell activity correlates with the severity of GVH reactions as assessed by development of histopathological lesions in non-lymphoid organs. The fact that the peak NK cell activity is observed at a time when both the T and B-cell function are severely suppressed suggests that the specific and non-specific immune responses have different mechanism(s) for suppression and/or activation. It is possible that early after GVH reaction induction the specific and non-specific immune responses play different roles at different stages of the GVH reaction.

It is generally assumed that the GVH-associated tissue damage is mediated by cytotoxic T-lymphocytes. Our results showing that the initial appearance and later severity of GVH-induced lesions correlates with the peak NK cell activity and with the time when peak NK cell activity is reached after GVH induction, respectively,(but not with the T and B-cell function), would suggest that NK cells may play some role in the induction of GVH-associated tissue damage. An active role for NK cells in GVH induced tissue damage is a likely

possibility since recent studies have reported that: cytotoxic T-lymphocyte (CTL) responses are severely suppressed as early as day 4 after GVH induction (Shearer and Polisson, 1980); CTLs may not be essential in GVH induced pathogenesis (Hamilton, 1984; Judas and Peck, 1983; Mason, 1981); and that antibodies against NK cells prevent GVH induced mortality (Charley et al., 1983; Varkila and Hurme, 1985). Thus, in light of the studies cited above, the results presented in chapter 3 regarding production by GVH-reactive lymphoid cells of factor(s) which induced NK cell like activity in normal lymphoid cells, and the data presented in this chapter, we propose that specific T-cell immune responses may be involved in the activation and/or recruitment of non-specific (NK-like) effector cells which may then either directly or indirectly be involved in the GVH-induced tissue damage. Whether the host and/or donor NK cells are activated and/or recruited following GVH induction is not clear from the data presented in this chapter.

The fact that 10 and 20×10^6 B6 PLC were not as efficient as a similar number of A PLC in inducing tissue damage and/or immunosuppression may be due to the rather rapid elimination of B6 cells from B6AF1 mice by the phenomenon of hybrid resistance. We have observed an early rapid elimination (within 36-48 hours) of fluorescein labelled B6 PLC , but not A PLC, from B6AF1 hybrids (Peres et al, 1983; Peres et al, J.Immunol. in press). Moreover, Shearer and Pollison (1980, 1981, and 1983) have previously reported that greater numbers of B10 (H-2b) PLC ,as compared to B10.A (H-2a) PLC, are

required to overcome the hybrid resistance mechanism(s) in B10xB10.A (H-2bxH-2a)F1 mice. These workers have demonstrated that $20x10^6$ B10 PLC (similar to the genotype of the B6 PLC used in the present study) were unable to induce complete suppression of CTL generation when injected into B10xB10.A F1 mice. In contrast $20x10^6$ B10.A PLC (the same H-2 genotype of A PLC used in the present study) induced severe suppression of CTL generation in B10xB10.A F1 mice. Shearer and Pollison (1980,1981) further showed that $40x10^6$ B10 PLC were sufficient to overcome the hybrid resistance mechanism of B10.B10 A F1 and induce complete suppression of CTL generation. The data presented in this chapter suggest that although the injection of $20x10^6$ and $10x10^6$ B6 PLC cause a significant, but delayed, increase in NK cell activity, these cell doses are unable to induce severe immunosuppression and moderate-severe lesions However, $30x10^6$ B6 PLC, a dose which is sufficient to overcome the F1 hybrid resistance, cause an early rapid increase in NK cell activity as well as severe immunosuppression and moderate-severe tissue lesions. Collectively, these data would suggest that when $10x10^6$ and $20x10^6$ B6 PLC are injected into B6AF1 mice the required critical number of donor cells may not acquire sufficient tenure to promote the development of complete immunosuppression and histopathological lesions.

It would, therefore, appear that although non-specific cytotoxic mechanism(s) of host and/or donor origin may be activated following GVH-induction, the development of histopathological lesions may be dependent upon a major donor

cell contribution. If donor and host non-specific cytotoxic cells are activated during GVH reactions, but the donor non-specific and not the host non-specific cytotoxic cells (NK cells) may be responsible for the tissue injury, an interesting question of specificity of the effector cells for F1 target cells is raised. To investigate this question of specificity for GVH-induced tissue damage different parent into F1 hybrid combinations of beige/beige (a mutant with a defect in NK cell activity) and +/beige mice were employed. The data are presented in chapter 6.

Thus, in this chapter we have shown a correlation between the appearance of early peak of NK cell activity and the development of moderate-severe lesions in non-lymphoid organs after GVH induction. However, this correlation was based upon studying NK cell activity in one organ (spleen) and the development of lesions in different organs (liver,pancreas, and salivary glands). In the following chapter we present data on NK cell activity and the development of lesions in the same organ (thymus).

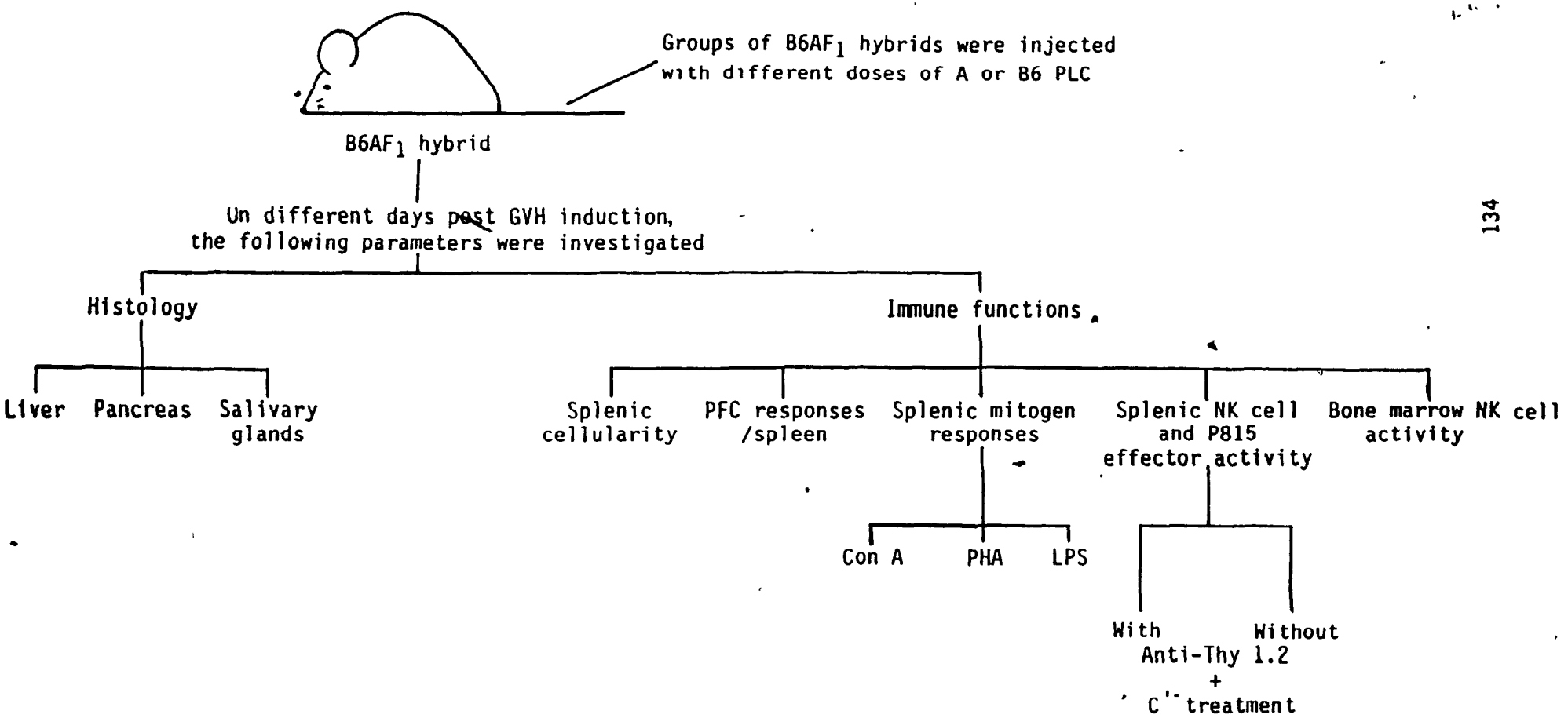


Figure 4.1 Experimental design used to investigate the relationship between splenic NK cell activity, T-and B-cell functions, and the development of histopathological alterations in the non-lymphoid organs.

Table 4.1a. PFC/spleen response to SRBC of B6AF1 mice injected with different doses of either A or B6 PLC.

Number and Strain of PLC injected[a]	Experiment Number	PFC ± S.E/Spleen x 10^{-3} (% of normal response) Days post GVH induction 4	12
	1	114.3 ± 4.5	98.0 ± 5.5
	2	183.6 ± 27.0	180.2 ± 7.9
$30x10^6$ A	1	5.3 ± 1.2 (4.6)	0
	2	40.3 ± 2.3 (21.9)	0
$20x10^6$ A	1	22.3 ± 5.9 (19.5)	0
	2	49.0 ± 12.6 (26.7)	0
$10x10^6$ A	1	61.7 ± 18.6 (53.9)	1.4 ± 1.4 (1.5)
	2	86.1 ± 10.9 (46.9)	2.2 ± 1.9 (1.2)
$30x10^6$ B6	1	54.0 ± 4.9 (47.2)	0
	2	90.2 ± 25.0 (49.1)	0
$20x10^6$ B6	1	64.7 ± 3.8 (56.6)	73.0 ± 5.2 (82.0)
	2	104.9 ± 14.0 (57.1)	-b
$10x10^6$ B6	1	64.1 ± 24.8 (56.0)	134.5 ± 16.6 (151.1)
	2	124.2 ± 16.1 (67.6)	297.3 ± 26.6 (164.9)

a -Three animals/group in experiment no.1 and four animals/group in experiment no.2 were randomly selected from a pool of mice. The data is presented as mean ± S.E.

-b = not done

Table 4.1b. PFC/10^6 spleen cell response to SRBC of B6AF1 mice injected with different doses of either A or B6 PLC.

Number and Strain of PLC injected[a]	Experiment Number	PFC ± S.E/10^6 spleen cells (% of normal response)	
		Days post GVH induction	
		4	12
	1	845.3 + 52.7	743.3 + 91.9
	2	1429.5 + 19.6	1678.3 + 107.4
$30x10^6$ A	1	40.6 + 10.2 (4.8)	0
	2	260.0 + 23.0 (16.7)	0
$20x10^6$ A	1	191.0 + 69.4 (22.6)	0
	2	333.9 + 83.7 (23.3)	0
$10x10^6$ A	1	583.4 + 278.2 (69.0)	12.9 + 12.9 (1.7)
	2	612.0 + 120.0 (42.8)	14.9 + 12.6 (0.8)
$30x10^6$ B6	1	383.2 + 14.5 (45.3)	0
	2	655.5 + 170.8 (45.8)	0
$20x10^6$ B6	1	516.3 + 7.6 (61.0)	525.4 + 84.1 (70.6)
	2	880.5 + 159.0 (61.6)	-b
$10x10^6$ B6	1	566.8 + 294.4 (65.8)	975.3 + 48.1 (131.2)
	2	1149.4 + 218.3 (80.4)	2015.1 + 319.0 (120.0)

a Three animals/group in experiment no.1 and four animals/group in experiment no.2 were randomly selected from a pool of mice. The data is presented as mean ± S.E.

-b = not done

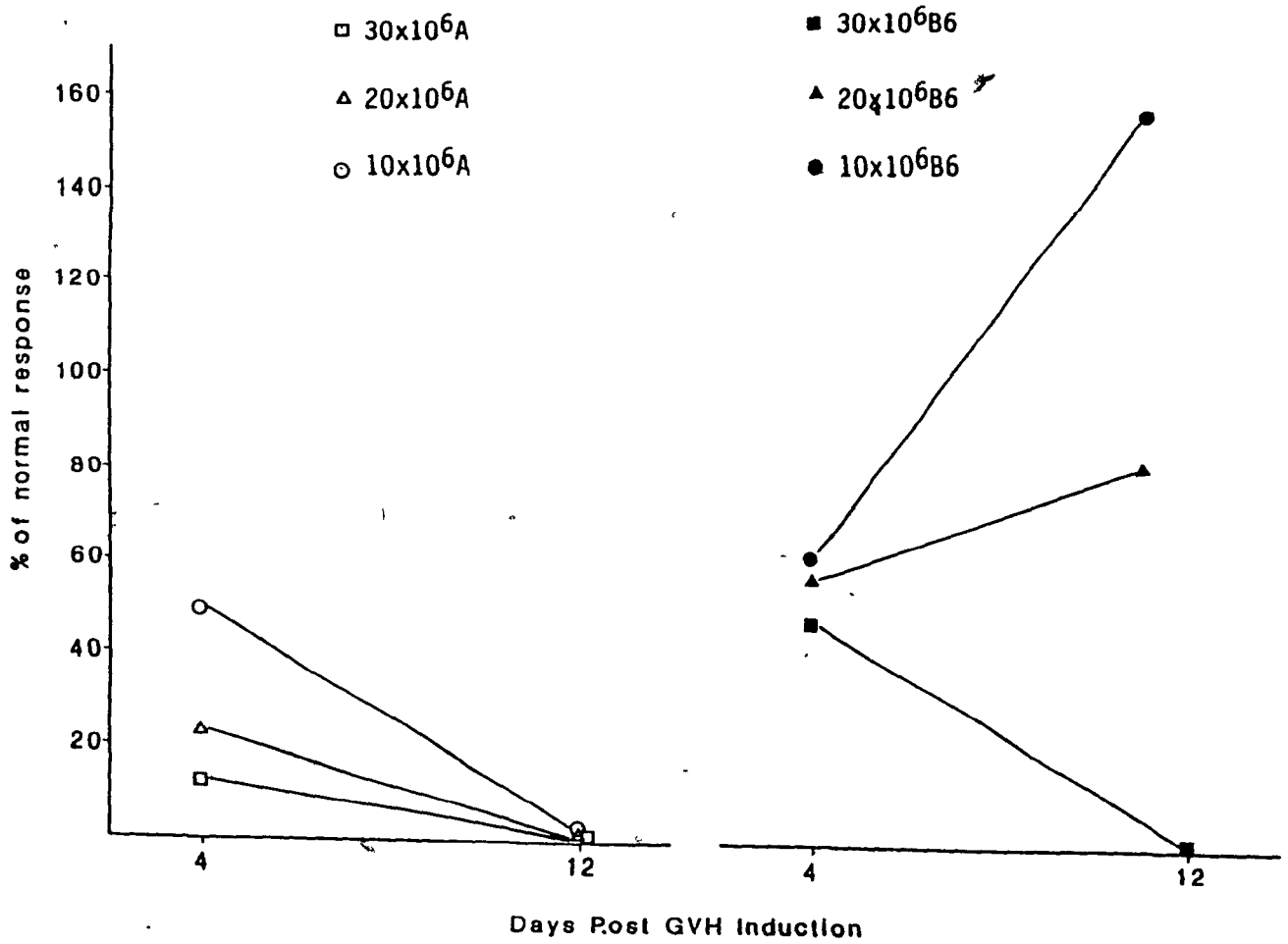


Figure 4.2a Summary of the PFC/spleen response to SRBC of B6AF1 mice injected with different doses of either A or B6 PLC. The % of normal response shown is the mean of two experiments (see table 4.1a)

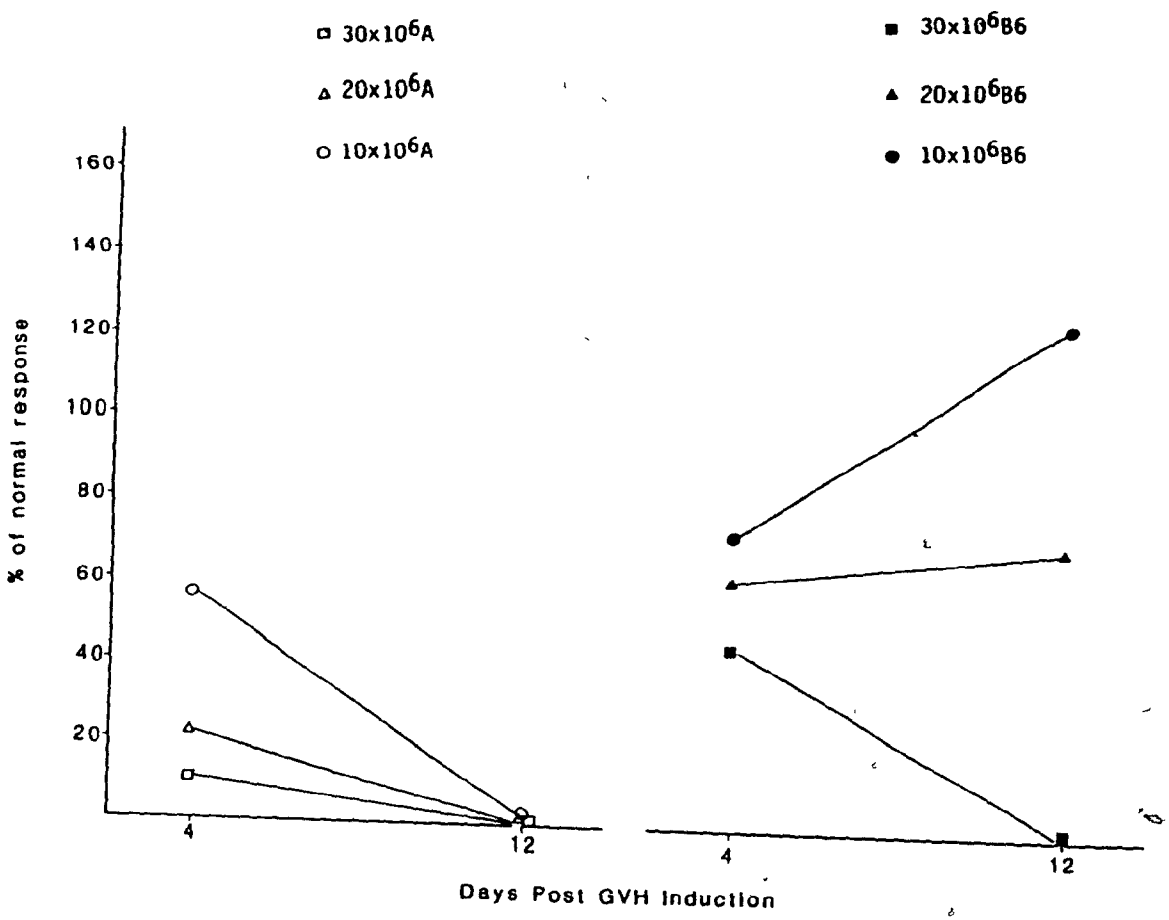


Figure 4.2b Summary of the PFC/10^6 spleen cell response to SRBC of B6AF1 mice injected with different doses of either A or B6 PLC. The % of normal response shown is the mean of two experiments (see table 4.1b).

Table 4.2a .Splenic ConA, PHA, and LPS mitogen responses of B6AF1 mice on day 8 after the injection of different doses of either A or B6 PLC.

Number ($x10^{-6}$) and Strain of parental lymphoid cells injected[a]	Expt. #	Mitogen Responses mean net cpm ± S.E. x 10^{-3} (% of normal response)		
		ConA	PHA	LPS
-	1	220.0 ± 19.0	133.7 ± 0.7	49.21 ± 0.74
	2	284.3 ± 13.2	235,1 ± 22.1	N.D.
30 A	1	5.2 ± 2.6 (2.39)	3.4 ± 2.0 (2.5)	0.8 ± 0.2 (1.7)
	2	5.3 ± 1.2 (1.89)	3.9 ± 0.9 (1.5)	N.D.
20 A	1	11.1 ± 3.6 (5.06)	9.4 ± 3.5 (7.0)	1.9 ± 1.2 (3.8)
	2	21.4 ± 3.6 (7.54)	20.8 ± 5.3 (8.7)	N.D.
10 A	1	36.4 ± 4.9 (16.57)	20.0 ± 5.0 (14.7)	19.5 ± 1.3 (39.8)
	2	49.4 ± 2.0 (17.50)	55.4 ± 11.7 (23.5)	N.D.
30 B6	1	9.2 ± 2.8 (4.26)	10.9 ± 2.8 (8.2)	1.2 ± 0.5 (2.4)
	2	18.2 ± 4.8 (6.50)	35.5 ± 12.9 (15.1)	N.D.
20 B6	1	27.9 ± 10.6 (12.69)	26.6 ± 1.0 (19.9)	8.2 ± 1.7 (16.8)
	2	75.5 ± 8.7 (26.75)	81.3 ± 4.6 (34.5)	N.D.
10 B6	1	61.8 ± 4.5 (28.11)	73.0 ± 12.1 (54.7)	10.7 ±-2.03 (21.8)
	2	117.3 ± 5.1 (41.50)	95.8 ± 11.8 (40.7)	N.D.

[a] Three animals/group in Experiment no. 1 and four animals/group in Experiment no. 2 were randomly selected from a pool of mice The data is presented as mean netcpm ± S.E. x 10- .[3]

Table 4.2b. Splenic ConA, PHA, and LPS mitogen responses of B6AF1 mice on day 16 after the injection of different doses of either A or B6 PLC.

Number ($\times 10^6$) and Strain of parental lymphoid cells injected[a]	Expt. #[a]	Mitogen Responses mean net cpm ± S.E. $\times 10^{-3}$ (% of normal response)		
		ConA	PHA	LPS
-	1	236.0 ± 14.4	143.3 ± 4.5	45.1 ± 4.1
-	2	252.4 ± 19.8	212.2 ± 3.0	N.D.
30 A	1	1.9 ± 1.7 (0.8)	0.6 ± 0.7b(0.6)	0.8 ± 0.6 (1.9)
	2	0.4 ± 0.8[b](0.1)	1.2 ± 0.8 (0.6)	N.D.
20 A	1	2.5 ± 2.0 (1.0)	2.1 ± 1.8 (1.4)	1.4 ± 0.7[b](3.1)
	2	1.3 ± 0.8 (0.5)	1.7 ± 0.8 (0.8)	N.D.
10 A	1	24.4 ± 17.7 (10.3)	13.7 ± 14.5[b](9.5)	0.6 ± 4.5[b](1.3)
	2	6.6 ± 1.7 (2.6)	11.7 ± 2.8 (5.5)	N.D.
30 B6	1	13.0 ± 3.6 (5.5)	10.0 ± 1.9 (7.0)	0.6 ± 0.7[b](1.4)
	2	4.5 ± 1.4 (1.7)	4.1 ± 1.0 (1.9)	N.D.
20 B6	1	101.5 ± 33.5 (43.0)	78.8 ± 18.8 (54.9)	26.4 ± 3.5 (58.5)
	2	167.8 ± 8.5 (66.4)	149.8 ± 4.6 (70.5)	N.D.
10 B6	1	131.3 ± 9.3 (55.6)	109.9 ± 8.9 (76.6)	26.4 ± 2.8 (58.6)
	2	N.D.	N.D.	N.D.

a Three animals/group in Experiment no. 1 and four animals/group in Experiment no. 2 were randomly selected from a pool of mi... The data is presented as mean net cpm ± S.E. $\times 10^{-3}$.

b Some individual mice in these groups gave a negative net cpm value.

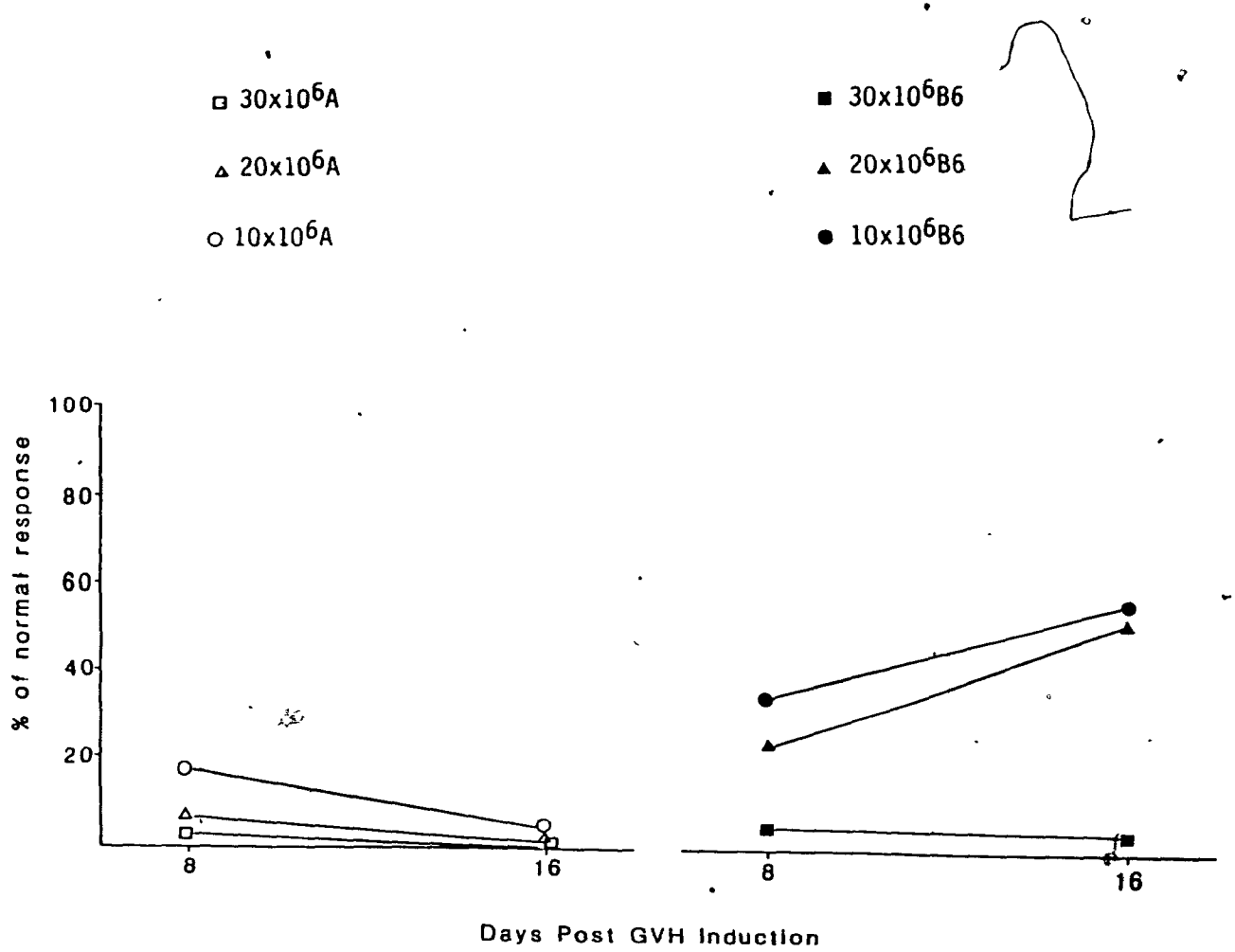


Figure 4.3a Summary of the splenic ConA responses of B6AF1 mice on days 8 and 16 after the injection of different doses of either A or B6 PLC. The % of normal responses presented is the mean of two experiments on each day (see tables 4.2a and 4.2b).

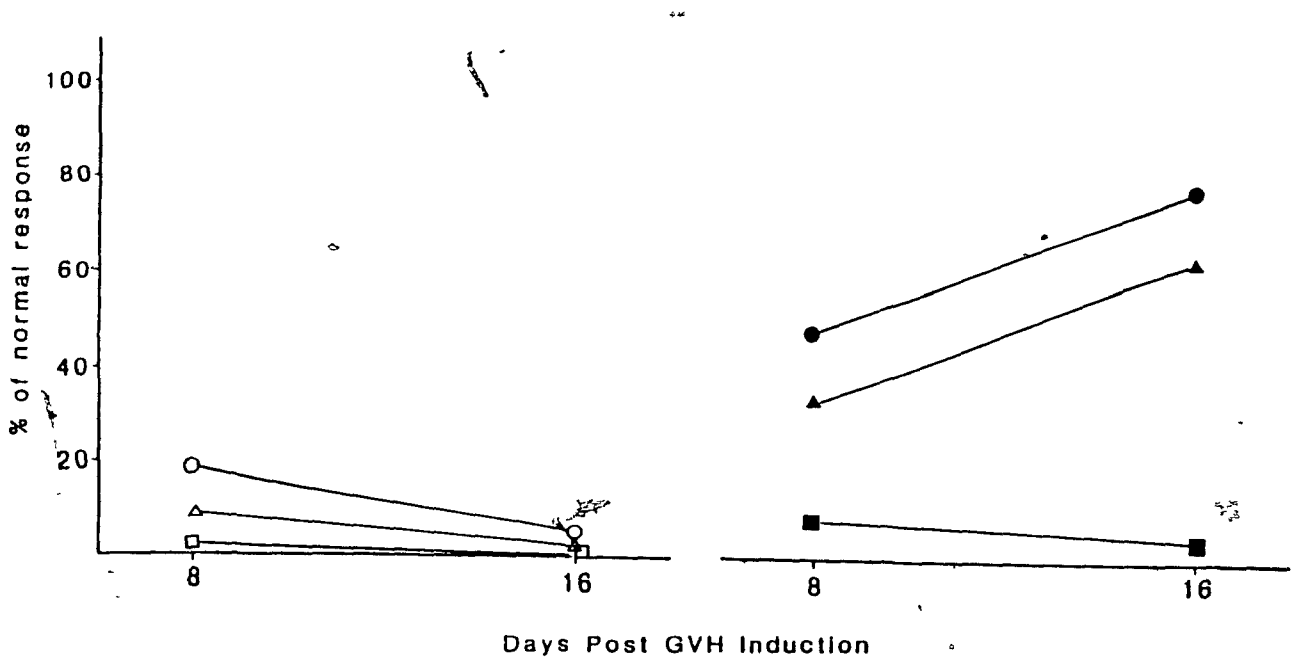


Figure 4.3b Summary of the splenic PHA responses of B6AF1 mice on days 8 and 16 after the injection of different doses of either A or B6 PLC. The % of normal responses shown is the mean of two experiments on each day (see tables 4.2a and 4.2b).

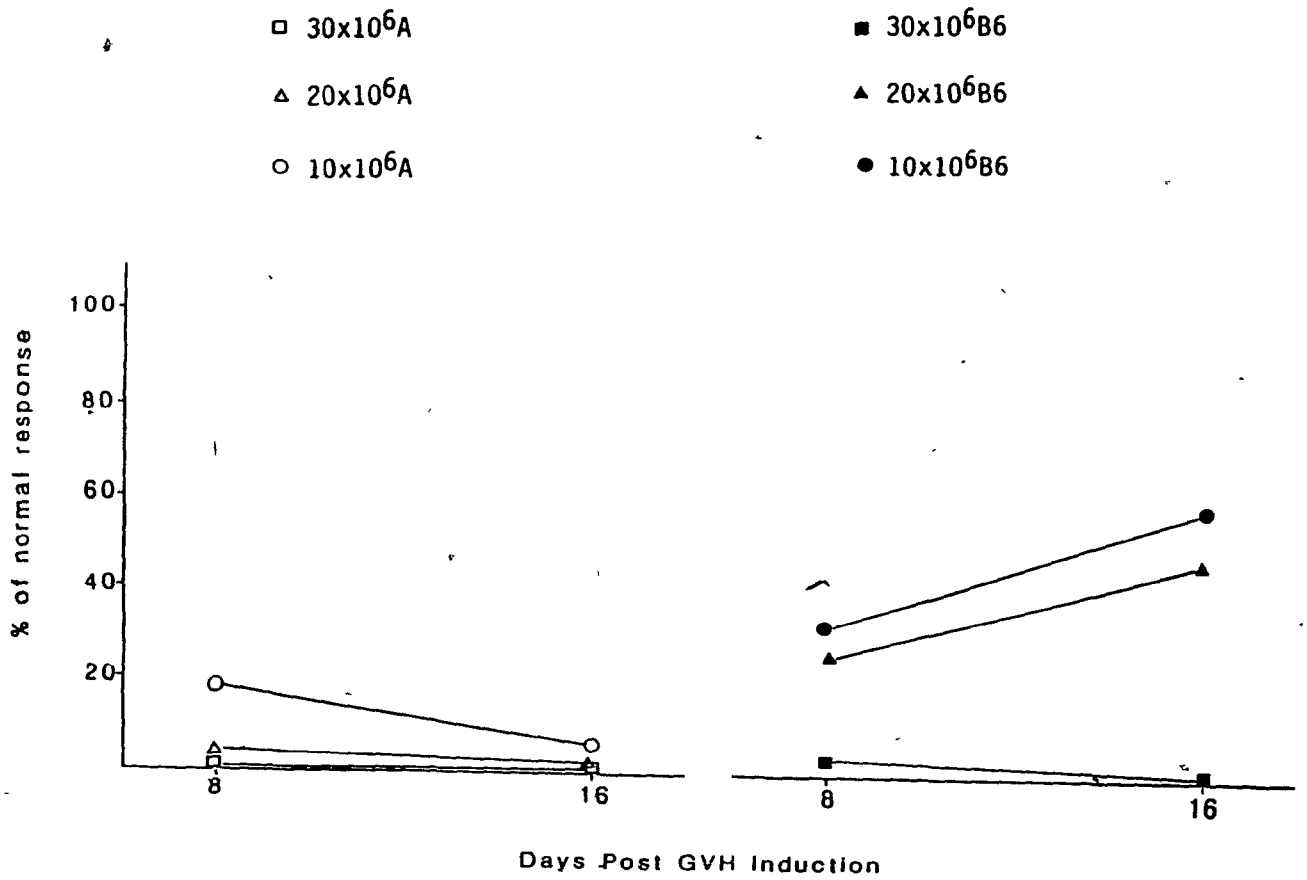


Figure 4.3c Summary of splenic LPS responses of B6AF1 mice on days 8 and 16 after the injection of different doses of either A or B6 PLC. The % of normal responses shown were calculated from the data shown in tables 4.2a and 4.2b.

Table 4.3 Splenic nucleated cell numbers of B6AF1 mice injected with different doses of either A or B6 PLC.

Number and strain of PLC injected	Experiment number	Cellularity ± S.E x 10^6 Days post GVH induction	
		8	16
	1	138.0 ± 19.8	139.5 ± 3.1
	2	128.0 ± 17.1	111.0 ± 8.8
$30x10^6$ A	1	130.8 ± 5.8 (102.0)	49.0 ± 9.9 (37.9)
	2	172.0 ± 13.5 (132.4)	49.8 ± 6.7 (49.8)
$20x10^6$ A	1	96.6 ± 34.1 (74.8)	55.3 ± 5.6 (42.8)
	2	137.5 ± 20.9 (106.4)	67.8 ± 2.4 (52.5)
$10x10^6$ A	1	87.0 ± 5.6 (67.4)	136.0 ± 15.6 (105.3)
	2	146.2 ± 13.6 (113.2)	139.8 ± 8.2 (108.3)
$30x10^6$ B6	1	140.5 ± 9.5 (108.8)	44.0 ± 4.7 (34.0)
	2	135.6 ± 6.6 (104.5)	35.5 ± 1.7 (27.4)
$20x10^6$ B6	1	125.3 ± 8.8 (97.0)	144.5 ± 17.6 (111.9)
	2	123.0 ± 9.2 (95.2)	-b
$10x10^6$ B6	1	133.0 ± 5.8 (103.0)	137.0 ± 10.4 (106.0)
	2	111.7 ± 10.7 (86.5)	154.8 ± 13.8 (119.9)

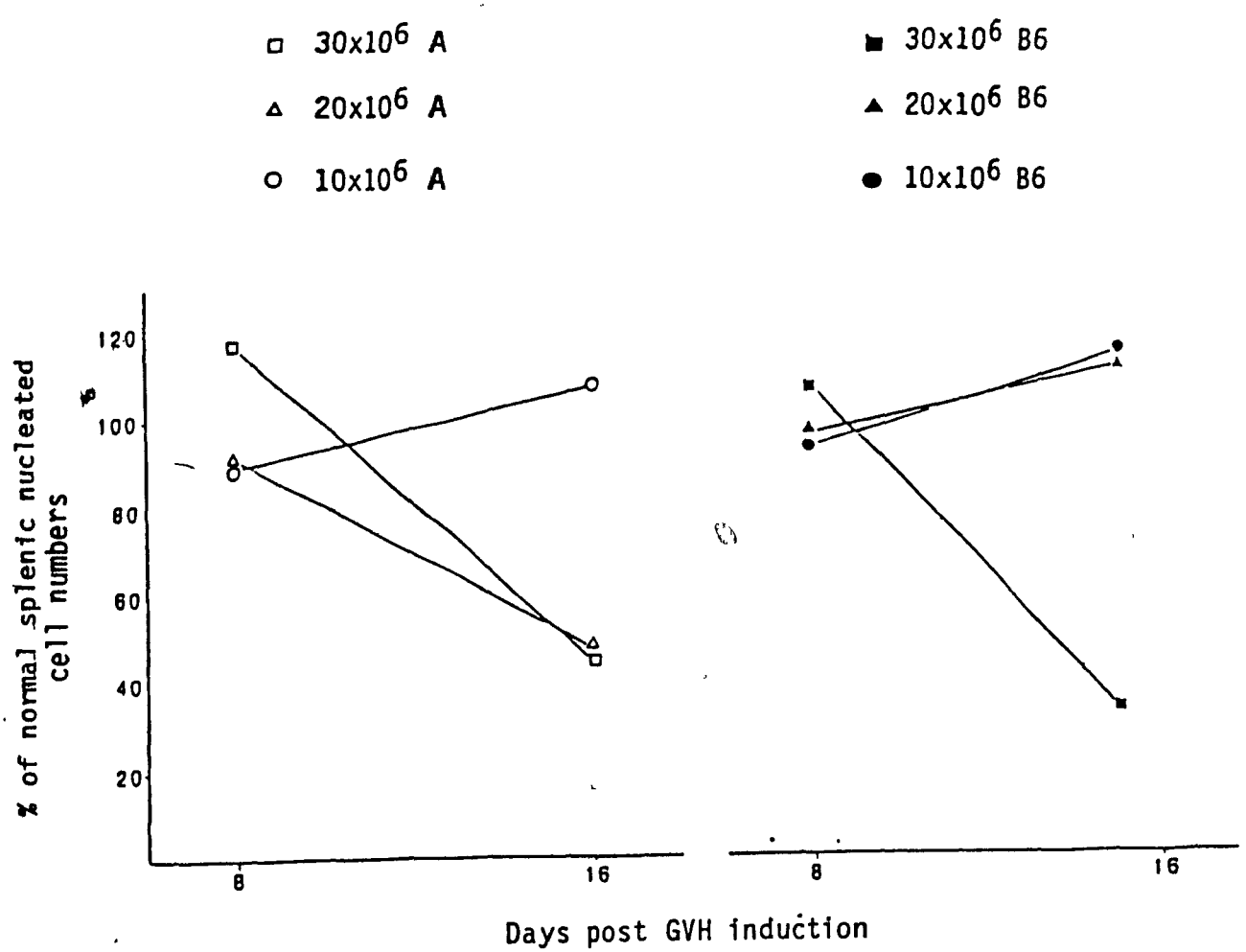


Figure 4.4. Summary of the splenic nucleated cell numbers of B6AF1 mice on days 8 and 16 after the injection of different doses of either A or B6 PLC. The % of normal splenic nucleated cell numbers shown are the mean of two experiments (see table 4.3).

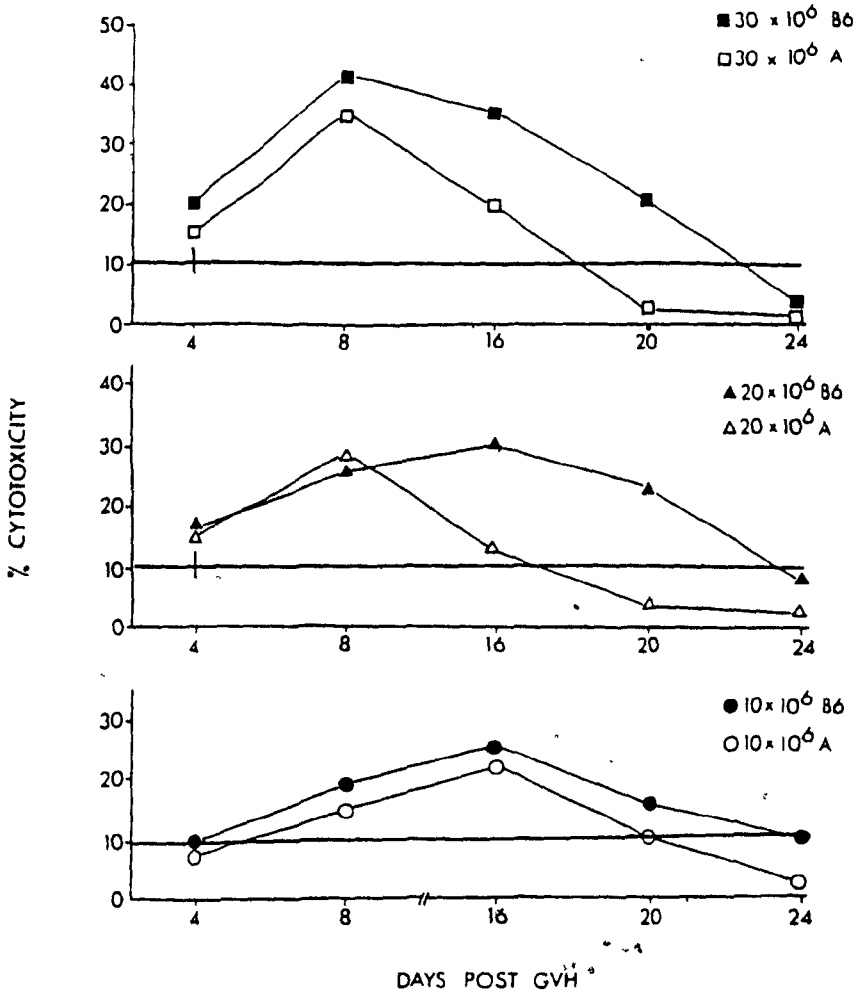


Figure 4.5 Kinetics of splenic NK cell activity against YAC targets of B6AF1 mice after the injection of different doses of either A or B6 PLC. Spleens from 3 animals/group/day were pooled. The experiment was performed three times. Each experiment showed similar kinetics. Results from one experiment are shown. The horizontal line is the mean (±S.E) NK cell activity of normal B6AF1 mice (3 animals/day). The effector:target cell ratio used was 50:1.

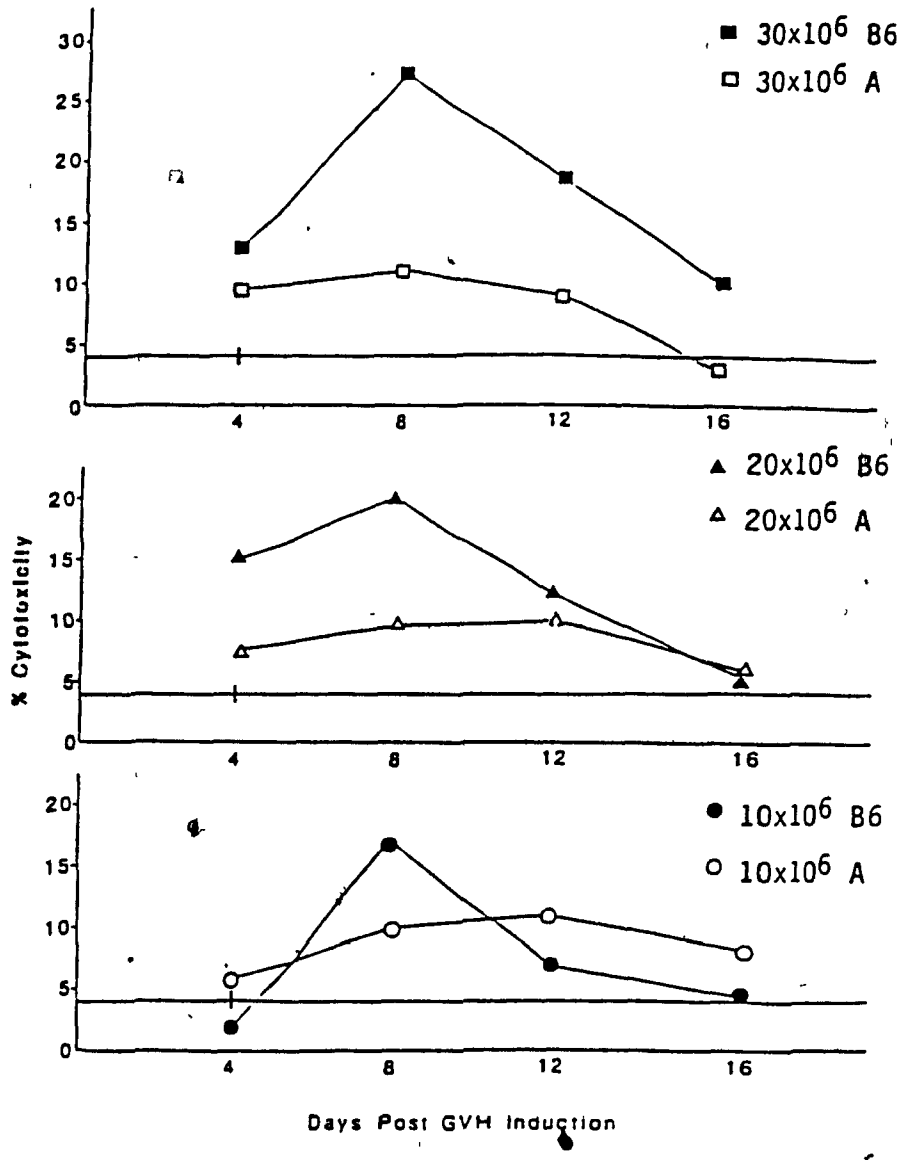


Figure 4.6. Kinetics of BM NK cell activity against YAC targets of B6AF1 mice after the injection of different doses of either A or B6 PLC. BM cells from 3 animals/group/day were pooled. The experiment was performed three times. Each experiment showed similar kinetics. The horizontal line is the mean (± S.E) Nk cell activity of normal B6AF1 mice (3 animals/day). The effector:target cell ratio used was 50:1.

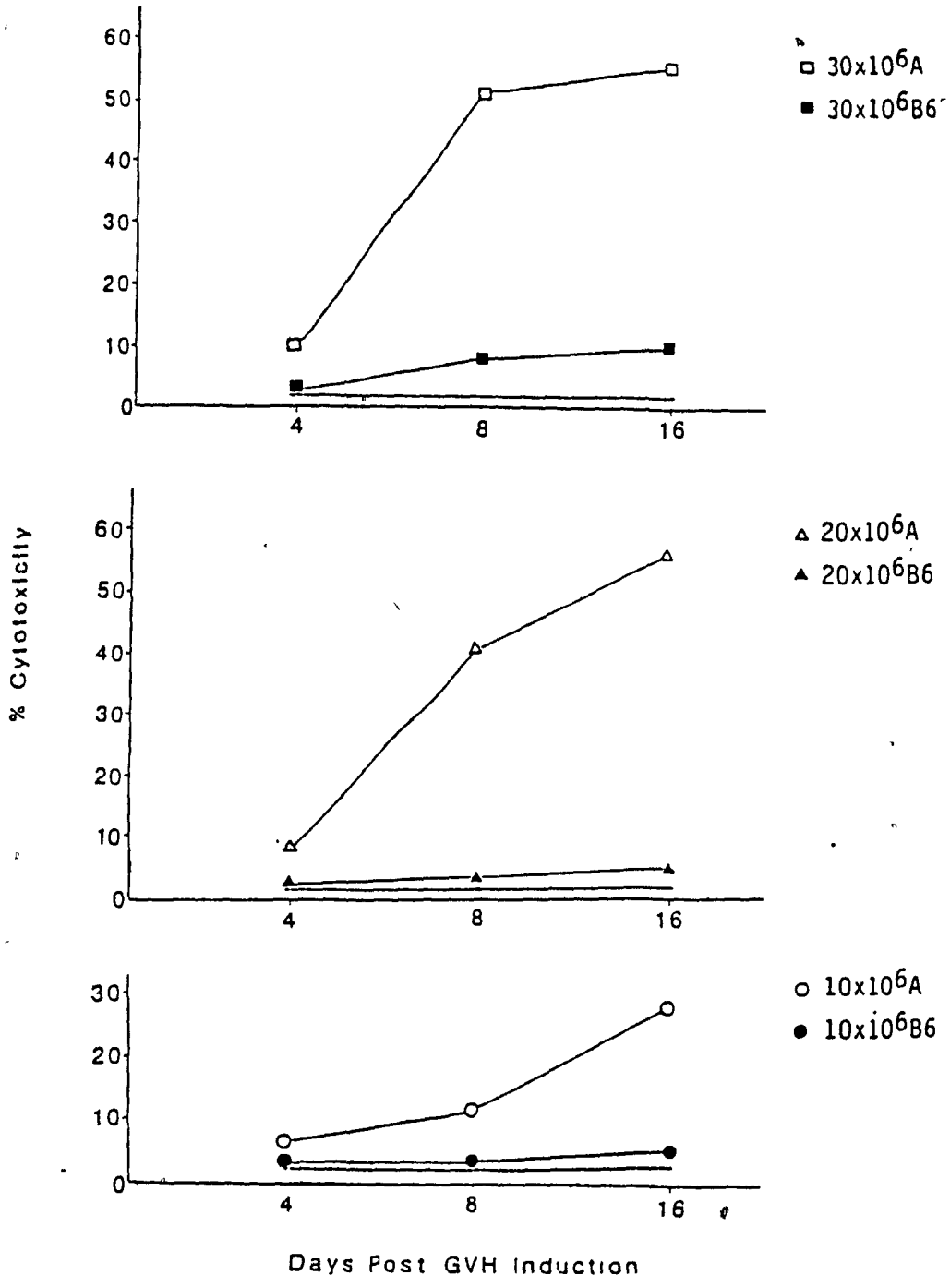


Figure 4.7 P-815 target cell killing by splenocytes of B6AF1 mice injected with different doses of either A or B6 PLC. Spleen cells from three animals/group/day were pooled. The experiment was performed three times. Each experiment gave similar results. Results of one experiment are shown. The effector: target cell ratio used was 50:1.
The dashed line is the mean P-815 target cell killing by normal B6AF1 splenocytes.

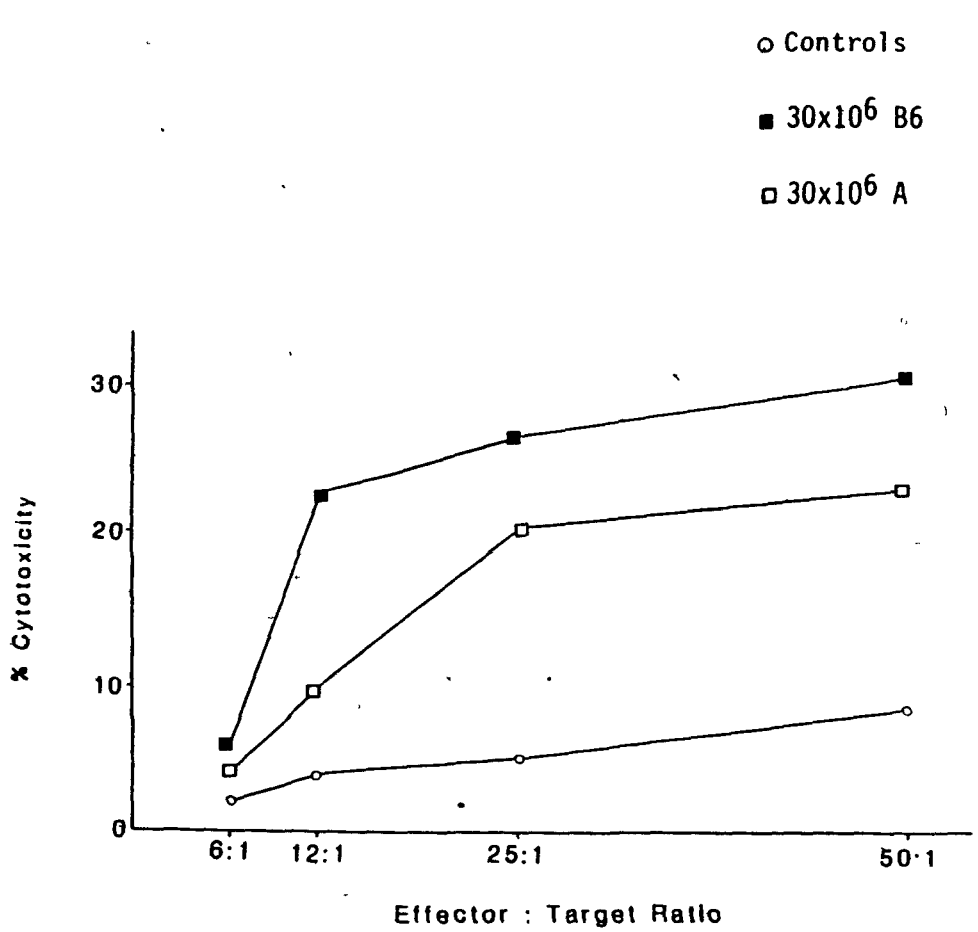


Figure 4.8 Eb target cell killing by splenocytes of B6AF1 mice injected with either $30x10^6$ B6 or $30x10^6$A PLC. Splenocytes from 3 animals/group were pooled on day 8 after PLC injection. The effector:target cell ratio used was 50:1.

Table 4.4 Percent cytotoxicity of $B6AF_1$ GVH splenocytes on day 16 post GVH induction against YAC-1 targets after treatment with anti Thy-1 + C'.[a]

NUMBER ($x10^6$) AND STRAIN OF DONOR CELLS INJECTED	TREATMENT OF EFFECTOR CELLS WITH ANTI Thy-1.2 + C' [b,c]	% CYTOTOXICITY E:T RATIO USED	
		50:1	25:1
-	-	10[d]	7[d]
	+	11[d]	7[d]
30 B6	-	41	22
	+	36	21
20 B6	-	36	28
	+	32	25
10 B6	-	16	11
	+	13	7
30 A	-	12	7
	+	6	4
20 A	-	17	11
	+	14	9.
10 A	-	N.D.[e]	N.D.[e]
	+	N.D.[e]	N.D.[e]

(a) GVH reactions were induced in $B6AF_1$ hybrids by injecting different doses; $30x10^6$, $20x10^6$, $10x10^6$ of either parental strain A or B6 lymphoid cells.
(b) Effector cells were used untreated (-) or treated with anti Thy-1 and C'(+). Protocol for treatment is described in detail in Materials and Methods.
(c) Splenocytes from 3 mice/group were pooled.
(d) Normal $B6AF_1$ mice age and sex matched were used as normal controls.
(e) N.D. not done.

Table 4.5 Percent cytotoxicity of $B6AF_1$ GVH splenocytes against P-815 targets after treatment with anti Thy-1 + C'.[a]

DONOR STRAIN USED TO INDUCE GVH REACTION[b]	NUMBER OF DONOR CELLS INJECTED x 10^6	TREATMENT OF EFFECTOR CELLS WITH ANTI Thy-1.2 + C'[c,d]	% CYTOTOXICITY TARGET CELL & E:T RATIO USED[e] P815 50:1	P815 25:1
B6	30	-	9	5
		+	11	9
B6	20	-	4	4
		+	-1	1
B6	10	-	3	3
		+	1	0
A	30	-	56	55
		+	59	56
A	20	-	57	57
		+	49	47
A	10	-	34	26
		+	27	22

(a) GVH reactions were induced in $B6AF_1$ hybrids by injecting different doses; 30 x 10^6, 20 x 10^6, 10 x 10^6 of either parental strain A or B6 lymphoid cells.
(b) Normal $B6AF_1$ mice age and sex matched were used as normal controls.
(c) Effector cells were used untreated (-) or treated with anti Thy-1 and C' (+).
(d) Splenocytes from 3 mice/group were pooled.
(e) Animals were sacrificed on day 16 after GVH induction.

Table 4.6: Intensity of histopathological lesions in the liver, pancreas and salivary gland (S.G.) of $B6AF_1$ mice on days 8 and 16 after the injection of different doses of either B6 or A PLC.[a]

Donor cell injected	Number of cells injected x 10^{-6}	Organ examined for lesions	Intensity of Lesions (Frequency/total) on days 8 and 16 after GVH induction: Normal 8	Normal 16	Mild 8	Mild 16	Moderate - Severe 8	Moderate - Severe 16
B6	30	Liver	4/7	0/7	3/7	1/7	0/7	6/7
		Pancreas	5/7	1/7	2/7	1/7	0/7	5/7
		S.G.	4/7	0/7	3/7	2/7	0/7	5/7
B6	20	Liver	5/7	6/7	2/7	1/7	0/7	0/7
		Pancreas	7/7	7/7	0/7	0/7	0/7	0/7
		S.G.	5/7	5/7	2/7	2/7	0/7	0/7
B6	10	Liver	6/7	6/7	1/7	1/7	0/7	0/7
		Pancreas	7/7	7/7	0/7	0/7	0/7	0/7
		S.G.	7/7	2/7	0/7	5/7	0/7	0/7
A	30	Liver	0/7	0/7	7/7	0/7	0/7	7/7
		Pancreas	1/7	0/7	6/7	0/7	0/7	7/7
		S.G.	2/7	0/7	5/7	1/7	0/7	6/7
A	20	Liver	1/7	0/7	4/7	0/7	2/7	7/7
		Pancreas	3/7	0/7	4/7	1/7	0/7	6/7
		S.G.	2/7	0/7	4/7	0/7	1/7	7/7
A	10	Liver	5/7	2/7	2/7	4/7	0/7	1/7
		Pancreas	6/7	2/7	1/7	4/7	0/7	1/7
		S.G.	5/7	2/7	2/7	4/7	0/7	1/7

[a] The different degrees of histopathological lesions are defined in section 4.3.6 and are shown in figures 4.9-4.17.

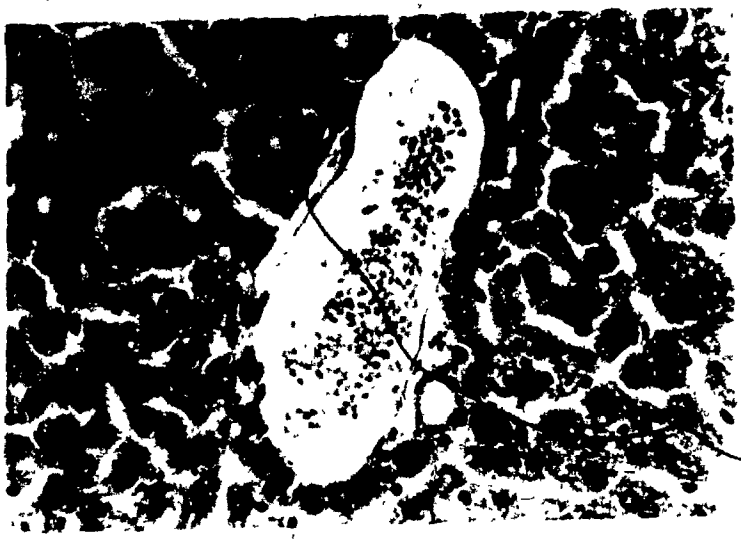


Figure 4.9. Photomicrograph of liver with normal architecture (HPS x200) Note the absence of lymphocytic infiltrates in the vicinity of the duct and vessel and the uniform liver parenchyma.
The liver was taken from B6AF1 mouse at day 16 after the injection of 20×10^6 B6 PLC.

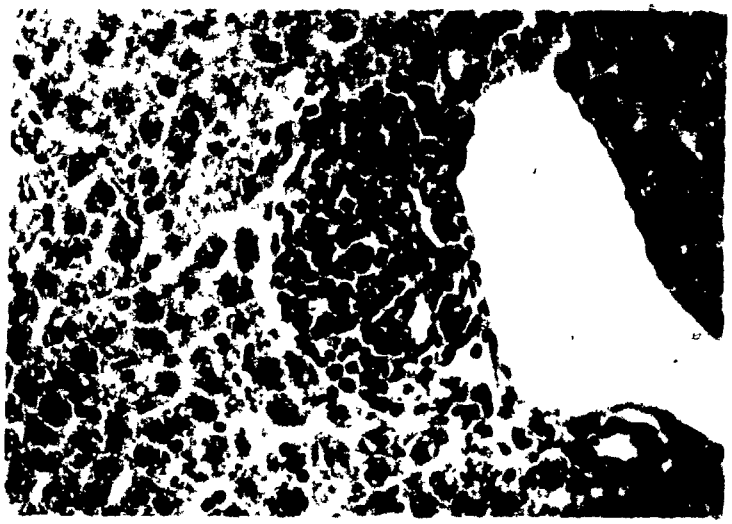


Figure 4.10 Photomicrograph of liver showing mild lymphocytic infiltrates (mild liver damage) (HPS x200). Note the presence of lymphocytes around the ducts (localized infiltrates) Also note the absence of lymphocytes in the liver parenchyma.
The liver was taken from B6AF1 mouse at day 16 after the injection of $10x10^6$A PLC.

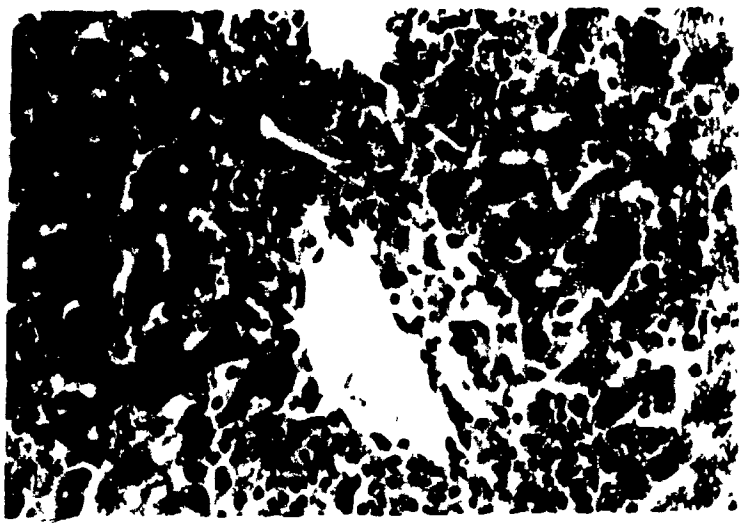


Figure 4 11 Photomicrograph of liver showing severe lymphocytic infiltrates (severe liver damage) (HPS x200) Note the presence of lymphocytic infiltrates around the ducts and in the liver parenchyma
The liver was taken from B6AF1 mouse at day 16 after the injection of 30×10^6A PLC.

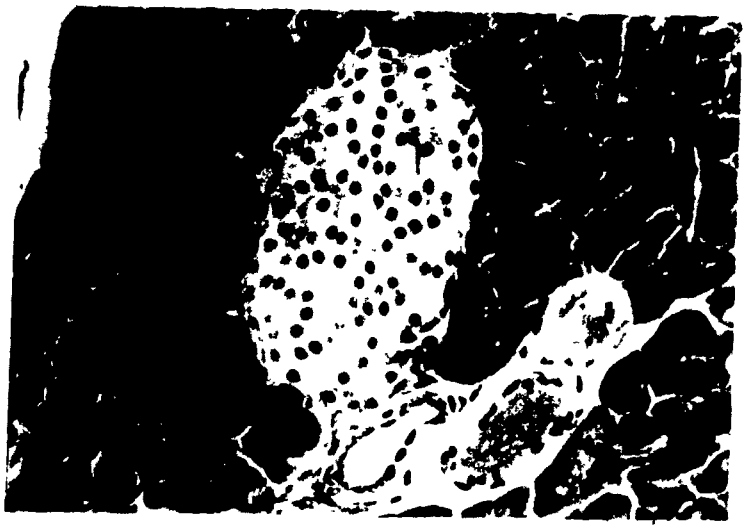


Figure 4 12 Photomicrograph of pancreas with normal architecture (HPS x200) Note the presence of an islet of langerhans. Also note the absence of lymphocytic infiltrates around the ducts and in the parenchyma.
The pancreas was taken from B6AF1 mouse at day 16 after the injection of $20x10^6$B6 PLC.

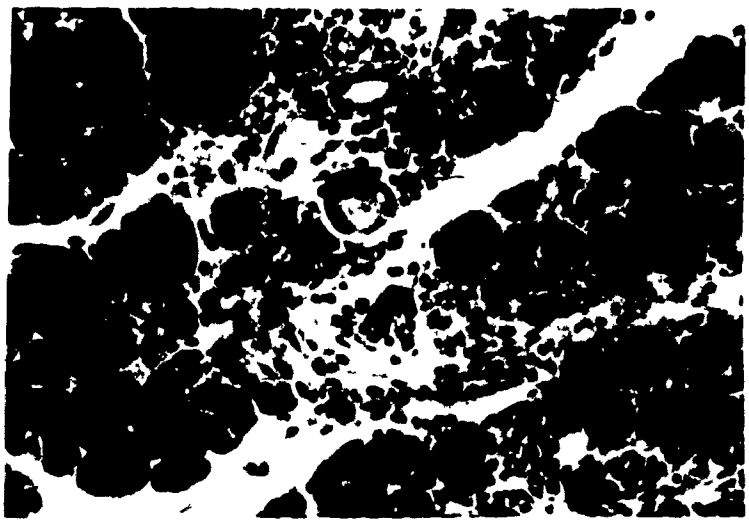


Figure 4.13 Photomicrograph of pancreas showing mild lymphocytic infiltrates (mild lesions) (HPS x200). Note the presence of lymphocytic infiltratres around the duct (localized infiltrates). Also note that most of the acinar cells surrounding the infiltrates are healthy.
The pancreas was taken from B6AF1 mouse at day 16 after the injection of $10x10^6$A PLC.

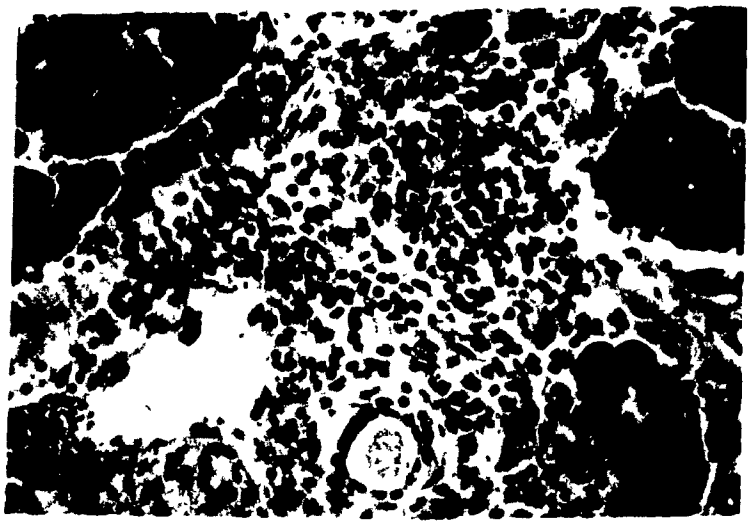


Figure 4.14 Photomicrograph of pancreas showing severe lymphocytic infiltrates (severe lesions) (HPS x200). Note the intense lymphocytic infiltrates around the ducts. Also note that the lymphocytes have moved into the acinar tissue.
The pancreas was taken from B6AF1 mouse at day 16 after the injection of $30x10^6$A PLC.

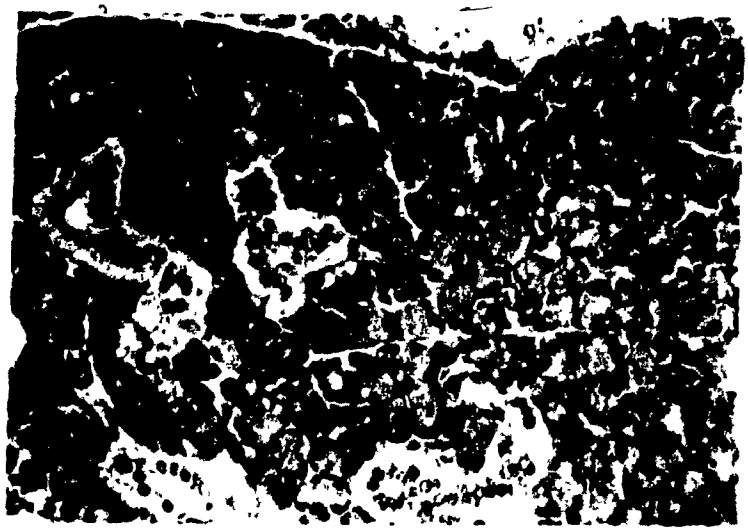


Figure 4.15 Photomicrograph of salivary gland with normal architecture (HPS x200). Note the absence of lymphocytic infiltrates around the ducts. Also note the uniform parenchyma of the salivary gland.
The salivary gland was taken from B6AF1 mouse at day 16 after the injection of $20x10^6$B6 PLC.

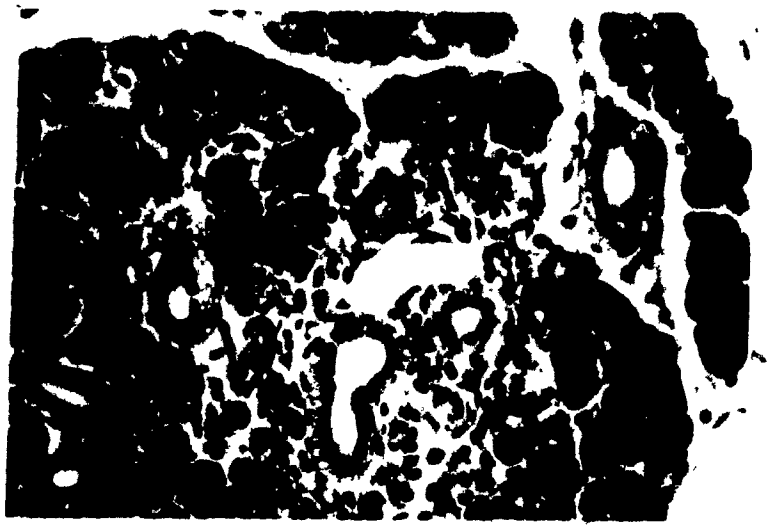


Figure 4.16 Photomicrograph of salivary gland showing mild lymphocytic infiltrates (mild lesions) (HPS x200). Note the presence of lymphocytic infiltrates around the ducts and localized damage to the parenchyma.
The salivary gland was taken from B6AF1 mouse at day 16 after the injection of $10x10^6$A PLC.

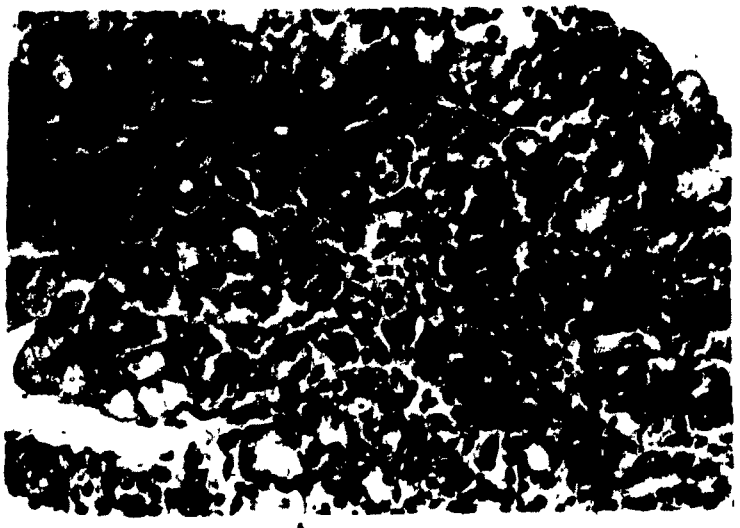


Figure 4.17 Photomicrograph of salivary gland showing severe lymphocytic infiltrates (severe lesions) (HPS x200). Note the presence of intense lymphocytic infiltrates around the ducts and in the salivary gland parenchyma.
The salivary gland was taken from B6AF1 mouse at day 16 after the injection of $30x10^6$A PLC.

CHAPTER FIVE

THE RELATIONSHIP BETWEEN THYMIC NK CELL ACTIVITY AND THE DEVELOPMENT OF THYMIC HISTOPATHOLOGICAL LESIONS AFTER THE INDUCTION OF GVH REACTIONS.

5.1 INTRODUCTION

In chapter three it was shown that the initial appearance of thymic NK cell activity was dependent upon the number of PLC injected into B6AF1 mice. However, it is not known whether there is any relationship between the thymic NK cell activity and the development of thymic histopathological lesions. In the previous chapter it was demonstrated that GVH-induced moderate-severe histopathological lesions in the non-lymphoid organs appeared in those groups of B6AF1 mice in which a greater and early peak of splenic NK cell activity was observed. In contrast, only mild or no histopathological lesions were observed in the groups of B6AF1 mice in which the peak of splenic NK cell activity was delayed. In this chapter we have investigated the relationship between the thymic NK cell activity and the degree of GVH-induced thymic histopathological alterations in B6AF1 mice injected with different doses of either B6 or A PLC.

5.2 EXPERIMENTAL DESIGN

The experimental design is presented in Figure 5.1. GVH reactions were induced in B6AF1 mice by injecting different doses , 30 , 20 , or $10x10^6$, of either parental strain B6 or A lymphoid cells. On different days post-PLC injections, 3-4 animals/group were sacrificed and their thymuses were removed to determine NK cell mediated killing of YAC tumor targets. Similary, 3-4 animal/group were also randomly selected at

different days post-PLC injections and their complete thymuses were fixed in 5% formalin for histology.

On day 8 post-PLC injections splenocytes from the animals in each group of B6AF1 mice whose thymocytes were tested for NK cell activity were tested for the suppression of Con A, PHA, and LPS responses to assess the induction of GVH reaction.

In the second series of experiments, GVH reactions were induced in B6AF1 mice by injecting $30x10^6$ B6 PLC (this PLC dose resulted in immunosuppression, which is a hallmark of GVH reaction induction). On different days post-GVH induction 3-4 GVH- reactive mice and 3-4 normal B6AF1 mice were sacrificed and their thymuses were removed. Single cell suspensions of GVH-reactive and normal thymuses were made separately and supernatants from these thymocyte populations were collected, as described previously (section 2.8). These supernatants were then tested for their ability to induce/augment NK cell activity (YAC killing) in normal B6AF1 thymocytes.

5.3. RESULTS

5.3.1 ASSESSMENT OF GVH INDUCTION : MITOGEN RESPONSES OF B6AF1 MICE INJECTED WITH DIFFERENT DOSES OF EITHER A OR B6 PLC

Table 5.1 shows the splenic Con A and PHA responses of B6AF1 mice injected with different doses of either A or B6 PLC. As can be seen severe suppression of T-cell mitogen resposnses was observed in all groups of B6AF1 mice that received different doses of A PLC. The degree of suppression

was dependent upon the dose of PLC injected. In contrast, severe suppression of ConA and PHA responses was only observed in F1 mice that received $30x10^6$ B6 PLC, whereas B6AF1 mice that received either $20x10^6$ or $10x10^6$ B6 PLC exhibited partial suppression only. These data confirm the results presented in chapter four (Tables 4.2a,b).

5.3.2 KINETICS OF APPEARANCE OF THYMIC HISTOPATHOLOGICAL LESIONS AFTER GVH INDUCTION

Table 5.2 shows the frequency of animals with mild and moderate-severe thymic lesions as well as the time of initial appearance and later severity of the histopathological changes that develop in the thymuses of B6AF1 mice following injections of different doses of either strain A or B6 PLC. In the groups of F1 mice injected with $30x10^6$ A and $20x10^6$ A strain PLC thymic histopathological changes started to appear by day 8 and were as graded mild; however, on day 16 after GVH reaction induction severe thymic alterations were observed in all animals in these two groups. However, the injection of $10x10^6$ A PLC resulted in only mild thymic lesions or no lesions at all on day 8 and 16 after GVH reaction induction. In contrast to the A-->B6AF1 GVH combinations, thymic lesions of moderate intensity developed only in B6AF1 mice that received $30x10^6$ B6 PLC on day 16 after GVH reaction induction. The majority of animals in the groups of F1 mice injected with $20x10^6$ B6 and $10x10^6$ B6 PLC did not show any signs of detectable thymic histopathological changes. The data in table

5.2 demonstrate that the degree of thymic alterations observed by day 16 post-GVH reaction induction depended upon the number and genotype of PLC injected to induce GVH reactions.

5.3.2.1 DESCRIPTION OF THYMIC HISTOPATHOLOGICAL CHANGES

Various degrees of thymic histopathologiacl alterations were recognized on different days post PLC injections in B6AF1 mice which received 30 , 20 , or $10x10^6$ PLC of either B6 or A origin. Thymic changes were graded as normal, mild, moderate, or severe. A brief description of various degrees of thymic lesions follows:-

(a) NORMAL: A normal B6AF1 thymus was characterized by a lobular configuration, sharp cortico-medullary delineation, and broad cortex packed with thymocytes. The medulla, in contrast, contained fewer thymocytes and was distinctive since it displayed clusters of pale epithelial cells aggregated to form Hassall's corpuscles, as well as "large" pale individual epithelial cells. Individual epithelial cells could be differentiated from thymocytes by their larger size, vesicular nuclei, abundant pale cytoplasm, polygonal shape, and occasionally, cytoplasmic processes. Photomicrographs of a normal thymus are shown in figures 5.2a and 5.2b.

(b) MILD LESIONS: The mild thymic histopathological lesions were characterized by only partial changes in the cortex and/or medulla, but the cortico-medullary demarcation was clearly visible. In some such mildly dysplastic thymuses partial depletion of lymphocytes was observed in the cortex, In the

medulla distinct "small" dark individual epithelial cells were visible, however, "large" pale individual epithelial cells, epithelial cell clusters or Hassall's corpuscles could not be recognized. A representative thymus is shown in figures 5.3a and 5.3b.

(c) **MODERATE LESIONS:** The moderate thymic histopathological alterations were characterized by a considerable depletion of cortical lymphocytes, involution (size) of the gland, complete loss of cortico-medullary demarcation, and total disappearance of "large" pale individual eplithelial cells and epithelial cell clusters (Hassal's corpuscles) in the medulla. However, rare "small" dark individual epithelial cells could be noted in the medulla. Such a moderately dysplastic thymus is shown in figures 5.4a and 5.4b.

(d) **SEVERE LESIONS :** The severe thymic histopathological lesions were characterized by a dramatic involution (size) of the gland, complete loss of cortico-medullary demarcation, and complete disappearance of Hassall's corpuscles and both the "small" and "large" individual epithelial cells in the medulla. Intense lymphocytic infiltrates were also visible in the medulla. The medulla of such thymuses was identifiable only by the presence of large vessels. A severely dysplastic thymus is shown in figures 5.5a and 5.5b.

A summary of various degrees of thymic histopathological changes is presented in table 5.3.

5.3.3 KINETIC OF THYMIC NK CELL ACTIVITY

The data presented in figure 5 7 show the kinetic of NK cell cytotoxicity against YAC targets by thymocytes of B6AF1 mice injected with different doses of either strain A or B6 PLC. Two distinct patterns of thymic NK cell activity are observed In the groups of B6AF1 mice injected with $30x10^6$ A, $20x10^6$ A, and $30x10^6$ B6 PLC, thymic NK cell activity was detected as early as day 4 post-GVH reaction induction, reached its peak by day 8 and then declined to control levels by day 20 after GVH-induction On the other hand, in the groups of F1 mice that received either $10x10^6$ A, $20x10^6$ B6, or 10x10 $B6^6$ PLC, the appearance of thymic NK cell activity was delayed It was first observed on day 8 post-GVH induction NK cell activity in these GVH-reactive groups reached a peak value by day 12 and then declined to undetectable levels by day 20 after GVH reaction induction. These data suggest that on the basis of the time of appearance of thymic NK cell activity following PLC injections, the GVH-reactive F1 hybrid mice can be divided into two groups. The first includes those animals in which thymic NK cell activity was observed on day 4 while the other includes those animals in which thymic NK cell activity is first detected on day 8 after GVH induction These results show that all doses of cells from both B6 and A strains induced thymic NK cell activity ; however, the initial appearance and magnitude of peak thymic NK cytotoxic activity attained following GVH induction are dependent upon the number of PLC injected within a given GVH combination.

5.3.4 EFFECT OF SUPERNATANTS FROM GVH THYMOCYTES ON NORMAL THYMOCYTE NK ACTIVITY

The data presented in figure 5 7 show that when normal B6AF1 thymocytes were treated with supernatants derived from GVH-reactive thymocytes, NK cell cytoxicity against YAC tumor targets was augmented and/or induced rapidly in normal B6AF1 thymocytes On the other hand, supernatants derived from normal B6AF1 thymocytes had no effect in augmenting and/or inducing YAC tumor cell killing in normal B6AF1 thymocytes These results suggest that NK cells or NK-like cells (which mediate killing of YAC targets) are present in the normal thymus and that the GVH thymocytes, but not normal thymocytes, produce factor(s) that can augment/induce YAC killing in normal thymocytes.

5.4 DISCUSSION

The data presented in this report demonstrate two distinct patterns of appearance of thymic NK cell activity following GVH reaction induction, namely an early appearance (day 4 post-GVH induction) and a late appearance (day 8 post-GVH induction) Similarly, different GVH-reactive groups can be divided into two catagories depending up on the severity of thymic lesions that develop following GVH reactions induction, namely moderate-severe or no lesions-mild lesions A comparison between the two patterns of appearance of thymic NK cell activity and the two patterns of thymic lesions shows that when

thymic NK cell activity appears early after GVH induction, moderate to severe thymic lesions (moderate lesions in F1 mice injected with $30x10^6$ B6, and severe lesions in F1 mice injected with $30x10^6$ A and $20x10^6$ A parental lymphoid cells) were observed On the other hand, when the appearance of thymic NK cell activity was delayed, mild thymic lesions or no thymic lesions were observed (mild lesions, in F1 mice injected with $10x10^6$ A cells, and no lesions in F1 mice injected with either $20x10^6$ B6 or $10x10^6$ B6 parental lymphoid cells) Moreover, thymic lesions appeared at the time when thymic NK cell activity was at its peak and/or highly augmented These data suggest that if NK cells are involved in GVH-induced thymic alterations, then the augmented thymic NK cell activity *per se* may not be important, but rather the time at which thymic NK cell activity appears may play the decisive role either directly or indirectly in GVH-associated thymic alterations.

The early appearance and rapid increase in thymic NK cell activity in F1 mice which show thymic lesions may be due to at least two possibilities, each of which may not be mutually exclucive. Firstly, this early appearance and rapid increase in thymic NK cell activity may be due to the entry of donor cells into the host thymus following GVH reaction induction. We have previously shown that there is a significant entry of both parental and F1 cells into the thymus of GVH mice, as measured by fluorescein isothiocyanate labelling (Gartner et al., 1984) and mitogen responses (Lapp and Kirchner, 1979). Entry of mature T-cells into the thymus has also been reported by other

workers (Naparstek et al., 1982). Moreover, we have previously proposed that NK cells may be present in the thymus of normal animals as a resident population (Roy et al., 1982) Recently, Zoller et al (1981) have demonstrated the presense of NK cells in the normal thymus. Furthermore, the data presented in figure 5.7 also suggest that NK and/or NK-like cells are present in the normal thymocyte population Thus, it is possible that the cells entering the thymus following GVH- induction may be either T-cells in different stages of maturation/ differentiation or even NK and/or NK-like cells present in the inoculum which enter the host thymus preferentially It has been proposed that NK effector cells may belong to the T-cell lineage (Herberman and Holden, 1978; Herberman et al , 1979, Kaplan , 1984) Dokhelar et al (1981) and Clancy et al (1983) have suggested that NK or NK-like effector cell observed during GVH reactions may represent a T-cell at a certain stage of its maturation/differentiation following alloantigen-induced activation. Neminin and Seksela (1984) have shown that NK effector function may reside in a precursor CTL population and Morretta et al. (1984) have shown that alloantigen activated CTL clones can exert NK-like killing Since the number of cells entrying the thymus is dependent upon the dose of cells injected i.v. and since spleen cells in the mouse contain progenator-T-cells (Kadish and Basch, 1976), the data showing an association between higher dose of parental cells injected, early appearance of thymic NK cell activity, and development of moderate-severe thymic alterations would suggest that early thymic NK cell activity may be due, at least in part, to the

entry of donor cells into the host thymus.

The second possibility for the early appearance and rapid increase in thymic NK cell activity may be the depletion of thymic and/or T-cell derived factor(s), as reported previously (Grushka and Lapp, 1971, 1974; Lapp et al., 1974). Thymic factor depletion may render the thymic environment condutive for the expression of NK and/or NK-like activity of donor as well as host cells. It has been reported that the resident thymic NK cell population is under the influence of T-suppressor cells (Zoller et al., 1981) and a functionally normal thymus exerts a suppressive influence on NK cell activity (Zoller et al , 1981; Bardos et al., 1982). It is plausible that the rate of thymic and/or T-cell derived factor depletion may depend upon the number and genotype of parental cels injected to induce GVH reactions (namely, intensity of the GVH reaction).

On the other hand, the reasons for the lack of thymic alteration in GVH-reactive F1 mice that received either $10x10^6$ or $20x10^6$ B6 PLC are not clear. However, at least two reasons may exist each of which may not be mutually exclusive. The first may be the rapid elimination of B6 donor cells by the B6AF1 hybrids, due to the hybrid resistance phenomenon, as discussed in the previous chapter (section 4.4). This rapid elimination of B6 donor cells by the B6AF1 hybrid would reduce the size of the inoculum (and possibly the size of the effector cell population), thereby decreasing the effectiveness of the donor cells to induce GVH reactions, as assessed by immuno-

suppression (Table 5.1) and thymic injury (Table 5 2). The induction of immunosuppression and thymic injury in F1 hybrids injected with $30x10^6$ B6 lymphoid cells would suggest that this cell dose can override the B6AF1 hydrid resistance mechanism(s) and enough donor cells survive to induce thymic lesions. The second reason for delayed thymic NK cell activity and the lack of development of thymic lesions could be due to the activation of only host thymic NK cells in response to circulating lymphokines eg , interferon (Zawatsky et al , 1979), released following PLC injections It is possible that donor NK cell activity, but not host thymic NK cell activation, may play an important role in inflicting/initiating thymic injury

The mechanism(s) responsible for the GVH-induced histopathological alterations of the thymus is not yet clear However, if a certain cell type is to be assigned the mediator of GVH-associated tissue damage it is important to show that the effector cell is present in host organs at the time when tissue damage is observed. Recently Borland et al. (1983) have demonstrated NK cell activity by lymphocytes present in the intestinal epithelial cells of GVH-reactive mice. Intestinal epithelial cells are one primary target of GVH reactions (Ferguson and Parrott, 1972). The data presented in this report show an augmented NK cell activity in the thymus, an organ which also serves as a target for the GVH reaction (Seemayer et al., 1977, 1978; Lapp et al., 1985), at the time when thymic lesions are developing. These results suggest that even if thymic NK cells are not directly involved in GVH reaction

induced thymic damage, the kinetics of their appearance following GVH reaction induction may predict the severity and/-or development of thymic injury during the course of GVH reactions in mice.

The studies presented in the previous chapter showed that the early appearance of peak splenic NK cell activity following GVH induction correlated with moderate-severe alterations in the non-lymphoid organs. A comparison of data presented in this chapter (chapter 5) and data presented in the previous chapter (chapter 4) shows a close parallel between the initial appearance and the later severity of histopathological lesions in the lymphoid and non-lymphoid organs. Thus, the data presented in this chapter and in chapter 4, collectively suggest that the kinetics of splenic NK effector cells that mediate the killing of YAC targets in vitro , may be able to predict the severeity of GVH-associated tissue damage that would develop in different organs (both the lymphoid and non-lymphoid) following GVH-induction.

Although the data presented in this chapter and in the previous chapter suggest that NK cells may either directly or indirectly play an important role in the development of histopathological lesions (or NK cell activity can at least predict the development of lesions), the findings raise three important questions :

(1) Do tissue lesions occur in the absence of NK cell activity?

(2) What is the origin of NK cell activity, i.e., host origin, donor origin, or both host and donor origin ?

(3) Is it the host, donor or both host and donor NK cell

activity that correlates with the development of lesions ? These questions are addressed in the following chapter.

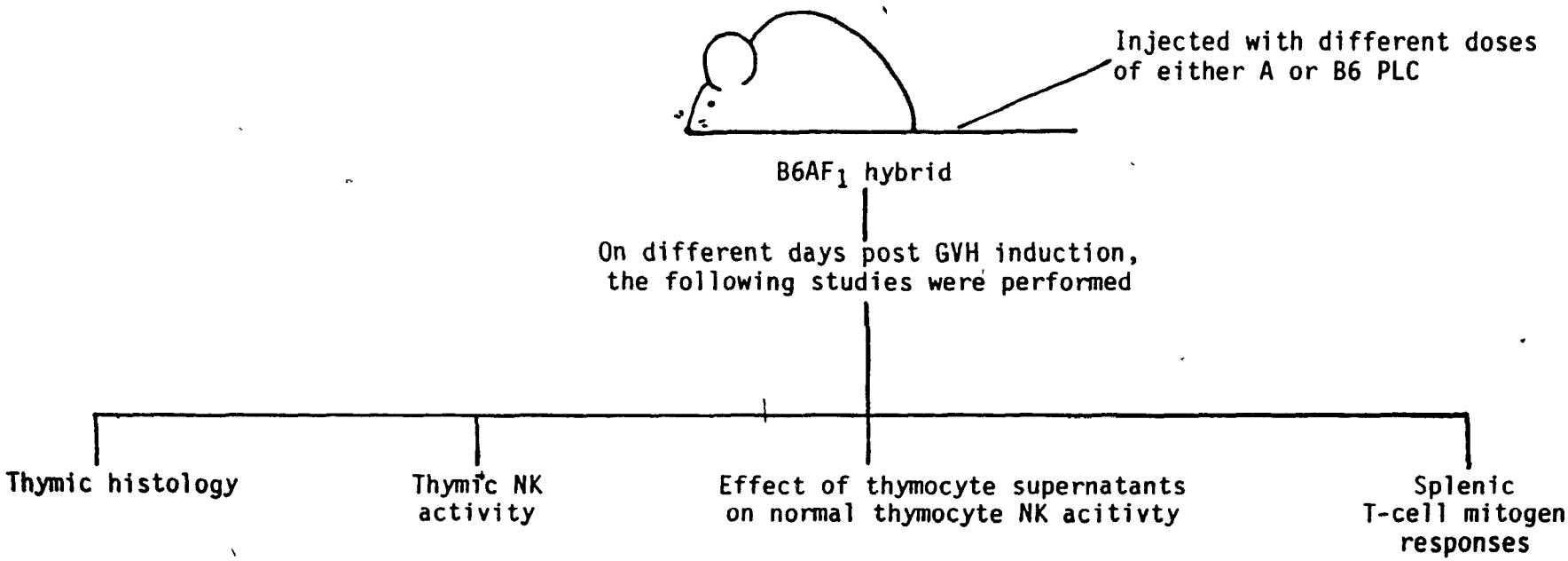


Figure 5.1 Experimental design used to investigate the relationship between thymic NK cell activity and the development of thymic histopathological alterations.

Table 5.1 Splenic Con A and PHA responses of B6AF1 mice injected with different doses of either A or B6 PLC.

Number and strain of PLC injected[a]	Mitogen responses cpm ± S.Ex10^{-3} (% of normal response)		
	Blank	ConA	PHA
	2.1 ± 0.4	191.2 ± 5.8	162.9 ± 0.1
$30x10^6$ A	0.4 ± 0.1	2.8 ± 0.2 (1.2)	7.3 ± 0.1 (4.3)
$20x10^6$ A	0.3 ± 0.1	21.4 ± 3.1 (11.2)	30.1 ± 1.8 (18.5)
$10x10^6$ A	17.9 ± 0.5	48.8 ± 1.8 (16.3)	59.2 ± 1.9 (25.6)
$30x10^6$ B6	4.2 ± 0.5	6.3 ± 0.8 (1.1)	7.2 ± 0.3 (1.8)
$20x10^6$ B6	12.5 ± 1.1	133.5 ± 1.4 (64.0)	82.0 ± 0.6 (43.2)
$10x10^6$ B6	5.7 ± 1.2	108.2 ± 6.6 (54.2)	92.7 ± 12.4 (54.1)

a Spleens from three animals/group were pooled. Splenocytes were tested for mitogen responses 8 days after PLC injection.

Table 5. 2: Intensity of histopathological lesions in the thymus of B6AF1 mice on days 8 and 16 after the injection of different doses of either B6 or A PLC.

Donor cells injected	Number of cells injected x 10^6	Intensity of lesions (frequency/total) on days 8 and 16 after GVH induction					
		Normal		Mild		Moderate-severe	
		8	16	8	16	8	16
B6	30	3/7	0/7	4/7	0/7	0/7	7/7
B6	20	5/7	5/7	2/7	2/7	0/7	0/7
B6	10	6/7	7/7	1/7	0/7	0/7	0/7
A	30	2/7	0/7	5/7	0/7	0/7	7/7
A	20	1/7	0/7	5/7	0/7	0/7	7/7
A	10	3/7	0/7	4/7	6/7	0/7	1/7

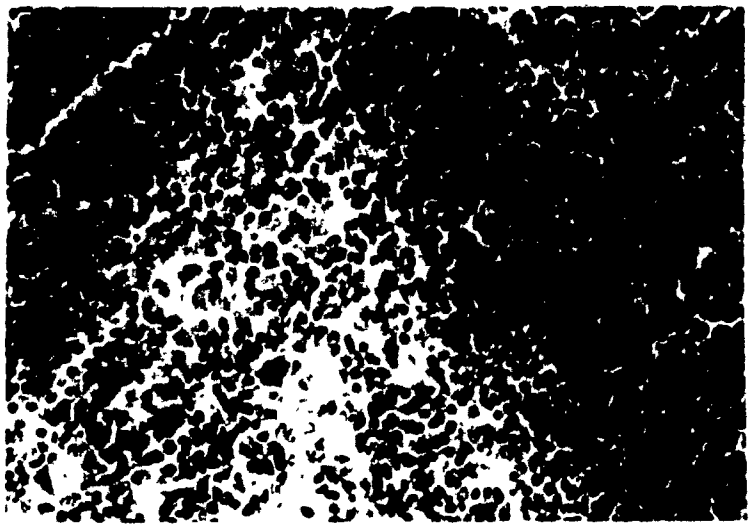


Figure 5.2a Photomicrograph of a thymus showing normal architecture (HPS x200). Note the densely packed cortex and a distinct cortico-medullary demarcation.
The thymus was taken from B6AF1 mouse at day 16 after the injection of 20×10^6 B6 PLC.

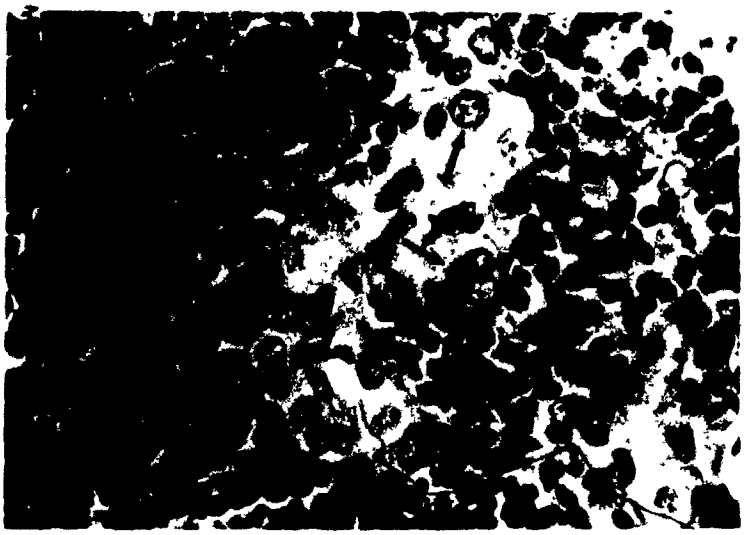


Figure 5.2b Photomicrograph of a thymus showing a normal architecture (HPS x400). Note the presence of pale epithelial cell clusters and "large" pale healthy individual epithelial cells (arrows) in the medulla. Hassall's corpuscles were also observed in the section (not shown in the photomicrograph).
The thymus was taken from B6AF1 mouse at day 16 after the injection of 20×10^6 B6 PLC.

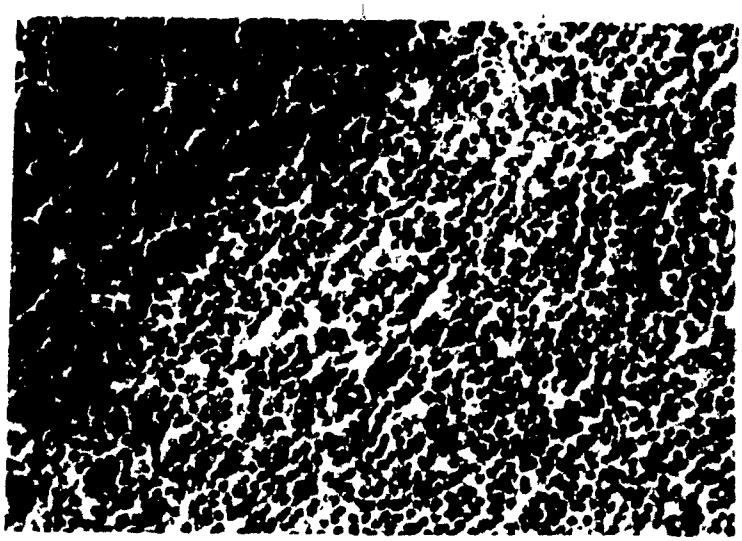


Figure 5.3a Photomicrograph of a thymus showing mild thymic dysplasia (HPS x200). Note the presence of a distinct cortico-medullary demarcation.
The thymus was taken from B6AF1 mouse at day 16 after the injection of $10x10^6$A PLC.

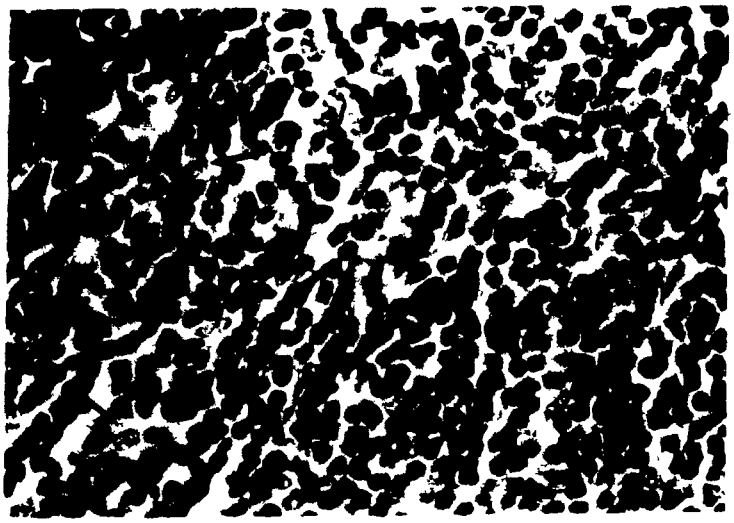


Figure 5.3b Photomicrograph of a thymus showing mild thymic dysplasia (HPS x400). Note the absence of epithelial cell clusters and "large" pale individual epithelial cells. Also note the presence of "small" dark individual epithelial cells (arrows) in the medulla. Such mildly dysplastic thymuses were also devoid of Hassall's corpuscles.
The thymus was taken from B6AF1 mouse at day 16 after the injection of $10x10^6$A PLC.

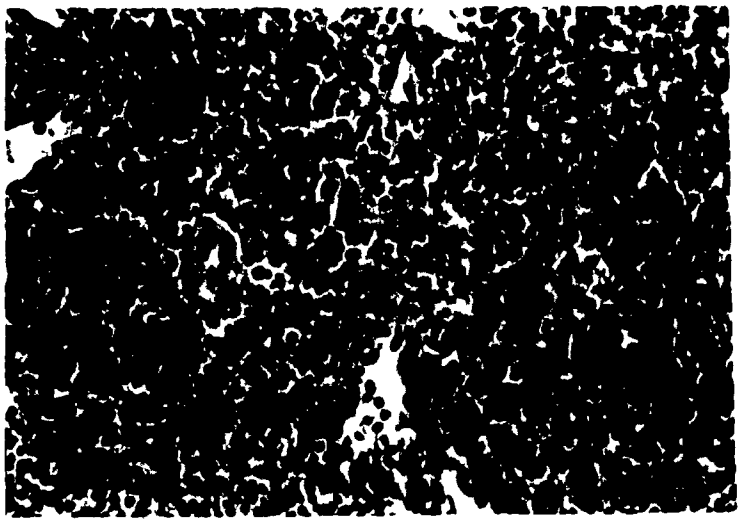


Figure 5.4a Photomicrograph of a thymus showing moderate thymic dysplasia (HPS x200). Note the complete loss of cortico-medullary demarcation.
The thymus was taken from B6AF1 mouse at day 16 after the injection of $30x10^6$B6 PLC.

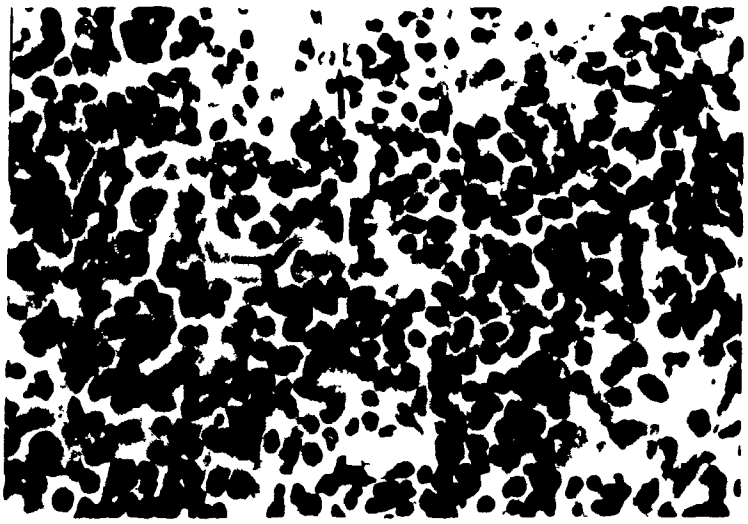


Figure 5.4b Photomicrograph of a thymus showing moderate thymic dysplasia (HPS x400). The thymic section is devoid of "large" pale individual epithelial cells, epithelial cell clusters, and Hassall's corpuscles. However, a few "small" dark individual epithelial cells (arrows) can be recognized.
The thymus was taken from B6AF1 mouse at day 16 after the injection of 30×10^6 B6 PLC.

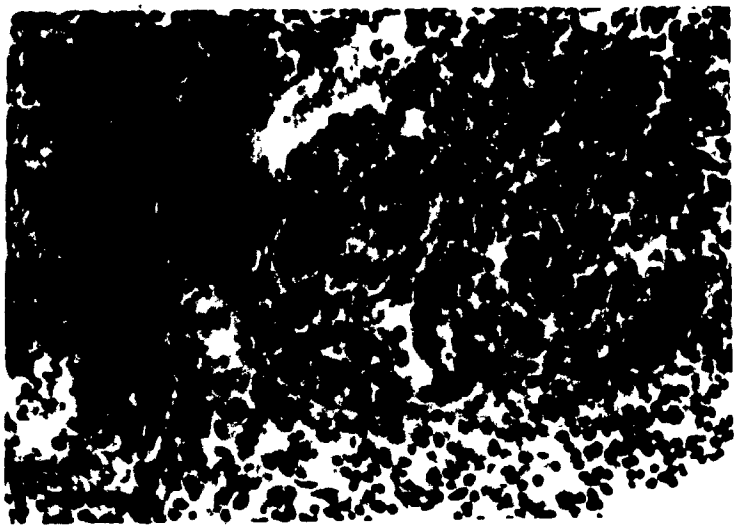


Figure 5.5a Photomicrograph of a thymus showing severe dysplasia (HPS x200). Note the complete loss of cortico-medullary demarcation and intense lymphocytic infiltrates around the vessels in the medulla.
The thymus was taken from B6AF1 mouse at day 16 after the injection of 30×10^6A PLC.

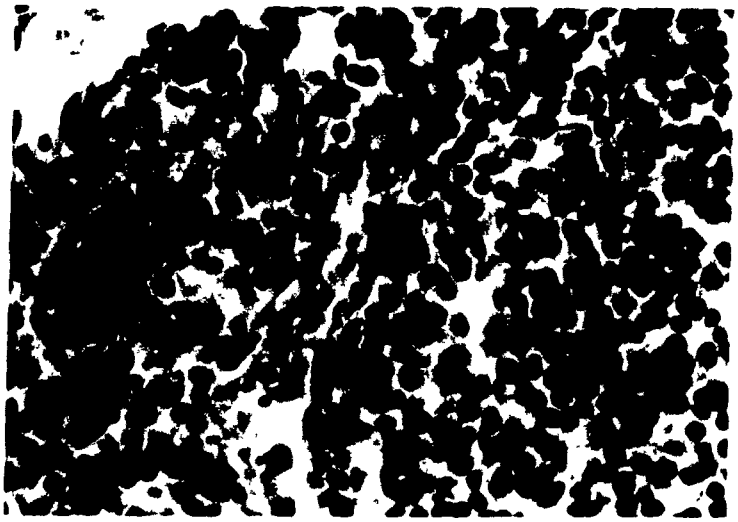


Figure 5.5b Photomicrograph of a thymus showing severe dysplasia (HPS x400). Note the complete loss of individual epithelial cells, epithelial cell clusters, and Hassall's corpuscles. The medulla is recognized only due to the presence of large vessels.
The thymus was taken from B6AF1 mouse at day 16 after the injection of 30×10^6A PLC.

Table 5.3 Summary of characteristics of various degrees of thymic lesions observed in B6AF1 mice undergoing GVH reactions of different intensities

Degree of Thymic Lesions	Thymic Size	CHANGES IN THE CORTEX		Cortico - Medullary Demarcation	CHANGES IN THE MEDULLA			
		Partial Depletion	Severe Depletion		Epithelial Cells		Hassall's Corpuscles	Degree of Lymphocytic Infiltrates
					Clusters	Individual		
Normal	++++	-	-	+	+	+	+	-
Mild	++++	+	-	+	-	+	-	-
Moderate	++	-	+	-	-	±	-	-
Severe	+	-	+	-	-	-	-	+

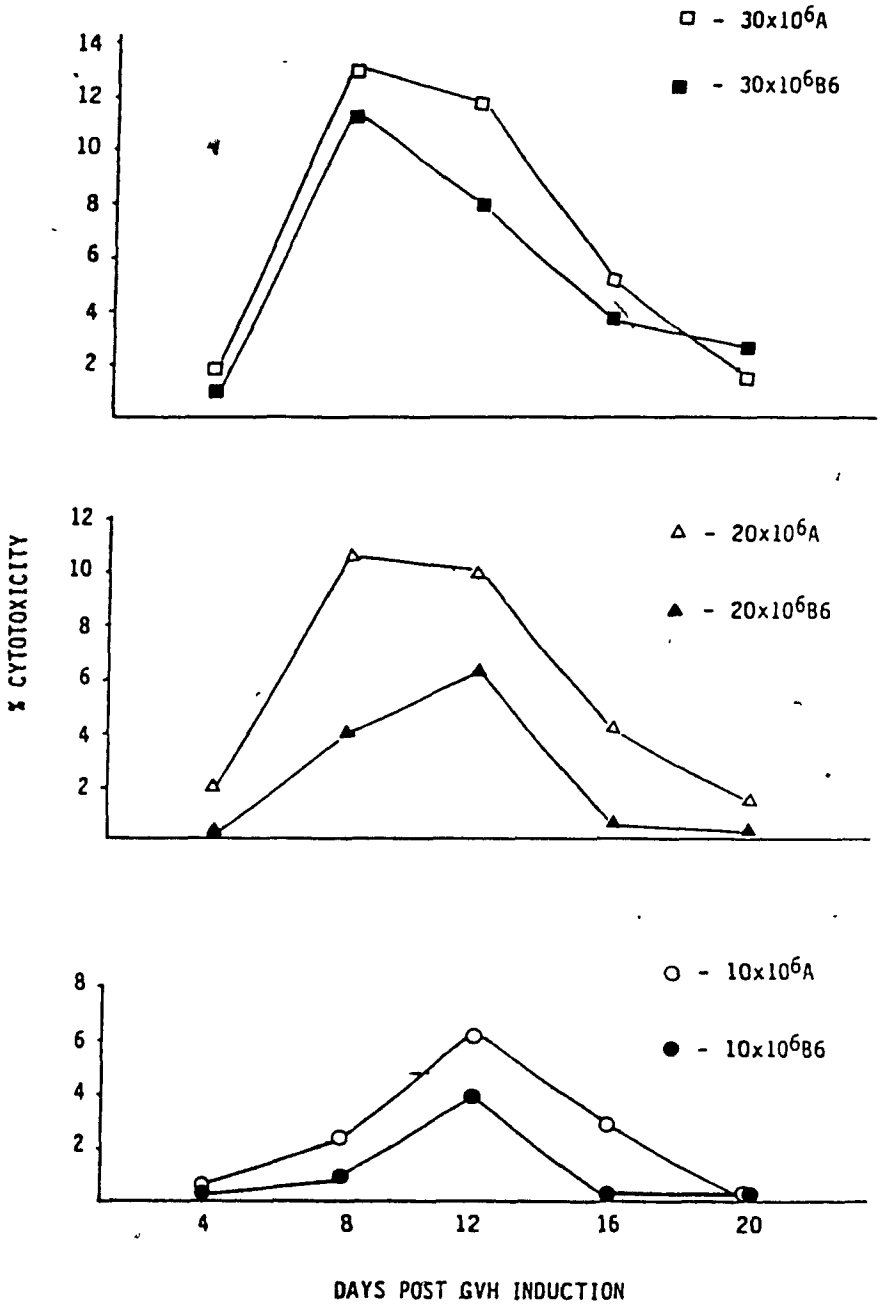


Figure 5.6 Kinetics of thymic NK cell activity against YAC targets of B6AF1 mice injected with different doses of either A or B6 PLC. The experiments were repeated three times. Each experiment gave similar results. Results of one experiment are shown. The effector:target cell ration used was 50:1.

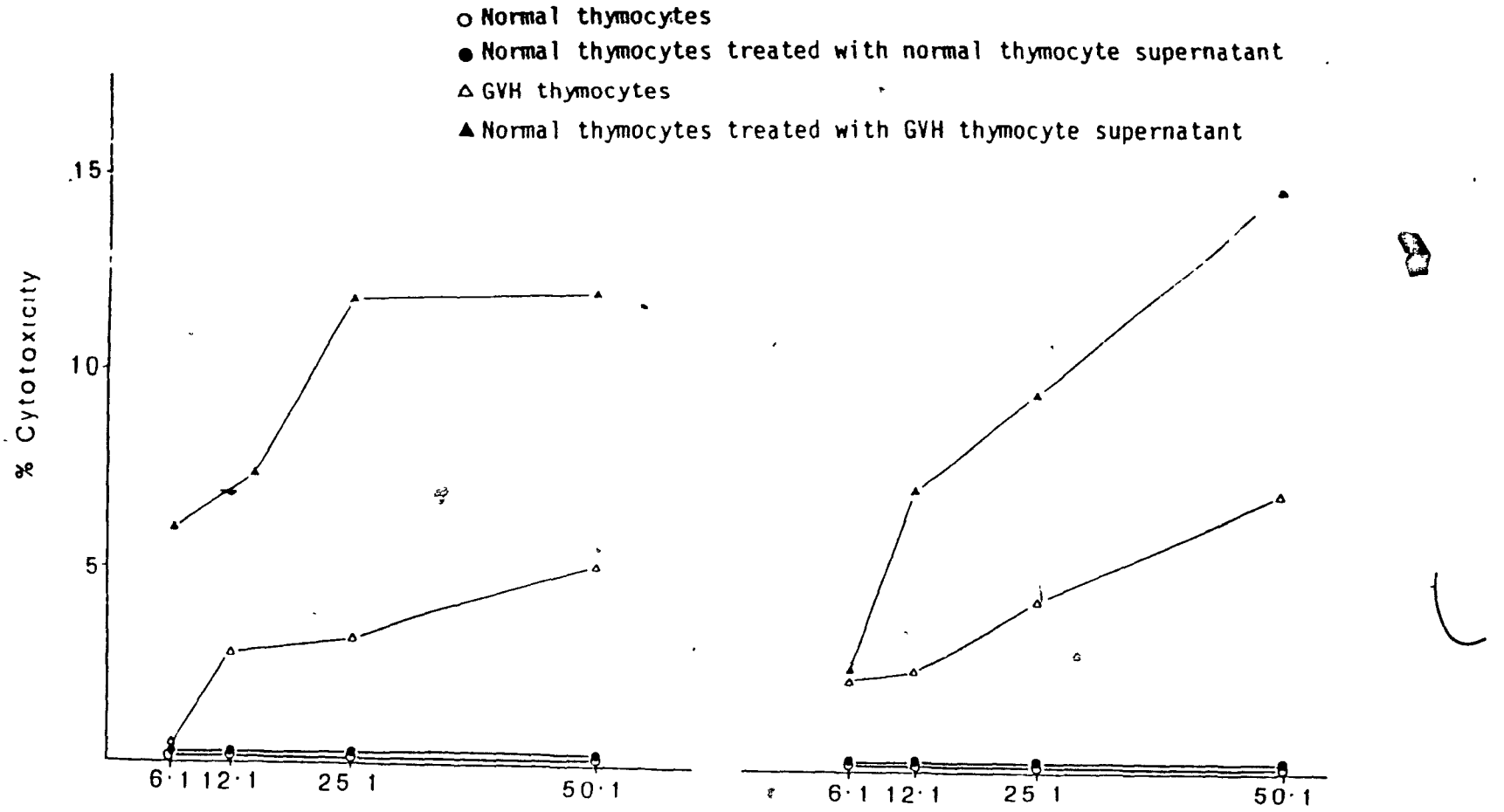


Figure 5.7 Effect of supernatants derived from normal and GVH reactive thymocytes on normal thymocyte NK cell activity against YAC targets Left panel· The supernatants were obtained from thymocytes of B6AF1 mice 5 days after the injection of $30x10^6$B6 PLC; day 5 GVH thymocytes were also tested for NK cell activity. Right panel· The supernatants were derived from thymocytes of B6AF1 mice 8 days after the injection of $30x10^6$B6 PLC; day 8 GVH thymocytes were also tested for NK cell activity.

CHAPTER SIX

THE RELATIONSHIP BETWEEN NK CELL ACTIVITY OF HOST AND DONOR ORIGIN AND THE DEVELOPMENT OF HISTOPATHOLOGICAL LESIONS.

6.1 INTRODUCTION

The results presented in the previous chapters (4 and 5) suggested an association between NK cell activity and GVH-induced pathological changes. However, these studies did not elucidate whether NK cell activity was essential for the devlopment of histopathological lesions, nor did they elucidate the origin of NK cell activity (host versus donor) or determine the origin of NK cells that correlated best with the development of histopathological lesions In this chapter we have investigated these issues by employing different parent into F1 hybrid GVH combinations carrying the beige/beige mutation (beige mice are deficient in NK cytolytic function, whereas +/beige mice possess normal NK cell cytolytic function).

6.2 EXPERIMENTAL PROTOCOL

The experimental protocol is presented in figure 6.1 Recent studies have shown that the beige (bg/bg) mutant mice may also be deficient in cytotoxic T-cell function against some targets (Halle-Pannenko, 1985; Seksena et al , 1982), but not against other targets (McKinnon et al., 1981), in addition to their defect in NK cell cytotoxic activity (Roder, 1979; Roder et al., 1979a,b). Since functional T-cells are required (not necessarily CTL) for the initiation/induction of GVH reactions (Korngold and Sprent, 1978, 1982), we investigated T-cell

function of the B6 bg/bg and B6 +/bg donor mice prior to the induction of GVH reactions by evaluating skin graft rejection, Con A and PHA mitogen responsiveness, interleukin-2 (IL-2) production in response to Con A and PHA, and the PFC response to SRBC antigens. The skin grafts were derived from C3H/Hej +/bg (H-2k) donor animals and the H-2k haplotype of B6C3HF1 recipients provided the stimulus for the initiation/induction of GVH reaction for the B6(H-2b) donor (parental) cells.

Prior to GVH induction, NK cell activity was also assessed in the B6 bg/bg and B6 +/bg donor mice and in B6C3HF1 bg/bg and B6C3HF1 +/bg recipient mice employed in this study.

GVH reactions were induced in B6C3HF1 bg/bg (bg/bgF1) and B6C3HF1 +/bg (+/bgF1) mice by injecting either 50x10 B6 bg/bg or 50x10 B6 +/bg PLC. Thus, four different GVH combinations were studied: (i) bg/bg PLC injected into bg/bgF1 mice. (ii) bg/bg PLC injected into +/bgF1 mice. (iii) +/bg PLC injected into +/bgF1 mice, and (iv) +/bg PLC injected into bg/bgF1 mice.

On different days after GVH induction, splenomegaly , splenic NK cell activity, splenic Con A and PHA responses, the PFC response to SRBC, and histological changes in the thymus, liver, and pancreas were evaluated.

6.3 RESULTS

6.3.1 IMMUNE FUNCTION OF PARENTAL B6 bg/bg AND B6 +/bg DONOR

MICE

6.3.1.1 SPLENIC NK CELL ACTIVITY OF B6 bg/bg AND +/bg PARENTAL AND bg/bgF1 AND +/bgF1 MICE

The results presented in Table 6.1 demonstrate that splenic NK cell activity was markedly deficient in B6 bg/bg animals when compared with B6 +/bg litermate controls Table 6.1 further shows that bg/bgF1 animals were also markedly deficient in splenic NK cell activity when compared with +/bgF1 controls. These results show that the bg/bgF1 recipients that we have used in the present study, were indeed homozygous for the beige gene as defined by both the functional defect in the NK cell activity and the lightness of coat colour (as determined by visual examination)

6.3.1.2 T-CELL FUNCTIONS IN THE B6 bg/bg AND B6 +/bg DONOR MICE

Table 6.2 demonstrates that no significant differences were observed in the survival time of C3H/Hej +/bg skin grafts between B6 +/bg and B6 bg/bg mice. Mice of both genotypes rejected their skin grafts by day 11. Similarly, there was no difference in the *in vivo* PFC response to SRBC between the B6 bg/bg and B6 +/bg mice. These results suggest that the effector cell function for allograft rejection and T-helper cell function of B6 bg/bg mice were similar to B6 +/bg

mice *in vivo*. Table 6.2 further shows the proliferative responses to T and B-cell mitogens. As shown in Table 6.2, B6 bg/bg cells gave a greater proliferative response to both Con A and PHA than B6 +/bg littermate control. The reasons for these differences in mitogen responsiveness are not clear. Moreover, table 6.2a shows that IL-2 production in response to Con A and PHA by B6 bg/bg splenocytes was also greater than IL-2 production by B6 +/bg mice. These results suggest that B6 bg/bg splenocytes produce at least as much, if not more, IL-2 than +/bg littermate controls.

Thus the data presented in tables 6.1, 6.2 and 6.2a show that the B6 bg/bg parental and bg/bgF1 mice employed in the present study, were clearly deficient in their NK cell function (as assessed by YAC killing), whereas *in vivo* and *in vitro* T-cell functions of B6 bg/bg donor mice were normal as assessed by skin graft rejection, the PFC response to SRBC, T-cell mitogen responses, and IL-2 production in response to T-cell mitogens.

6.3.2 MORPHOLOGICAL, FUNCTIONAL, AND HISTOLOGICAL STUDIES OF GVH REACTIVE bg/bg AND +/bg MICE:

GVH reactions induce marked splenomegaly, severe suppression of T and B-cell function, histopathological alterations of lymphoid and non-lymphoid organs, and generalized augmentation of NK cell activity early after GVH induction (for details, see Chapter 1). Studies were performed

to compare the efficacy of +/bg and bg/bg PLC in inducing these GVH-associated changes in either +/bgF1 or bg/bgF1 recipients.

6.3.2.1 SPLENOMEGALY

The results presented in table 6.3 demonstrate that when GVH reactions were induced in bg/bgF1 or +/bgF1 mice by injecting either B6 bg/bg or B6 +/bg PLC, splenomegaly was observed as early as day 4 and persisted up to day 12 post-GVH induction. Since functional T-cells are required for the initiation/induction of GVH reactions and since splenomegaly is a classical feature of GVH reaction induction, the results presented in Table 6.3, further suggest that the B6 bg/bg mice that we have employed to induce GVH reactions possessed functional T-cells.

6.3.2.2 THE PFC RESPONSE TO SRBC

Table 6.4 shows the data obtained for the *in vivo* PFC response to SRBC in different GVH combinations. As can be seen, on day 8 after GVH induction both the bg/bgF1 and +/bgF1 mice injected with either B6 bg/bg and B6 +/bg PLC were severely immunosuppressed for the PFC response to SRBC. However, complete suppression of the PFC response to SRBC was observed only in bg/bgF1 and +/bgF1 mice that received B6 +/bg PLC. Although the PFC response in both bg/bgF1 and +/bgF1 mice injected with B6 bg/bg cells was severely suppressed, none of

the animals in either group was totally suppressed On day 26 after GVH induction bg/bgF1 mice that received bg/bg donor cells showed partial recovery from the early suppression of the PFC response, (Table 6.4) however, +/bgF1 mice that received bg/bg PLC showed continued severe persistent suppression of the PFC response to SRBC (Table 6 4) In contrast, neither the +/bgF1 mice nor the bg/bgF1 mice inoculated with B6 +/bg donor cells responded to SRBC on day 26 after GVH induction (Table 6 4)

6.3.2.3 MITOGEN RESPONSES

Table 6 5 shows the results for the in vitro T-cell mitogen responsiveness of splenocytes taken from bg/bgF1 and +/bgF1 mice injected with either B6 bg/bg or B6 +/bg donor cells The mitogen data were obtained from the same animals used for the PFC assays in table 6 4 Table 6 5 demonstrates that when B6 bg/bg donor cells are injected into bg/bgF1 or +/bgF1 mice only partial suppression of Con A and PHA responses was observed on both days 12 and 30 post-GVH induction. In contrast, bg/bgF1 and +/bgF1 mice that received B6 +/bg donor cells showed persistent severe suppression of both Con A and PHA responses up to day 30 after GVH-induction The results presented in tables 6.4 and 6.5 collectively suggest a disassociation between the suppression of the PFC response to SRBC and the suppression of mitogen responses. The data also suggest that both host and donor cells may be involved in inducing GVH-associated early immunosuppression.

6.3.2.2 HISTOPATHOLOGICAL CHANGES

Table 6 6 demonstrates the frequency of animals displaying different degrees of histopathological lesions in thymus, liver, and pancreas in various GVH combinations The results clearly show that when B6 bg/bg PLC were injected into either bg/bgF1 or +/bgF1 mice, nearly all mice displayed either mild lesions or no lesions at all (78%) In contrast, moderate-severe lesions in the thymus, liver, and pancreas were observed at a high frequency (80%) in both bg/bgF1 and +/bgF1 mice that received B6 +/bg PLC These results demonstrate that B6 bg/bg PLC, but not +/bg PLC, lack an effective mechanism to induce moderate-severe GVH-associated lesions in lymphoid and non-lymphoid organs

6.3.2.5 SPLENIC NK CELL ACTIVITY

The data presented in figure 6 2 and table 6 7 show that when bg/bg PLC were injected into bg/bgF1 mice, only slightly augmented splenic NK activity was observed (about 10%) on day 4 after GVH induction. However, when bg/bg PLC were injected into +/bgF1 mice markedly augmented splenic NK cell activity was evident at day 4 post-GVH induction, suggesting that the observed NK cell activity in this GVH group was of host origin. Conversely, splenic NK cell activity was also observed by day 4 after GVH induction when +/bg parental

lymphoid cells were injected into bg/bgF1 hybrids, suggesting that in this group NK cell activity was derived from the donor cells. The highest NK cell activity was observed in +/bgF1 mice that received +/bg PLC. These data indicate that NK cells of both host and donor origin are activated during the course of GVH reactions.

6.3.2.6 SPLENIC P-815 EFFECTOR CELL ACTIVITY

Table 6 8 demonstrates the P-815 effector cell activity in the spleens of bg/bgF and +/bgF1 mice that received either B6 bg/bg or B6 +/bg PLC As can be seen, the P-815 effector cell activity was augmented to approximately the same degree (10%) in all GVH groups on day four after GVH induction These data suggest that the bg/bg or +/bg genotype of either the host or the donor had no effect on the degree of augmented P-815 effector cell activity.

6.4 DISCUSSION

In this study we have investigated the role of host and donor NK cells in inducing GVH-associated moderate-severe tissue damage, and the effects of host and donor NK cell deficiency on GVH-induced immunosuppression and splenomegaly. The results show that: (i) GVH-induced splenomegaly and tissue damage are separate events mediated by distinct mechanisms; (ii) The PFC response to SRBC (T-cell and B-cell cooperative response) is more susceptible to GVH-induced immunosuppression

than T-cell mitogen responses (proliferative response) (iii) GVH reactions activate NK cells of both host and donor origin, (iv) activated (and perhaps recruited) NK cells of donor origin may play an important role in inducing GVH-associated moderate-severe tissue damage

The data presented in this study show that PLC with (+/bg) or without (bg/bg) NK cytolytic potential are able to induce splenomegaly as well as early partial suppression of the PFC response to SRBC However, NK deficient PLC, unlike PLC with normal NK function, clearly lack the capacity to induce GVH-associated moderate-severe tissue damage as well as severe persistent immunosuppression of T-cell mitogen responses Previous data from this laboratory have demonstrated two distinct phases of immunosuppression during GVH reaction, namely, an early and a late phase each caused by different mechanisms (Lapp et al , 1985) During the early phase, GVH reactions caused a quantitative increase in splenic macrophages (the principal cause of splenomegaly) (Elie and Lapp, 1976,1977), which produced copious amounts of PGE and suppressed T-helper cell function (Lapp et al , 1980) Since splenomegaly results as a consequence of donor T-cell induced recruitment and accumulation of host macrophages and macrophage -like cells, the results presented in this report showing that NK deficient PLC are able to induce splenomegaly and early partial immunosuppression suggest that NK deficient PLC can activate mechanisms responsible for the recruitment of host cells as well as early partial immunosuppression.

In contrast to the early phase of immunosuppression, the late phase of GVH-induced immunosuppression (severe-persistent) was found to be due , at least in part, to a T-helper cell maturational arrest resulting from GVH-induced thymic dysplasia (Seddik et al , 1979,1980) Our recent studies showed that thymic dysplasia resulted in a depletion of IL-2 producing, but not IL-2 responding, cells (Mendez et al., 1985a,b). IL-2 production is essential for T-cell responses (both mitogenic and antigenic) The data presented in the present study show that the PLC with normal NK cell activity, which induced severe prolonged immunosuppression of T-cell mitogen responses, also induced moderate-severe thymic dysplasia. In contrast, NK deficient bg/bg PLC failed to cause severe prolonged immunosuppression and thymic dysplasia. Collectively, these results strongly suggest that splenomegaly and early immunosuppression are mediated by mechanisms distinct from those responsible for inducing tissue damage (thymic dysplasia) and late immunosuppression.

A comparison of data presented in this report on the PFC response to SRBC (Table 6.4) and T-cell responses (Table 6.5) shows several interesting points regarding the early phase and late prolonged phase of immunosuppression during GVH reactions. The data suggest that cells involved in the PFC response (Table 6.4) and T-cell mitogen responses (Table 6.5) have different susceptibilities to suppression during the early phase of GVH reactions (compare GVH groups; bg/bg-->+/bgFl, bg/bg-->

bg/bgF1, and +/bg-->bg/bgF1). On the other hand, it is possible that distinct suppressor mechanisms for PFC and mitogen suppression are activated during the early phase of GVH reactions Both host and/or donor cells may contribute to the suppression of the PFC response, whereas severe suppression of the mitogen responses is dependent upon a donor component The NK deficient mice (bg/bg) might lack or may not possess an effective suppressor component for the suppression of mitogen responses as present in the mice with normal NK cell function (+/bg) The data presented in this report (Tables 6 4 and 6 5) show that lymphoid cells deficient in NK cell cytolytic function can induce early severe immunosuppression of the PFC response to SRBC regardless of the NK activity of the reciepient However, immunosuppression of T-cell mitogen responses is dependent upon the presence of functional NK cells

The present study also demonstrates a strong correlation between severe suppression of T-cell mitogen responses (Table 6.5) and GVH-associated tissue damage (Table 6.6). The data presented demonstrate that both bg/bgF1 and +/bgF1 mice that were injected with +/bg PLC showed severe suppression of mitogen responses and the majority of these mice developed moderate-severe histopathological lesions. In contrast, F1 mice that received bg/bg PLC showed only partial suppression of T cell mitogen responses and the majority of these F1 mice did not develop moderate-severe lesions. These results suggest that the donor component which induces severe early and persistent

suppression of mitogen responses may also play a central role in GVH associated tissue damage. However, the possibility that the suppression of mitogen responses is secondary to thymic injury cannot be ruled out.

The data presented in this chapter also demonstrate that there is no correlation between the P-815 effector cell activity (Table 6.8) and the severity of GVH-associated lesions (Table 6.6) which develop after GVH induction. However, data presented in chapter 4 showed that, in groups of GVH mice in which moderate lesions appeared, only slightly augmented P-815 target cell killing and highly augmented NK activity was observed. In contrast, in GVH mice which demonstrated severe lesions highly augmented P-815 target cell killing as well as highly augmented NK cell activity was observed. These results of chapter 4 suggested that P-815 effector cells may be playing a role in the induction of moderate-severe lesions. The results presented in this chapter, however, show that in bg/bgF1 and +/bgF1 mice that received either bg/bg or +/bg PLC, the degree of P-815 effector cell killing was approximately the same (about 10% on days 4 and 12, see Table 6.8), whereas marked differences in the degree of histopathological lesions (Table 6.6) were observed. The severity of GVH associated lesions correlated with NK cell activity of donor origin. Thus, the data presented in this chapter strongly suggest that P-815 effector cell activity after GVH reaction induction cannot predict the degree of GVH-associated lesions.

The precise mechanism(s) responsible for the GVH-induced

histopathological lesions in the thymus as well as in the non-lymphoid organs is (are) not yet clear (Elkins, 1978). In the present study a comparison of NK cell activity (Figure 6 2) and the development of moderate-severe histopathological lesions (Table 6.6) suggests that the activation of donor cells may play a pivotal role in the pathogenesis of acute GVH disease In contrast, the role of activated host NK cells in this process may only be minimal, if any The data presented in this report demonstrate that when bg/bg PLC were used to induce GVH reactions in bg/bgF1 mice, only slight augmentation of NK cell activity (Figure 6 2) and either none or slight histopathological alterations were observed (Table 6 6) However, when B6 bg/bg PLC were injected into +/bgF1 mice highly augmented NK cell activity was observed, but either none or only mild histopathological lesions were observed. These results suggest that in this GVH combination (bg/bg-->+/bgF1) the augmented NK cell activity is of host origin and that the host NK cell activity does not play an active role in GVH induced moderate-severe lesions. In contrast, when B6 +/bg PLC were injected into bg/bgF1 mice, highly augmented NK cell activity as well as moderate-severe GVH associated histopathological changes were observed. Thus, the data suggest that in this GVH combination (+/bg-->bg/bgF1) augmented NK cell activity is of donor origin and this augmented donor NK cell activity correlates best with the GVH- associated moderate-severe histopathological alterations. The data presented in the preceding chapters (chapters 4 and 5) also suggested that donor cells may play a critical

role in the induction of GVH-associated tissue damage.

Finally, we would like to comment on the slight augmentation of NK cell activity observed in bg/bgF1 mice that received bg/bgPLC. This slight augmentation of NK cell activity, to a level similar to that observed in control +/bg mice (Table 6.1), may reflect the activation of both host and donor NK cells. This slight augmentation of NK cell activity in the bg/bg parent-->F1 GVH combination may be due to the production of interferon during the GVH reaction (Zawatsky et al , 1979; also see chapter 9). Other workers have reported slight augmentation of NK cell activity in bg/bg mice, to levels similar to that observed in unifected +/bg mice, after viral infections which is known to induce interferon production (McKinnon et al., 1981). Moreover, bg/bg mice produce the same amounts of interferon as +/bg mice (McKinnon et al., 1981). The data presented in this report, however, show that in the bg/bg PLC-->bg/bgF1 GVH combination the level of activated host and donor NK cell activity is much lower than that observed in +/bgF1 mice that received +/bg PLC (9% vs 64%) (Figure 6.2). It is of interest to note that moderate-severe pathological lesions were rarely seen in those mice (bg/bgF1 or +/bgF1) that received bg/bg PLC. It would appear that the near absence of moderate-severe lesions is due to the deficiency in the cytotoxic capacity of the bg/bg donor NK cells.

The fact that the histopathological lesions appeared in GVH combinations in which NK cell activity was of donor origin (+/bg-->bg/bgF1), as opposed to host origin (bg/bg-->+/bgF1)

suggests a degree of specificity for donor NK cells in the development of GVH-induced histopathological lesions One can but speculate at this time on the relevance for donor as opposed to host NK cytotoxic activity in the development of GVH-induced histopathological lesions. One possibility is that the NK cell sub-population responsible for the GVH-induced histopathological lesions may be of T-cell lineage (Herberman and Holden, 1978, Herberman et al , 1979, Kaplan, 1985) It has been documented that some allospecific T-cell clones could be induced to undergo a change to NK-like non-specific cytotoxicity in vitro (Seelay et al , 1979, Pawlec et al , 1982, Brook, 1983, Morretta et al., 1984) It has also been suggested that the MLC-induced NK-like cells and MLC-induced allospecific cytotoxic effector cells have a common precursor and may probably represent different stages of differentiation (Neiminin and Seksela, 1984). More recent studies by Morretta et al. (1984) have shown that alloantigen activated T-cell clones can exhibit antigen specific killing as well as killing of NK-sensitive targets. Thus, it is possible that the donor NK cell activity responsible for both YAC killing and GVH effector function may be derived from either (i) the same sub-population of T-cells that contain CTL precursors (ii) alloantigen activated T-cells with specificity for dual recognition (specific and NK sensitive target structures) as shown by Morretta et al. (1984), or (iii) donor T-cells with "incomplete" maturation and/or differentiation following their activation after GVH reaction induction.

Since T-cell maturation and differentiation are dependent upon adequate amounts of thymic factors (Stutman, 1977) and since earlier GVH studies suggested a deficiency of such factors (Grushka and Lapp, 1971, 1974; Elie et al., 1974; Lapp et al., 1974), it seems plausible that the GVH reaction may be conducive to the production of specific NK cells. Dokhelar et al. (1981) and Clancy et al. (1983) have also suggested that cells (T-cells) involved in GVH disease mechanisms may exert NK cell like activity at some stage of their maturation/ differentiation.

Recent studies by Pennenko and Bruley-Rosset (1985) showed that the parental lymphoid cells of C57BL/6 Pas orl-bg/bg mice possessed a decreased potential to induce GVH-associated mortality in x-irradiated (C57BL/6xDBA/2)F1 hybrids as compared to lymphoid cells of C57BL/6 parental mice. These workers also showed that the bg/bg donor mice employed to induce GVH reactions were totally incapable of generating specific cytotoxic T-cell responses against P-815 tumor targets in vitro. Similarly, Sexsena et al. (1982) have reported deficient cytotoxic T-cell responses against P-815 targets in the B6 bg/bg mice. On the other hand, normal cytotoxic T-cell responses have been reported against vesicular stomatitis virus in B6 bg/bg animals (McKinnon et al., 1981). Sexsena et al. (1982) have suggested that the development of cytotoxic T-cell responses in B6 bg/bg animals might depend upon the antigen employed (and also probably on the route of sensitization and the strength of antigen employed). However, in the present

study B6 bg/bg animals that we have employed as donors, were normal in T-cell function as assessed by skin graft rejection, in vivo PFC response to SRBC, T-cell mitogen responses to Con A and PHA, and IL-2 production. It is also important to note that the skin grafts were derived from C3H (H-2k) mice, and the same H-2k haplotype served as the stimulator/initiator for the GVH reaction induction. Furthermore, the results show that B6 bg/bg donor cells possess the capacity to induce splenomegaly (Table 6.3) and early immunosuppression of at least PFC responses to SRBC (Table 6.4), suggesting that the mechanism(s) responsible for the activation of a GVH reaction (which is T-cell dependent (Korngold and Sprent, 1978, 1982)) was (were) intact in the bg/bg donor cells. Moreover, B6 bg/bg donor cells when injected into +/bgF1 mice were also able to activate NK cells of the recipient (Figure 6.2). However, bg/bg donor cells were clearly deficient in inducing GVH-associated tissue damage (Table 6 6), and GVH-associated severe prolonged T-cell immuno deficiency (Table 6.4). Our data suggest that the bg/bg mutation may be a useful model to study the complex interactions involved in the early and the late phases of GVH reaction induced immunosuppression, at least in the parent into F1 hybrid GVH model

The results presented in this chapter showed that : (i) NK cells of donor origin may play an active role in GVH induced pathological lesions; (ii) splenomegaly and tissue damage are two distinct features of the GVH reaction; (iii) the PFC response to SRBC is more sensitive to the immunosuppressive effects of the GVH reaction than the T-cell proliferative

responses; and, (iv) severe persistent immunosuppression of T-cell proliferative functions is observed only in those groups in which thymic medullary injury occurs, whereas the suppression of the PFC response can be observed in the absence of thymic dysplasia. In the following chapter the relationship betweeh splenomegaly, thymic injury, and the duration of T-cell immunodeficiency is further investigated in the non-beige mice.

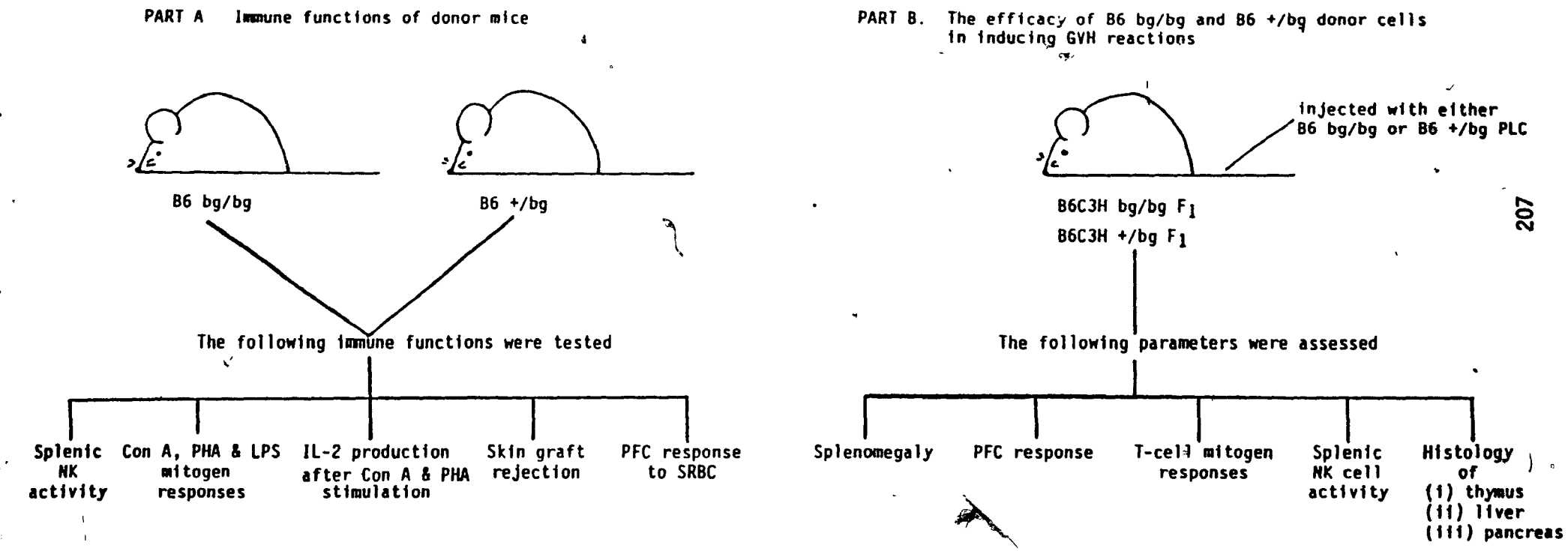


Figure 6.1 Experimental design used to investigate the role of host and donor NK cells in inducing GVH-associated histopathological alterations.

Table 6.1. Splenic NK cell cytotoxicity of parental B6 +/bg and bg/bg and recipient B6C3H F_1 +/bg and bg/bg mice.

Genotype	Number of animals[a]	% Cytotoxicity against YAC[b,c]
B6 +/bg	6	10.26 ± 0.50
B6 bg/bg	6	1.68 ± 0.37
F_1 +/bg	7	9.16 ± 0.77
F_1 bg/bg	5	0.85 ± 0.32

a. The animals used were between the ages of 12-16 weeks.

b. Animals were randomly selected from a pool of mice and NK cytotoxicity performed on individual animals. The mean ± SE is presented for each group.

c. The effector to target cell ratio was 50:1

Table 6.2. Immune function of B6 +/bg and B6 bg/bg mice used as donors to induce GVH reactions.

Genotype	Immune Function (Number of Animals)				
	Skin graft survival time[a]	PFC to SRBC $\bar{X} \pm$ S.E. $\times 10^{-3}$/per spleen	Mitogen Response net cpm + S.E. $\times 10^{-3}$ Con A	PHA	LPS
B6 +/bg	11 (8)	96.8 ± 15.4 (6)	32.8 ± 9.9 (7)	26.5 ± 6.1 (7)	50.4 ± 12.3 (7)
B6 bg/bg	11 (7)	92.8 ± 18.2 (6)	86.0 ± 20.9 (7)	89.9 ± 20.4 (7)	28.5 ± 13.7 (7)

a. Animals were grafted with C3H skin. Graft dressings were removed on day 10 and all grafts were rejected by day 11 in both groups.

Table 6.2a. Lymph node and splenic cellularity and splenocytes IL-2 production in response to Con A, PHA, and LPS by B6 +/bg and B6 bg/bg mice.

GENOTYPE[a]	CELLULARITY ± S.E.x10^6		Proliferation of CTLL cells to IL-2 containing supernatants obtained from mitogen stimulated splenocytes			
			H^3 - Thymidine incorporation (cpm ± S.E. x10^{-3}) for different mitogens.			
	Lymph nodes	Spleen	0	Con A	PHA	LPS[b]
B6 +/bg	32.0 ± 0.7	106.6 ± 11.1	0.6 ± 0.1	60.8 ± 9.1	30.1 ± 10.8	0.6 ± 0.1
B6 bg/bg	28.6 ± 1.4	127.3 ± 25.7	0.5 ± 0.1	96.1 ± 10.8	75.0 ± 16.1	0.6 ± 0.1

a - 7 animals of each genotype were used. For each parameter tested, the data is presented as Mean ± S.E.

b - LPS was used as control.

Table 6.3. Spleen indices of B6C3H F_1 +/bg and bg/bg mice injected with either B6 bg/bg or +/bg parental lymphoid cells.

Donor Genotype	Recipient Genotype	Spleen Index at 4 and 12 days after GVH induction[a]	
		4	12
+/bg	+/bg	1.44 ± 0.03	1.95 ± 0.14
+/bg	bg/bg	2.36 ± 0.37	1.99 ± 0.17
bg/bg	bg/bg	1.88 ± 0.29	1.68 ± 0.35
bg/bg	+/bg	1.45 ± 0.07	1.70 ± 0.06

a. A minimum of 3 animals/group/day were used.

Table 6.4. PFC response to SRBC of B6C3H F_1 +/bg and bg/bg mice injected with either B6 +/bg or bg/bg PLC

GVH Combination		PFC/spleen ± S.E. x 10^3		PFC/10^6 spleen cells ± S.E.	
Donor Genotype	Recipient F_1 Genotype	Days after GVH[a] (% of normal response)		Days after GVH[a] (% of normal response)	
		8	26	8	26
	bg/bg	64.5 ± 5.6	74.0 ± 9.1	372.2 ± 44.6	517.5 ± 64.1
	+/bg	79.4 ± 8.2	86.6 ± 4.7	580.9 ± 151.9	635.5 ± 111.6
+/bg	+/bg	1.1 ± 1.1 (1.3)	0.1 ± 0.1 (0.1)	8.5 ± 5.7 (1.4)	0.9 ± 0.9 (0.1)
+/bg	bg/bg	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
bg/bg	bg/bg	10.1 ± 2.0 (15.6)	49.4 ± 14.8 (76.6)	86.2 ± 25.6 (23.1)	283.3 ± 44.1 (64.0)
bg/bg	+/bg	19.4 ± 12.4 (24.4)	5.5 ± 2.6 (6.9)	82.7 ± 56.3 (14.2)	52.0 ± 28.7 (8.98)

a. A minimum of 3 animals/group/day were randomly selected from a pool of animals. The data presented is the mean ± S.E.

Table 6.5. Splenic Con A and PHA mitogen responses of B6C3H +/bg and bg/bg F_1 mice injected with either B6 +/bg or bg/bg PLC.

GVH Combination		Con A response[a] on different Days after GVH (% of normal)		PHA response[a] on different Days after GVH (% of normal)	
Donor Genotype	Recipient F_1 Genotype	Days After GVH		Days After GVH	
		12	30	12	30
-	+/bg	93.8 ± 5.1	115.6 ± 0.9	137.3 ± 1.9	84.8 ± 3.6
-	bg/bg	105.6 ± 1.3	122.5 ± 7.9	154.1 ± 4.4	140.4 ± 2.1
+/bg	bg/bg	9.2 ± 8.8 (9.8)	10.7 ± 5.7 (9.2)	23.8 ± 22.7 (17.3)	13.1 ± 5.9 (15.5)
+/bg	+/bg	9.3 ± 4.0 (8.8)	19.1 ± 18.1 (15.5)	6.7 ± 1.5 (4.3)	20.0 ± 19.7 (14.2)
bg/bg	bg/bg	72.2 ± 11.7 (68.3)	102.1 ± 8.2 (83.3)	62.9 ± 2.5 (40.8)	89.3 ± 9.1 (63.6)
bg/bg	+/bg	55.3 ± 29.6 (58.9)	62.9 ± 14.1 (40.4)	55.9 ± 27.3 (40.6)	51.2 ± 4.6 (60.4)

a. A minimum of 3 animals/group/day were randomly selected from a pool of animals. The data is presented as the mean net cpm ± S.E. x 10^{-3}.

Table 6.6 Frequency of bg/bgF1 and +/bgF1 mice showing different intensities of histopathological lesions in the thymus, liver, and pancreas after the injection of B6 bg/bg or B6 +/bg PLC.

GVH Combinations[a]		Organs Examined for Lesions[b]	Intensity of Lesions (Frequency/Total)		
Donor	Recipient		Normal	Mild	Moderate-Severe
bg/bg	bg/bg F_1	Thymus	5/12	6/12	1/12
		Liver	9/12	3/12	0/12
		Pancreas	10/12	2/12	0/12
bg/bg	+/bg F_1	Thymus	5/6	0/6	1/6
		Liver	6/6	0/6	0/6
		Pancreas	6/6	0/6	0/6
+/bg	bg/bg F_1	Thymus	0/9	0/9	9/9
		Liver	0/9	2/9	7/9
		Pancreas	1/9	2/9	6/9
+/bg	+/bg F_1	Thymus	0/13	2/13	11/13
		Liver	2/13	4/13	7/13
		Pancreas	3/13	4/13	6/13

a GVH reactions were induced by injecting 50 x 10^6 B6 bg/bg or B6 +/bg PLC.

b Organs were removed on days 12 and 30 after GVH induction.

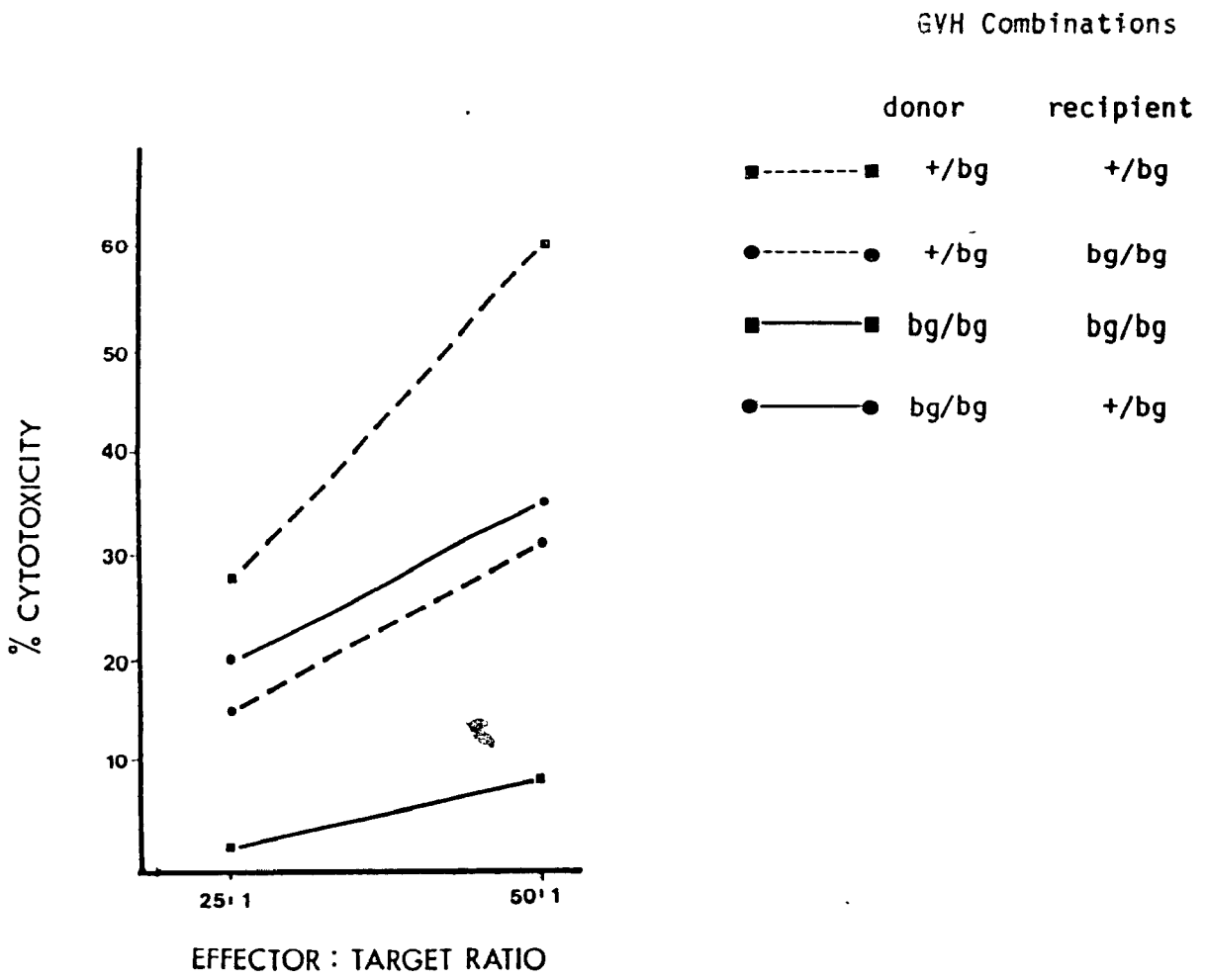


Figure 6.2. Splenic NK cell activity of bg/bgF1 and +/bgF1 mice injected with either B6 bg/bg or B6 +/bg PLC against YAC targets. Splenocytes were taken from F1 mice 4 days after the injection of PLCs. The effector:target cell ratio used was 50:1.

Table 6.7. Splenic NK cell activity observed against YAC targets in bg/bg F_1 and +/bg F_1 mice on different days after the injection of either B6 bg/bg or B6 +/bg PLC.

GVH Combinations[a]		% Cytotoxicity		
		Days Post-GVH Induction		
Donor Genotype	Recipient Genotype	4	12	30
+/bg	+/bg	61.26	10.67	5.67
+/bg	bg/bg	31.48	12.33	6.92
bg/bg	bg/bg	8.75	3.33	1.08
bg/bg	+/bg	35.20	9.33	4.33

a. Spleens from 4 animals/group/day were pooled. On each day, splenocytes from all the GVH groups and normal bg/bg F_1 & +/bg F_1 splenocytes were tested in the same cytotoxicity assay. The mean ± S.E. of the NK activitity of normal bg/bg F_1 and +/bg F_1 splenocytes on different days was 0.85 ± 0.32% and 9.07 ± 0.73%, respectively. The effector:target cell ratio used was 50:1.

Table 6.8 : Splenic P815 effector cell activity in bg/bg F_1 and +/bg F_1 mice on different days after the injection of either B6 bg/bg or B6 +/bg PLC.

GVH Combinations[a]		% Cytotoxicity	
Donor Genotype	Recipient Genotype	Days Post-GVH Induction	
		4	12
+/bg	+/bg	12.42	6.33
+/bg	bg/bg	11.38	9.33
bg/bg	bg/bg	12.78	8.00
bg/bg	+/bg	10.46	5.00

a. Spleens from four animals/group/day were pooled. On each day, splenocytes from all the GVH groups and normal bg/bg F_1 and +/bg F_1 splenocytes were tested in the same cytotoxicity assay. The mean + S.E. of P815 effector cell activity of normal bg/bg F_1 and +/bg F_1 splenocytes on different days was 0.97 + 0.68% and 2.75 + 0.68% respectivtly. The effector : target ratio used was 50 : 1.

CHAPTER SEVEN

THE ROLE OF SPLENOMEGALY AND THYMIC DYSPLASIA IN DETERMINING THE DURATION OF T-CELL IMMUNODEFICIENCY: THE KINETICS OF IMMUNE FUNCTIONAL RECOVERY AFTER GVH INDUCTION.

7.1 INTRODUCTION

The data presented in chapter 6 demonstrated that splenomegaly and thymic dysplasia observed during the course of GVH reactions may be unrelated events mediated by separate mechanisms. In addition, thymic injury, but not splenomegaly, may be important in inducing severe prolonged T-cell immunodeficiency.

In chapter 3 we demonstrated that, depending upon the number and genotype of PLC injected to induce GVH reactions, varying degrees of thymic dysplasia could be induced In this chapter we have further investigated the contribution of splenomegaly and thymic histopathological alterations in determining the duration of GVH-induced severe prolonged immunosuppression. We also describe the kinetics of regeneration of T- and B-cell immune function following GVH-induced severe immunosuppression.

7.2 EXPERIMENTAL DESIGN

The experimental design is outlined in Figure 7.1 GVH reactions were induced in B6AF1 hybrids by injecting different doses, 30, 20, or $10x10^6$, of either parental strain A or B6 lymphoid cells. Since the long-term effects of GVH reactions on immunosuppression were to be investigated, we initially studied the mortality of GVH-reactive B6AF1 mice in different groups.

In the next series of experiments the relationship between

early splenomegaly (days 8 and 16) and the severity of thymic injury was studied. Moreover, the association between early splenomegaly and the degree of thymic lesions was also investigated in relation to the duration of immunosuppression. During the studies on the mechanism(s) responsible for the duration of immunsuppression it was observed that animals that survived the initial moderate-severe thymic injury and severe immunosuppression started to recover from both the thymic damage and immunosuppression. However, during the immune recovery phase different numbers of PFC/spleen to SRBC were noted in these GVH mice. Since the PFC assay measures T-cell dependent B-cell responses, the gradual recovery of the PFC response to SRBC provided a model to study the kinetics of recovery of T-and B-cell function. We have, therefore, further investigated the relationship between the PFC response to SRBC, T-cell proliferative function, mitogen induced IL-2 production by T-cells, and B-cell proliferative function on individual mice during the immune recovery phase of the GVH reactions.

7.3 RESULTS

7.3.1. MORTALITY IN B6AF1 MICE INJECTED WITH DIFFERENT DOSES OF EITHER A OR B6 PLC

Table 7.1 shows the percent of B6AF1 mice surviving up to day 35 after the injection of different doses of either A or B6 PLC. As can be seen, at a given cell dose A strain PLC induced greater mortality than B6 PLC. By day 35 after PLC

injection, 20% of mice that received $30x10^6$A PLC and 80% of the mice that received $20x10^6$A PLC survived. However, no mortality was observed in the group of B6AF1 mice that were treated with $10x10^6$A PLC. On the other hand, 88% of the B6AF1 that were injected with $30x10^6$B6 PLC survived, whereas no mortality was observed in the groups that received either 20 or $10x10^6$B6 PLC.

7.3.2. DEGREE OF SPLENOMEGALY AND THYMIC LESIONS OBSERVED IN B6AF1 MICE INJECTED WITH DIFFERENT DOSES OF EITHER A OR B6 PLC:

Tables 7.2-7.4 and 7.5-7.7 demonstrate the degree of splenomegaly and thymic lesions observed in individual B6AF1 hybrids injected with different doses of either parental strain A or B6 lymphoid cells, respectively. A summary of the degree of splenomegaly and thymic lesions observed in different GVH combinations is presented in figure 7.2.

7.3.2.1 THE DEGREE OF SPLENOMEGALY OBSERVED IN DIFFERENT GVH COMBINATIONS

As can be seen, on day 8 after PLC injection, the degree of splenomegaly is dependent upon the number and genotype of PLC injected (Figure 7.2). The highest degree of splenomegaly on day 8, within a GVH genetic combination, is observed in the groups injected with either $30x10^6$ A or $30x10^6$ B6 PLC (Figure 7.2). When equal numbers of either A or B6 PLC are injected in B6AF1 mice, A PLC induce a greater degree of splenomegaly than B6 PLC. However, on day 16 post-PLC

injection, the degree of splenomegaly in the groups of B6AF1 hybrids injected with either $20x10^6$ A or $10x10^6$A PLC increased (as compared to the splenomegaly observed on day 8 post-PLC injections), and attained levels observed in the group of B6AF1 mice injected with $30x10^6$ A PLC (Figure 7.2) In contrast, in B6AF1 mice that received $30x10^6$ B6 PLC the degree of splenomegaly declined by day 16 post-PLC injection (as compared to that observed on day 8 post PLC injection), and reached the level observed in groups injected with $20x10^6$ or $10x10^6$ B6 PLC, which was close to normal (Figure 7 2).

7.3.2.2 THE DEGREE OF THYMIC LESIONS OBSERVED IN DIFFERENT GVH COMBINATIONS

Tables 7.2-7.4 and 7 5-7 7 also demonstrate the degree of thymic lesions observed in individual B6AF1 hybrids injected with different doses, $30x10^6$; $20x10^6$; and $10x10^6$, of either parental strain A or B6 lymphoid cells, respectively The data ARE summsrized in figure 7.2. As can be seen (Figure 7.2), the initial appearance of thymic lesions was dependent upon the dose and strain of PLC employed Thymic histopathological lesions in B6AF1 hybrids injected with either $30x10^6$ or $20x10^6$ A PLC started to appear by day 8 post-PLC injection and were graded as mild. In contrast, the thymuses of F1 hybrids injected with $10x10^6$ A PLC appeared to be normal (Figure 7.2). No thymic alterations were observed on day 8 post-PLC injection in the majority of B6AF1 hybrids that received either 30 , 20 , or $10x10^6$ B6 PLC (Figure 7.2 and Tables 7.5-7.7).

However, on day 16 post-PLC injection, severe lesions were observed in F1 hybrids injected with either $30x10^6$ or $20x10^6$ A strain lymphoid cells. Only mild thymic lesions were observed in F1 mice injected with $10x10^6$ A PLC (Figure 7 2) In contrast, moderate to severe thymic lesions were observed in F1 mice that received $30x10^6$ B6 PLC. The groups of F1 hybrids that received either $20x10^6$ or $10x10^6$ B6 lymphoid cells showed no thymic changes (Figure 7.2)

On day 30 after GVH induction severe thymic lesions persisted in F1 mice that received $20x10^6$ and $30x10^6$ A PLC, whereas F1 mice that received $10x10^6$ A PLC displayed a completely normal thymic architecture (mice in this group displayed mild thymic lesions on day 16 post-PLC injection) (Figure 7.2). On day 30 after PLC injection, moderate thymic lesions were observed only in the group of F1 mice that received $30x10^6$ B6 PLC, whereas completely normal thymuses were observed in F1 mice that received either 10 or $20x10^6$ B6 PLC (Figure 7.2). The characteristics of the different degrees of thymic lesions are described in detail in chapter 5 (Section 5.3 3.1).

Collectively, the data presented in tables 7 2-7.7 and summarized in figure 7.2 show that Although the degree of early splenomegaly may be able to predict the severity of thymic lesions, this relationship may be true only in a given GVH combination, i.e., the degree of early splenomegaly in one GVH combination may not be able to predict the severity of thymic

lesions in a different GVH combination. Furthermore the data also suggest that the maximum degree of splenomegaly cannot predict the severity of thymic lesions. It would therefore appear that splenomegaly and thymic lesions may be mediated by separate mechanisms

7.3.3. THE KINETICS OF REGENERATION OF THYMIC ARCHITECTURE FOLLOWING GVH-INDUCED MODERATE-SEVERE LESIONS

Figure 7.3 demonstrates the kinetics of thymic regeneration following GVH induced thymic dysplasia Figure 7.3 shows that the GVH dysplastic thymuses recovered and regained a completely normal architecture, with time post-PLC injections. The time required for the thymuses to recover was dependent upon the initial severity of the thymic lesions [compare thymic recovery of F1 mice injected with $10x10^6$ A (Figure 7.2), $30x10^6$ B6, and $20x10^6$ A PLC (Figure 7.3)]. The groups of F1 hybrids injected with ~~$10x10^6$~~ A PLC showed only mild initial thymic lesions (day 16) and their thymuses recovered structure by day 30 post-PLC injections (Figure 7.2). The group of F1 hybrids that received $30x10^6$ B6 PLC showed moderately severe lesions initially (days 16 and 30) and their thymuses recovered by day 130 post-PLC injection (Figure 7.3). Finally, the group of F1 mice that received $20x10^6$ A strain PLC showed severe thymic lesions on days 16 and 30 post-PLC injection and these thymuses took the longest period of time to recover, almost 160 days post-PLC injection (Figure 7.3).

7.3.3.1 DESCRIPTION OF THYMIC HISTOPATHOLOGICAL CHANGES OBSERVED DURING THYMIC REGENERATION:

In chapter 5 we described moderate and severe thymic dysplasia In both moderate and severe thymic dysplasia a complete loss of cortico-medullary demarcation was observed Furthermore, in severely dyplastic thymuses intense lymphocytic infiltrates were observed in the medulla on day 16 after GVH-induction. However, on day 30-35 after GVH induction, the moderate and severe dysplastic thymuses became depleted of lymphocytes Representative photomicrographs of such a hypocellular thymus are shown in Figures 7 4a and 7 4b Beyond day 30-35 after GVH-induction, various stages of thymic regeneration were observed (Figure 7 3) The different stages of thymic regeneration were characterized as follows

(i) **FIRST STAGE:** These thymuses demonstrated an increase in size following drastic prior involution. This increase in size was attributed to repopulation of the cortex with lymphocytes The lymphocytes were densely packed and the cortex resembled that of a normal thymus and was clearly distinct from the medulla The medulla of such thymuses (stage 1) was still hypocellular (depleted) and no individual epithelial cells, epithelial cell clusters, or Hassall's corpuscles were observed. Thus, the stage 1 of the regeneration process was characterized by a complete regeneration of the thymic cortex, whereas the medulla displayed characteristic GVH-associated dysplasia. A representative thymus showing stage 1 of regeneration is shown in figure 7.5a.

(ii) SECOND STAGE: These thymuses were characterized by the following changes: increased size, densely-packed cortex with lymphocytes, and distinct cortico-medullary delineation. In localized areas of the medulla of such thymuses, although "large" pale individual epithelial cells, and epithelial cell clusters were rarely observed, several "small" dark irregularly shaped individual epithelial cells were observed No Hassall's corpuscles were visible. The "small" dark individual epithelial cells appeared similar to epithelial cells that were observed in the medulla of mildly dysplastic thymuses as shown in chapter 5, (Figure 5.4b) The medulla of the thymuses in stage 2 of regeneration was hypocellular. The above described changes suggested that the process of medullary epithelial cell regeneration had started. Thus, in stage 2 of the regeneration process, partial regeneration of the thymic medulla, besides complete regeneration of the cortex was observed. A representative medulla of such a thymus is shown in figure 7.5b.

(iii) THIRD STAGE: The GVH thymuses in this stage of recovery possessed, besides a normal cortex and a distinct cortico-medullary junction, abundant "large" pale individual epithelial cells and epithelial cell clusters in the medulla. However, only a few sparcely distributed "small" dark individual epithelial cells were observed. Also, only rare Hassall's corpuscles were observed. The medulla of thymuses in stage three of regeneration also showed repopulation with

lymphocytes. A representative thymus showing stage three of regeneration is shown in figures 7.6a and 7.6b.

(iv) **FOURTH STAGE:** This stage was characterized by complete regeneration of both the cortex and the medulla. In such thymuses, abundant "large" pale epithelial cells, epithelial cell clusters, and Hassall corpuscles were distributed throughout the thymic medulla Thus, the architecture of such thymuses was indistinguishable from that of a normal (non-GVH) thymus. A representative thymus showing stage four of regeneration is shown in figure 7 7.

The characteristics of various stages of thymic regeneration are summarized in table 7.8

7.3.4. THE KINETICS OF SUPPRESSION AND SPONTANEOUS RECOVERY OF PFC RESPONSE TO SRBC IN B6AF1 MICE INJECTED WITH DIFFERENT DOSES OF EITHER A OR B6 PLC:

Table 7.9 shows the PFC response to SRBC of normal B6AF1 mice used as controls on different days after GVH induction. Tables 7.10-7.12 and 7.13-7.15 demonstrate the kinetic of early suppression and the later spontaneous recovery of the PFC response to SRBC of B6AF1 mice that received different doses ($30x10^6$, $20x10^6$, or $10x10^6$) of either A or B6 PLC. The data are summarized in figure 7.8. As can be seen, by day 4 post-PLC injection, the suppression of PFC response to SRBC in F1 hybrids is dependent upon the number and strain of PLC injected. However, the degree of suppression of the PFC

response to SRBC in the F1 hybrids injected with different doses of A strain lymphoid cells was greater than the degree of suppression in F1 hybrids injected with the same dose of B6 lymphoid cells (Figure 7.8). Furthermore, by day 12 post-PLC injection, complete suppression of the PFC responses to SRBC was observed in all groups of F1 mice injected with A strain lymphoid cells On the other hand, total suppression of the PFC response to SRBC was observed only in the group injected with $30x10^6$ B6 PLC (Figure 7.8).

The data presented in figure 7.8 further show that F1 hybrids in different GVH-reactive groups which had become totally suppressed earlier after GVH induction for the PFC response to SRBC, started to respond to SRBC at various times post-PLC injection. The group of F1 hybrids injected with $10x10^6$ A PLC recovered from total immunosuppression and started to respond to SRBC by day 60 post-PLC injection. F1 hybrids that received $30x10^6$ B6 PLC started to show recovery of the PFC response to SRBC by day 120-130, and on day 150 post-PLC injection all mice in this group responded to SRBC. Similarly, the group of F1 mice injected with $20x10^6$ A PLC started to recover from total suppression by day 150-160, and on day 180 after PLC injection all animals in this group responded to SRBC in the PFC assay.

7.3.5. SPLENIC CELLULARITY OF B6AF1 MICE INJECTED WITH DIFFERENT DOSES OF EITHER A OR B6 PLC:

Table 7.16 shows the splenic cellularity of B6AF1

mice used as controls on different days after GVH induction Tables 7.17-7.19 and 7.20-7.22 show the kinetics of depletion and repopulation of splenic mononuclear cellularity of B6AF1 mice following the injection of various doses of either A or B6 PLC, respectively. The data are summarized in figure 7 9 As can be seen, the splenic cellularity of B6AF1 mice that received $30x10^6$ A and $20x10^6$ A PLC started to decline by day 12 post-PLC injection and reached approximately 30% of the normal splenic cellularity by day 34 post PLC injection (Figure 7 9) On the other hand, splenic mononuclear cellularity of B6AF1 mice that received $10x10^6$ A PLC increased slightly on day 16, but then declined to approximately 60% of the normal splenic cellularity by day 34 post-PLC injection (Figure 7.9) In the groups of B6AF1 mice that received different doses of B6 PLC, depletion of splenic cellularity was observed only in the group that received $30x10^6$ PLC (approximately 28% of the normal) on day 34 post-PLC injection (Figure 7.9).

Beyond day 34 post-PLC injection, various degrees of repopulation of spleens with mononuclear cells were observed. The time required by the B6AF1 mice that received $10x10^6$ A, $20x10^6$ A, or $30x10^6$ B6 PLC, to achieve normal/near-normal splenic repopulation varied. B6AF1 mice that received $10x10^6$ A showed normal splenic cellularity by day 64; B6AF1 mice that received $30x10^6$ B6 PLC displayed normal/near-normal splenic cellularity by day 124-154; and B6AF1 mice that received $20x10^6$ A PLC required 184 days post-PLC injection to achieve normal splenic mononuclear cellularity (Figure 7.9).

Since the recovery of the PFC response to SRBC and splenic cellularity was gradual, i.e., variations in the number of PFC response to SRBC and splenic cellularty were observed in individual mice on a given day during the recovery of the PFC response (see table 7.11 day 150-160 and table 7.13 days 120-130), we investigated the relationship between splenic cellularity, T-cell proliferative responses to Con A and PHA, IL-2 production by T-cells following stimulation with Con A and PHA, and B-cell proliferative responses to LPS in GVH-reactive B6AF1 mice (that received $30x10^6$ B6 PLC) that possessed different numbers of PFC/spleen during the immune recovery phase

7.3.6 SPLENIC CELLULARITY AND THE PFC RESPONSE TO SRBC OF NORMAL B6AF1 MICE

Table 7.23 shows splenic mononuclear cellularity and PFC/spleen and PFC/10^6 spleen cell response to SRBC of normal B6AF1 mice used as controls for different experiments. Nine separate experiments were performed. The splenic cellularity and the PFC data for individual experiments presented in table 7.23 were used to calculate the % of normal cellularity and PFC response of GVH mice for a given experiment. This protocol was employed to standardize the variations observed in normal mice from experiment to experiment.

7.3.7 SPLENIC CON A, PHA, AND LPS RESPONSES OF NORMAL B6AF1

MICE.

Table 7.24 shows the splenic Con A, PHA, and LPS responses of normal B6AF1 mice that were used as controls. The mitogen data presented are from the same mice for which the splenic cellularity and the PFC data are presented in Table 7.23. Splenocytes from 3-4 normal mice were pooled The data represent the mean ± S E of triplicate cultures These mitogen responses for a given experiment were used to calculate the % of normal Con A, PHA, and LPS responses of GVH splenocytes in that experiment

7.3.8 RELATIONSHIP BETWEEN SPLENIC CELLULARITY, PFC RESPONSE TO SRBC, AND CON A, PHA, AND LPS RESPONSES OF GVH MICE DURING IMMUNE RECOVERY

The GVH mice were divided into four groups since variations in the numbers of PFC/spleen response to SRBC were observed during the immune recovery phase of the GVH reaction. In one group all the mice in different experiments that did not respond to SRBC were pooled. In the other group the mice, in different experiments, that responded to SRBC to normal/near-normal levels (30.30-upx10^{-3} PFC/spleen) were pooled. All remaining mice that possessed 0.3-30.30x10^{-3} PFC/spleen (intermediate range) were divided into two separate groups.

The data obtained for splenic cellularity, PFC response to SRBC, and the Con A, PHA, and LPS responses from GVH mice with different numbers of PFC/spleen are presented in Tables 7.25-

7.28 The data for each of the parameters of individual animals are presented as % of normal response. The % of normal responses was measured by using the response of normal mice in that experiment as 100% for each parameter.

Table 7.25 shows the splenic cellularity and Con A, PHA, and LPS responses of GVH mice that possessed no PFC to SRBC The data show that when cellularity and mitogen responses of individual mice were studied, a lack of correlation between these two parameters was observed. The data in this group of GVH mice (no PFC) also show that in 11/21 mice, although the Con A and PHA responses were less than 30%, the LPS responses varied between 62-235% of normal. Furthermore, when the % of normal response for each parameter was pooled and expressed as the mean in this group of GVH mice (0 PFC) the following was observed: cellularity 60%, Con A response 23%, PHA response 9% and LPS response 90% of normal (Table 7.25). The pooled data for each parameter are summarized in figure 7.10.

Table 7.26 demonstrates the relationship between splenic cellularity, PFC/10^6 spleen cell response to SRBC, and Con A, PHA, and LPS responses of GVH mice that possessed 0.3-15.00 x 10^{-3} PFC/spleen. The data presented in table 7.26 show a lack of correlation between the splenic cellularity and the PFC/10^6 spleen cell response to SRBC in individual mice. Moreover, no correlation between the PFC/10^6 spleen cell response to SRBC and Con A, PHA, and LPS responses was observed. When the percent of normal response of different parameters for

individual mice in this group ($0.3\text{-}15.0\times10^{-3}$ PFC/spleen) was pooled and expressed as the mean response, the following pattern was observed: splenic cellularity 110%, PFC/10^6 spleen cell response to SRBC 6%, Con A response 82%, PHA response 44%, and LPS response 112% of normal. The pooled data are shown in figure 7.10.

Table 7.27 shows the relationship between splenic cellularity, PFC/10^6 spleen cell response to SRBC, and Con A, PHA, and LPS responses of GVH mice that possessed 15 $30\text{-}30.00\times10^{-3}$ PFC/spleen. A comparason of data (% of normal response) of different parameters of an individual animal in this group again shows a lack of correlation between splenic cellularity and PFC/10^6 spleen cell responses and between PFC/10^6 spleen cell responses and Con A, PHA, and LPS responses. The mean results for various parameters in this group show the following: splenic cellularity 123%, PFC/10^6 spleen cell response 20%, Con A response 88%, PHA response 69%, and LPS response 108% of normal. The pooled data for each parameter are expressed in figure 7.10.

Table 7.28 shows the relationship between splenic cellularity, PFC/10^6 spleen cells, Con A, PHA, and LPS responses of GVH mice that possessed 30.30 and above x 10^{-3} PFC/spleen to SRBC. In this group a lack of correlation between splenic cellularity and PFC/10^6 spleen cell response to SRBC and between the PFC/10^6 spleen cell response to SRBC and Con A, PHA, and LPS responses in individual animals was also observed. The mean data for various parameters in this group (30.30 and

up x 10^{-3} PFC/spleen) show the following: splenic cellularity 133%, PFC/10^6 spleen cell response to SRBC 93%, Con A response 90%, PHA response 86%, and LPS response 102% of normal.

Figure 7.10, which summarizes the data presented in tables 7.25-7.28, shows the mean response of each parameter as a function of PFC/spleen response to SRBC. Collectively, the data in figure 7.10 suggest that during the immune recovery phase the kinetics of immune functional recovery may be as follows : splenic cellularity and LPS response, Con A responses, PHA responses, and, finally, PFC response to SRBC.

7.3.9 IL-2 PRODUCTION BY NORMAL B6AF1 SPLENOCYTES FOLLOWING CON A AND PHA STIMULATION

Table 7.29 shows the 3H-thymidine incorporation by IL-2 dependent CTL-L cell in the presence of IL-2 containing supernatants derived from normal splenocytes following their stimulation with either Con A or PHA. Supernatants derived from LPS stimulated normal splenocytes were also tested for their ability to support growth of CTL-L cells in some experiments and sereved as negative controls. The data presented in Table 7.29 were used to determine the % of normal response (3H-thymidine incorporation) of supernatants derived from GVH splenocytes after Con A or PHA stimulation. The data for each experiment (Table 7.29) are presented as the mean±S.E of triplicate cultures.

7.3.9 RELATIONSHIP BETWEEN SPLENIC CELLULARITY, PFC RESPONSE TO SRBC, AND IL-2 PRODUCTION BY GVH SPLENOCYTES FOLLOWING CON A AND PHA STIMULATION

Splenocyte IL-2 production was assessed in the same animals for which the cellularity, PFC response to SRBC, Con A, PHA, and LPS responses were studied (Tables 7.25-7.28). The data obtained for the proliferation (3H-thymidine incorporation) of CTL-L cells in the presence of IL-2 containing supernatants derived from GVH splenocytes following stimulation with Con A or PHA are presented in tables 7.30-7.32. The data are expressed as % of normal response

Table 7.30 shows the IL-2 production after Con A and PHA stimulation by GVH splenocytes of mice that expressed no PFC to SRBC. The data show that the % of normal amount of IL-2 produced after Con A stimulation was greater than that produced after PHA stimulation of splenocytes of the same animal (43% versus 17%, respectively). The pooled data are expressed in figure 7.11.

Tables 7.31 and 7.32 demonstrate IL-2 production following stimulation with Con A or PHA by GVH mice that possessed 0.3-15.00, 15.50 and more x 10^{-3} PFC/spleen, respectively, . The data show that in each of these group the Con A stimulated splenocytes produced greater amounts of IL-2 than PHA stimulated splenocytes. The pooled data are shown in figure 7.11.

7.4. DISCUSSION

The results presented in this chapter show that the duration of GVH-induced prolonged immunosuppression is closely associated with the severity of GVH-induced initial thymic histopathological changes, rather than with the degree of GVH induced splenomegaly. The GVH induced dysplastic thymuses gradually recover from the thymic injury with time after GVH induction. The thymic architectural recovery precedes the recovery of immune responses (PFC). The recovery of the immune response, like the thymic architectural recovery, is also gradual. The data further show that during the immune functional recovery the splenic B-cell proliferative function recovers earlier than the splenic T-cell proliferative function. Moreover, the T-cell population that is responsive to Con A recovers more rapidly than the PHA responsive T-cells The PFC responses reappear only after the Con A and PHA responses have recovered partially(80% and 45% of the normal, respectively). Thus, the kinetics of recovery of immune function following their severe suppression appear to be in the following order: LPS responsive B-cell proliferative function, Con A responsive T-cells proliferative function, PHA responsive T-cells proliferative function, and finally PFC response to SRBC.

The results presented in this chapter provide interesting information regarding the relationship between the degree of splenomegaly (Figure 7.2), thymic injury (Figure 7.2), and duration of GVH induced prolonged immunosuppression (Figure

7.8) (also see figure 7.12). The groups of F1 hybrids injected with either $10x10^6$ A or $20x10^6$ A PLC show that on day 8 post-GVH induction the degree of splenomegaly (Figure 7.2) is dependent upon the number of PLC injected. However, by day 16 after GVH induction the two groups of GVH-reactive B6AF1 mice exhibited approximately the same degree of splenomegaly but marked differences in both the degree of thymic injury (Figure 7.2) and duration of immunosuppression (Figures 7.8 and 7.12). Similarly, the maximum degree of splenomegaly in the groups of B6AF1 mice injected with $30x10^6$ B6 PLC was observed on day 8, whereas, by day 16 post-GVH induction, the degree of splenomegaly had declined. In contrast, mild splenomegaly was observed on day 8, but increased dramatically by day 16 post-GVH induction in the groups of B6AF1 mice injected with $10x10^6$ A PLC. Inspite of these differences in the kinetics of splenomegaly (Figure 7.2), the groups of B6AF1 mice that received $30x10^6$ B6 lymphoid cells exhibited moderately severe thymic lesions (Figure 7.2) by day 16 post-GVH induction and remained severely immunosuppressed for up to 150 days after GVH induction (Figures 7.8 and 7.12). In contrast, the groups of F1 mice that received $10x10^6$ A parental lymphoid cells showed only mild thymic histopathological lesions on day 16 post-GVH induction and no thymic lesions were detected by day 30 post-GVH induction. Moreover, these GVH reactive animals recovered from immunosuppression by day 60 post-GVH induction. These results suggest that in the GVH combinations used in the present study (A-->B6AF1 and B6-->B6AF1) the maximum degree

of splenomegaly may not be able to predict either the degree of thymic injury or duration of GVH induced immnuosuppression. The duration of prolonged immunosuppression of the PFC response to SRBC may be determined by the degree of initial thymic injury (Figure 7.12). However, the rapidity with which splenomegaly is induced following GVH induction may be a more useful indicator to predict the degree of thymic injury and, thus, the duration of immunosuppression within a GVH genetic combination. Furthermore, these data suggest that the mechanism(s) responsible for inducing splenomegaly and thymic injury may be totally independent. Continued splenomegaly may not influence the degree of GVH- induced thymic injury. These results support data presented in chapter 6 which also showed that the degree of splenomegaly could neither predict the degree of thymic lesions nor the induction of severe prolonged immunosuppression

The results presented in this chapter also show that the GVH induced thymic injury is not an all or none phenomenon, but the severity of GVH induced thymic lesions is dependent upon both the number and strain of PLC injected to induce GVH reactions (Figure 7.2; also see chapter 5). More important, the data presented in this chapter show that the GVH dysplastic thymuses recover structure gradually and regain a normal thymic architecture (cortico-medullary demarcation, reappearance of medullary epithelial cells and Hassall's corpuscles) with time post-GVH induction (Figure 7.3 and Figures 7.4-7.7). The time required for the dysplastic thymuses to regain normal

architecture is determined by the initial severity of thymic injury [compare thymic recovery in B6AF1 mice injected with $10x10^6$ A, $30x10^6$ B6, and $20x10^6$ A PLC, Figures 7.2 and 7.3] In GVH reactive groups in which complete destruction of thymic architecture was observed (complete depletion of the thymic cortex, obliteration of cortico-medullary demarcation, and complete loss of medullary epithelial cells and Hassall's corpuscles) i.e., the groups of B6AF1 mice that received $30x10^6$ B6 and $20x10^6$ A PLC, the time required for both cortical as well as medullary regeneration was determined by the initial severity of the thymic lesion (Figure 7.3). In B6AF1 mice that received $30x10^6$ B6 PLC the initial severity of thymic lesions was graded as moderate on day 30 post-GVH induction. The thymuses in this group displayed cortical regeneration (stage 1) by day 55 post-GVH induction (Figure 7.3). These thymuses required approximately 25 days for cortical regeneration. In addition, this combination ($30x10^6$ B6-->B6AF1) showed complete medullary regeneration by day 120-130 post -GVH induction Therefore, these thymuses required another 65-75 days for medullary regeneration after the cortex had regenerated (Figure 7.3). On the other hand, in B6AF1 mice that received $20x10^6$ A PLC the initial intensity of thymic lesions was graded as severe on day 30 post-GVH induction. These thymuses required approximately 45 days for cortical regeneration (stage 1) and another 85 days for complete medullary regeneration (Figure 7.3). In both the GVH combinations employed in this study it is evident that a longer time interval is needed for complete medullary regeneration after the cortical recovery than the

time interval needed for cortical regeneration after moderate-severe thymic dysplasia (Figure 7.3).

The kinetics of thymic regeneration as described in this chapter show that the process of thymic recovery occurs in the following sequence: cortical regeneration followed by medullary regeneration. This sequence of events that occurs during thymic regeneration is similar to events observed during thymic ontogeny during embryonic development in birds (LeDourin and Jotereau, 1975), mice (Moore and Owens, 1967; Owens and Ritter, 1969), sheep (Mackey et al., 1986), and man (Haynes, 1984). However, the time taken by the thymus to regenerate following GVH induced injury is significantly prolonged, as compared to that observed during ontogeny. These time disparities in the thymic development during the GVH reaction and fetal life may reflect the environmental differences under which the thymus develops. The explanation for the length of time required for complete thymic architectural recovery following GVH induced injury depends upon the initial severity of the thymic injury is not clear. However, it is possible that the initial intensity of the GVH reaction might determine the generation and intensity with which mechanism(s) responsible for inducing as well as maintaining the thymic lesions are induced during the early period after GVH induction. Our recent studies suggest that a radio-sensitive mechanism(s) is involved in maintaining thymic lesions following GVH reaction induction (Seddik et al., 1984b). The data presented in this report would suggest that when GVH reactions are induced by

injecting smaller doses of PLC and if these GVH-reactive animals survive for long periods of time, the mechanism(s) that maintains thymic lesions may subside gradually with time after GVH induction.

The observation that thymic medullary regeneration takes a longer time than cortical regeneration during the recovery phase is of particular interest in regards to the duration of GVH induced prolonged immunosuppression. Previous data reported from this laboratory strongly suggested that the GVH-induced prolonged T-cell immunodeficiency was, at least in part, associated with the GVH-induced thymic medullary injury that prevented T-helper cell maturation (Seddik et al , 1979,1980, 1984b). The results presented in this report show that the recovery of GVH induced thymic lesions precedes the recovery of the PFC response to SRBC, which is a T-cell dependent B-cell response (Figure 7.12). The observation would sugggest that perhaps complete thymic medullary recovery may be the limiting factor in the recovery of the PFC response to SRBC and that a normal thymic architecture may be essential for the T-cell dependent B-cell responses.

However, a comparison of the kinetics of thymic recovery from injury and recovery of the PFC response to SRBC from GVH-induced immunosuppression shows a "lag period" between the thymic architectural recovery and recovery of the PFC response to SRBC (Figure 7.12). We have observed that although the thymuses may appear architecturally normal, the animals may or

may not respond to SRBC. The F1 mice that were injected with either $20x10^6$ A PLC (Table 7.11; days 150-160, after GVH induction) or $30x10^6$ B6 PLC (Table 7.13; days 120-130 after GVH induction) displayed a normal thymic architecture, however, only a few animals responded to SRBC in the PFC assay, while others did not. The precise reasons for the presense of the "lag period" (lack of PFC response although the thymuses have regained a normal architecture) and the variations in the number of PFC to SRBC observed during the immune recovery phase are not clear. However, at least, two possibilities should be considered: (i) the defect may be in the functional potential of the T-cell and/or B-cell, since the PFC response to SRBC is a T-cell dependent B-cell response, (ii) that although the thymuses recover structurally from the GVH induced thymic injury, probably they do not resume their hormonal function, (i.e., functional recovery of the thymus may be gradual) which may take a longer time to recover.

To understand the reasons for the delay in the recovery of the PFC response as well as the variations observed in the PFC responses, we have also investigated the kinetics of recovery of B-cell and T-cell function and their relationship to the PFC response to SRBC during the immune recovery phase of the GVH reactions.

A comparison of B-and T-cell proliferative responses during the immune recovery phase of the GVH reaction demonstrates that the B cell proliferative responses recover earlier than the T-cell proliferative responses (Figure 7.10),

following their earlier severe suppression. We have previously demonstrated that the prolonged B-cell suppression following GVH induction is due to an arrest in the production of pre-B and B-cells in the bone marrow and severe depletion of B-cells in the spleen (Xenacostas et al., 1986, in press). On the other hand, the prolonged suppression of T-cell function is, at least in part, due to the GVH induced thymic dysplasia (Seddik et al., 1979,1980). The thymic dysplasia results in a maturational arrest of IL-2 producing T-helper cells (Mendez et al., 1985a, 1985b). Collectively, these studies suggest that the GVH associated immunosuppression of both the B- and T-cell function is due to the GVH-associated alterations in the primary lymphoid organs which are responsible for the production and maturation of B- and T-cells. It would therefore appear that, for the B-and T-cells to resume function following severe immunosuppression, the primary event/process must be the regeneration of architecture and resumption of function of the primary lymphoid organs responsible for the production and maturation of these cells.

Recent studies from this laboratory have shown that by day 60-80 after GVH induction a spontaneous, but gradual, increase in the number of pre-B and B-cells is observed in the bone marrow, suggesting the recovery of B-cell genesis following its cessation. The gradual recovery in bone marrow pre-B and B-cell numbers coincide with the recovery of B-cells in the spleen (Xenocostas et al., 1986). These "new" splenic B-cells were responsive to LPS (Xenacostas et al., 1986; Ghayur et al.,

1986a,b). This sequence of increase in bone marrow pre-B and B-cell numbers, as well as the recovery of splenic LPS responsive B-cells, suggests that the recovery of B-cell genesis in the bone marrow may be the limiting factor in the recovery of B-cell function (proliferative responses).

Since the production and maturation of T-cells is dependent upon a normal thymus (Stutman, 1975,1977, 1978), the data presented in this chapter showing a gradual recovery of T-cell proliferative function (Figure 7.10) would suggest that after the recovery/regeneration of thymic architecture the resumption of its function may also be gradual. The data presented in this chapter show that the rate of regeneration of Con A responsive T-cells is faster than the rate of regeneration of PHA responsive T-cells (Figure 7.10), suggesting that these two T-cell sub-populations may have different degrees of requirements/dependence upon the thymus. It is believed that Con A responsive T-cells are less mature than the PHA responsive T-cells (Schlesinger et al., 1975; Irle et al., 1978; Cantor and Weissman, 1976; Stobo and Paul, 1973). Scollay (1982,1984) and Scollay et al (1984) have shown that the T-cells that leave the thymic medulla and populate the peripheral lymphoid organs exhibit properties of mature T-cells. Moreover, several workers (Kaislerlian et al., 1981; Takiguchi et al., 1971; Folch and Waksman, 1972; Anderson et al., 1974) reported that PHA responsive T-cells are more sensitive to thymectomy than Con A responsive T-cells. Collectively, these studies suggest that the PHA responsive T-

cells may be more dependent upon an intact thymus or thymic medulla. Whether these PHA responsive cells require both thymic medullary epithelial cell contact as well as thymic hormones or thymic hormones alone, is not clear. However, in light of the studies mentioned above, the data presented in this chapter showing that the recovery of PHA responsive cells is slower than recovery of Con A responsive cells would suggest that the PHA responsiveness during the recovery phase may reflect the gradual functional regeneration of the thymus (thymic medulla?).

A comparison of the recovery of the T-cell and B-cell proliferative function and the recovery of the PFC response to SRBC also shows that the PFC response to SRBC remains severely suppressed even though the T-and B-cell proliferative function have regenerated to normal/near-normal levels (Figure 7.10) Moreover, the data presented in Table 7.25 show that in some mice which had 0 PFC, the Con A and PHA responses were still severely suppressed, yet B-cell proliferative responses were normal/near-normal and, in some instances highly augmented (Table 7.25). Furthermore, when the T-and B-cell proliferative responses were plotted as a function of PFC/spleen response to SRBC, it was observed that although Con A and PHA proliferative responses were approximately 90% and 70% of normal, the PFC/10^6 spleen cell response was still severely suppressed (about 20% of the normal) (Figure 7.10). However, after the initial recovery of T-cell responses (about 90% for Con A and 70% for

PHA of the normal resposnes) the further increase (recovery) in T-cell responses was less dramatic but the increase (recovery) in the PFC response was very dramatic (Figure 7.10). These results suggest that probably after the recovery of proliferative function , the regenerating immune system requires further maturation to acquire the capability to ellicit T-B-cell cooperative responses. On the other hand, it is also possible that :(i) the T-cells cells that respond to Con A and PHA and the B-cells that respond to LPS, respectively, are different (separate lineage) from those T-cells and B-cells that are required for the T-B cell cooperative responses; (ii) the T-cells and B-cells may be normal, however, the defect may be in antigen presenting cells The data presented in this chapter do not provide information for or against either of these possibilities.

However, in light of the observation that the gradual thymic architectural recovery precedes the recovery of the PFC response (Figure 7.12) and the data showing that the T-and B-cell proliferative function recover earlier than T-B cooperative function (7.10), it would appear that perhaps the limiting factor in the recovery of the PFC response to SRBC may be the functional maturation of T-cells that can cooperate and/or provide help to B-cells. The functional maturation of T-cells that can cooperate with the B-cells would depend upon the the gradual functional recovery of the thymus following its architectural recovery.

In brief, these data demonstrate that the GVH induced

In brief, these data demonstrate that the GVH induced thymic injury and splenomegaly are probably mediated by separate mechanisms. The GVH-induced thymic injury is not an "all or none" phenomenon. Since thymic recovery preceedes recovery of the PFC response to SRBC, the data also suggests that the GVH-induced thymic injury may, at least in part, determine the duration of GVH-induced T-cell immunosuppression. The studies presented in this chapter also suggest that the kinetics of immune functional recovery in an adult animal may be as follows : LPS responses, Con A responses, PHA responses, and finally T-cell dependent B-cell responses.

The data presented in this chapter showing a gradual regeneration of thymic architecture and immune function suggest that the thymic abnormalities, either structural or functional or both, may be involved in GVH-induced prolonged T-cell immunosuppression (and possibly other immunodeficiency states) The gradual histological recovery of the thymus followed by immune functional recovery provides an *in vivo* model to study thymic ontogeny in an adult animal as well as its role in the production of immunocompetent T-cells.

In the following chapter we have investigated whether the immunocompetent T-cells that respond to Con A and PHA require only the physical presense of thymic medullary epithelial cells or that the functional maturation of the mitogen responsive T-cells requires both the presense of medullary epithelial cells as well as some factor(s).

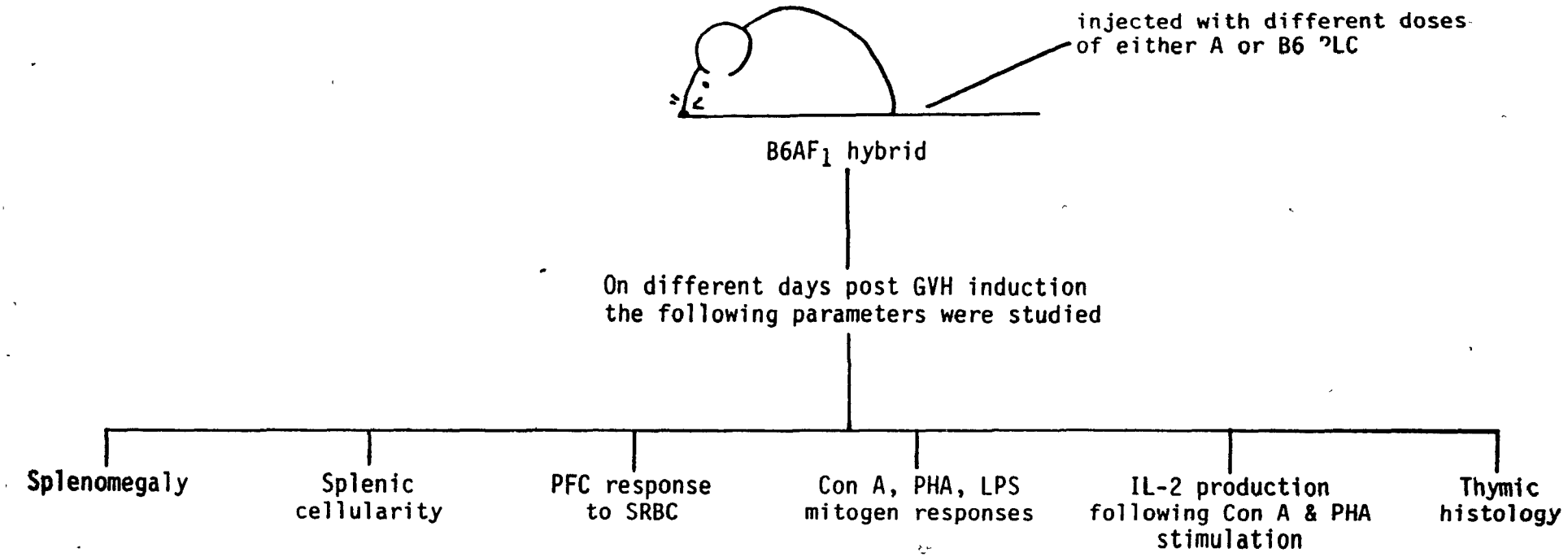


Figure 7.1 Experimental design used to investigate the role of splenomegaly and thymic dysplasia in determining the duration of T-cell immunodeficiency during the course of GVH reactions.

Table 7.1 . Survival times of $B6AF_1$ mice injected with different doses of parental strains A or B6 lymphoid cells

Donor Strain	No. of donor cells injected	No. of $B6AF_1$ mice per group	No. of $B6AF_1$ mice surviving up to 35 days post GVH induction	% Survival
A ($H\text{-}2^a$)	30×10^6	25	5	20
A	20×10^6	25	20	80
A	10×10^6	25	25	100
B6 ($H\text{-}2^b$)	30×10^6	25	22	88
B6	20×10^6	25	25	100
B6	10×10^6	25	25	100

Table 7.2 The degree of splenomegaly and thymic lesions observed in $B6AF_1$ mice on different days after the injection of 30×10^6 A PLC

Degree of Splenomegaly and thymic lesions in individual animals after GVH induction					
	Day 8		Day 16		Day 30
	Splenomegaly	Thymic Lesions	Splenomegaly	Thymic Lesions	Thymic Lesions
	3.59	Mild	3 12	Severe	Severe
	3 25	Mild	2 85	Severe	Severe
	3.12	Mild	3.41	Severe	Severe
	3 72	Mild			Severe
	3 84	Mild			
	4 30	Normal			
	4.11	Normal			
Mean + S E	3 70 ± 0 16		3 13 ± 0 16		

Table 7 3 The degree of splenomegaly and thymic lesions observed in $B6AF_1$ mice on different days after the injection of 20×10^6 A PLC

	Degree of Splenomegaly and thymic lesions in individual animals after GVH induction				
	Day 8		Day 16		Day 30
	Splenomegaly	Thymic Lesions	Splenomegaly	Thymic Lesions	Thymic Lesions
	2 52	Mild	2 82	Severe	Severe
	2 32	Normal	2 85	Severe	Severe
	2 07	Mild	3 02	Severe	Severe
	2 84	Mild	3 81	Severe	Severe
	3 72	Mild	3 86	Severe	
	3 41	Normal	3 35	Severe	
	3 61	Mild	3 26	Mild	
Mean + S E	2 93 + 0 25		3 28 + 0		

Table 7.4: The degree of splenomegaly and thymic lesions observed in $B6AF_1$ mice on different days after the injection of 10×10^6 A PLC.

Degree of Splenomegaly and thymic lesions in individual animals after GVH induction					
	Day 8		Day 16		Day 30
	Splenomegaly	Thymic Lesions	Splenomegaly	Thymic Lesions	Thymic Lesions
	1.97	Normal	2.66	Mild	Normal
	1.23	Normal	3.36	Mild	Normal
	1.47	Normal	3.71	Moderate	Normal
	2.11	Normal	2.86	Mild	Normal
	2.19	Normal	2.96	Mild	
	1.99	Normal	3.57	Mild	
	2.26	Mild	2.75	Mild	
Mean + S.E.	1.89 ± 0.15		3 12 ± 0.16		

Table 7.5: The degree of splenomegaly and thymic lesions observed in $B6AF_1$ mice on different days after the injection of 30×10^6 B6 PLC.

	Degree of Splenomegaly and thymic lesions in individual animals after GVH induction				
	Day 8		Day 16		Day 30
	Splenomegaly	Thymic Lesions	Splenomegaly	Thymic Lesions	Thymic Lesions
	2.41	Normal	1.23	Moderate	Moderate
	2.40	Normal	1.52	Moderate	Moderate
	2 57	Normal	1.25	Moderate	Severe
	2.55	Mild	2 26	Moderate	Moderate
	2 81	Mild	2.26	Moderate	
	2.76	Normal	2.41	Severe	
	2.88	Normal	2.67	Moderate	
Mean + S.E.	2 63 ± 0.07		1.94 ± 0 22		

Table 7.6: The degree of splenomegaly and thymic lesions observed in B6AF$_1$ mice on different days after the injection of 20 x 10^6 B6 PLC.

Degree of Splenomegaly and thymic lesions in individual animals after GVH induction				
Day 8		Day 16		Day 30
Splenomegaly	Thymic Lesions	Splenomegaly	Thymic Lesions	Thymic Lesions
1.60	Normal	1.49	Normal	Normal
1.77	Normal	1.18	Normal	Normal
1.66	Normal	1.37	Normal	Normal
2.30	Normal	2.11	Normal	Normal
2.07	Normal	1.81	Mild	
1.86	Normal	1.71	Mild	
2.07	Normal	1.73	Severe	
Mean + S.E. 1.90 ± 0.10		1.63 ± 0.12		

Table 7.7: The degree of splenomegaly and thymic lesions observed in B6AF1 mice on different days after the injection of 10 x 10^6 B6 PLC.

	Degree of Splenomegaly and thymic lesions in individual animals after GVH induction				
	Day 8		Day 16		Day 30
	Splenomegaly	Thymic Lesions	Splenomegaly	Thymic Lesions	Thymic Lesions
	1.21	Normal	1.33	Normal	Normal
	1.25	Normal	1.43	Normal	Normal
	1.56	Normal	1.32	Normal	Normal
	2.19	Normal	1.81	Normal	Normal
	1.82	Normal	1.85	Normal	
	1.17	Normal	1.54	Normal	
	1.25	Normal	1.26	Normal	
Mean ± S.E.	1.49 ± 0.15		1.51 ± 0.09		

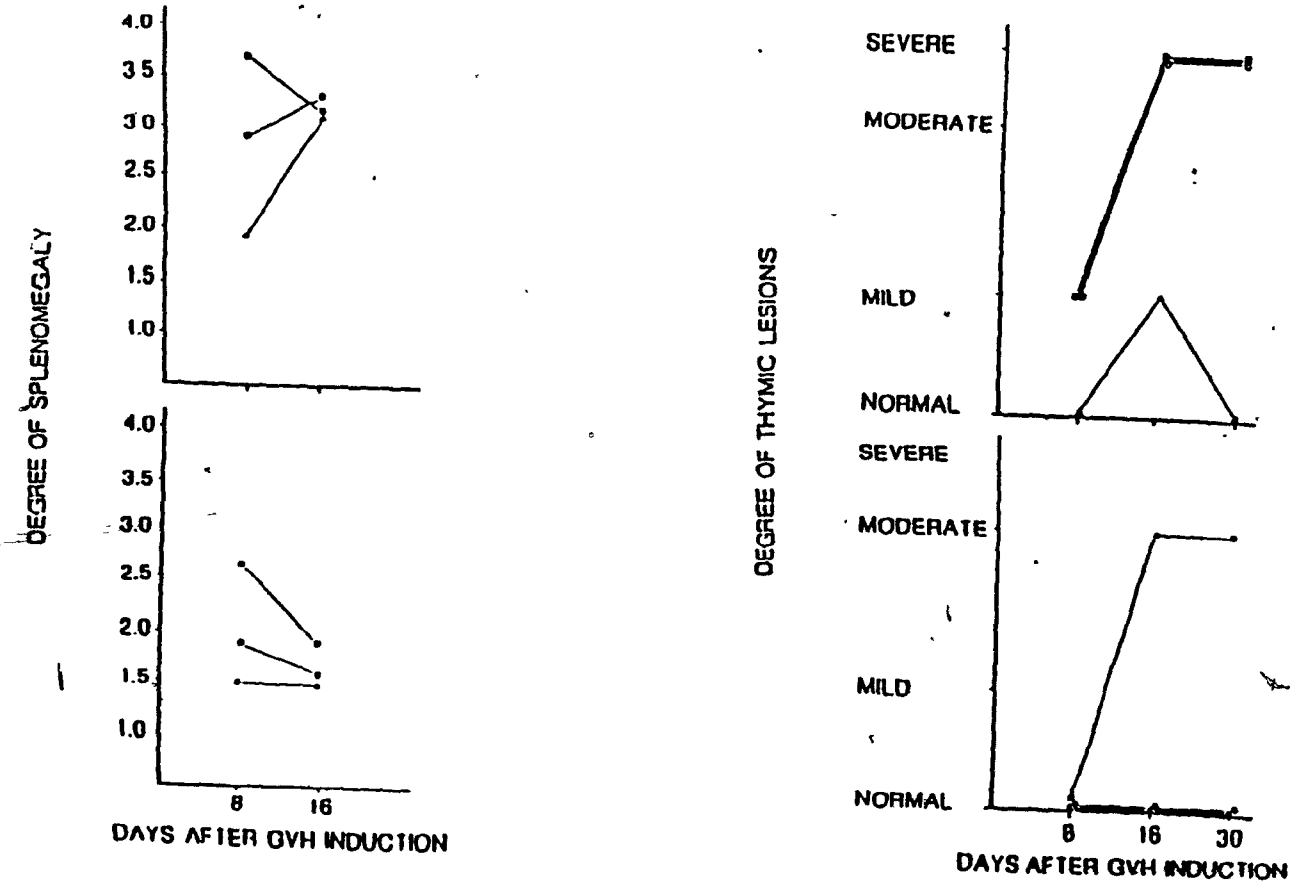


Figure 7.2 Summary of the degree of splenomegaly and thymic lesions observed in B6AF1 mice on different days after the injection of different doses of either A or B6 PLC. The degree of splenomegaly and thymic lesions observed in individual animals in each group are shown in tables 7.2-7.6.

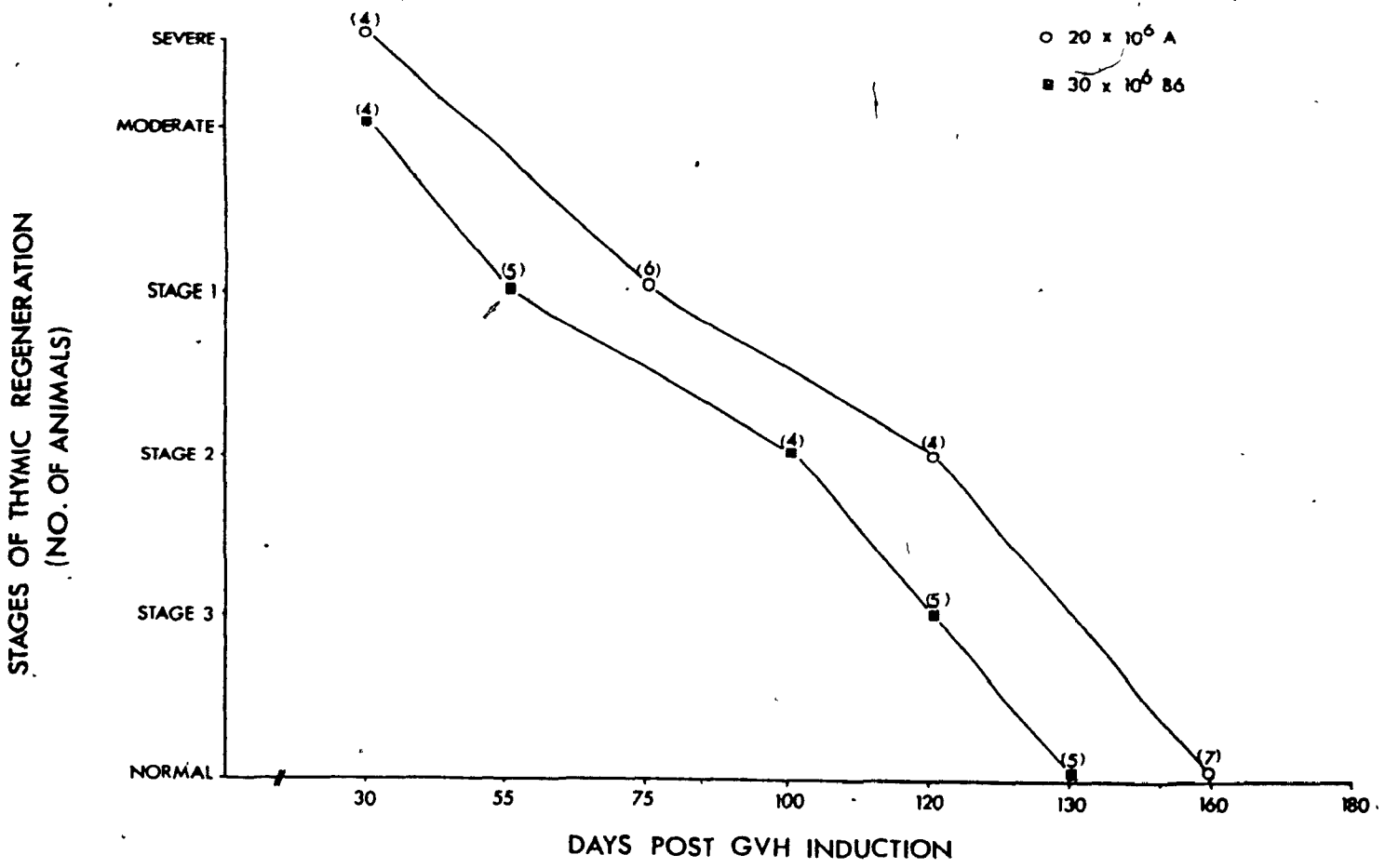


Figure 7.3 Kinetics of thymic regeneration observed in B6AF1 mice injected with either $20x10^6$ A or $30x10^6$B6 PLC.

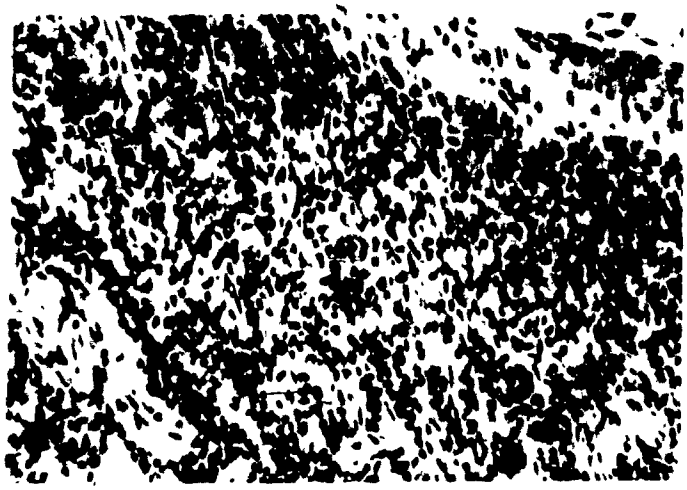


Figure 7.4a Photomicrograph of a thymus showing severe dysplasia (HPS x200) Note the complete absence of cortico-medullary demarcation and severe hypocellularity of the organ.
The thymus was taken from B6AF1 mouse at day 30 after the injection of 20×10^6A PLC.

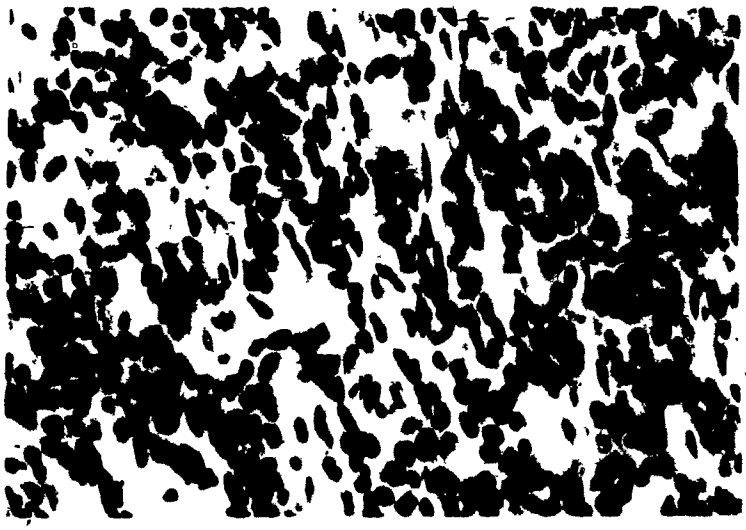


Figure 7.4b Photomicrograph of a thymus showing severe dysplasia (HPS x400). Note the complete absence of individual epithelial cells, epithelial cell clusters, and Hassall's corpuscles in the medulla. Also note that the medulla is severely hypocellular.
The thymus was taken from B6AF1 mouse at day 30 after the injection of 20×10^6A PLC.

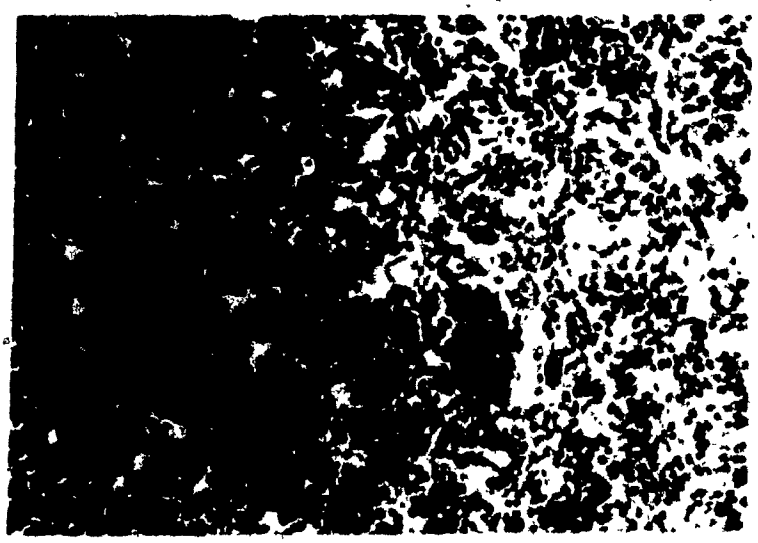


Figure 7.5a Photomicrograph of a thymus showing the stage 1 of thymic regeneration (HPS x200). Note the complete repopulation of the cortex with lymphocytes and reappearance of sharp cortico-medullary demarcation. Also note that the medulla is still hypocellular.
The thymus was taken from B6AF1 mouse at day 75 after the injection of $20x10^6$A PLC.

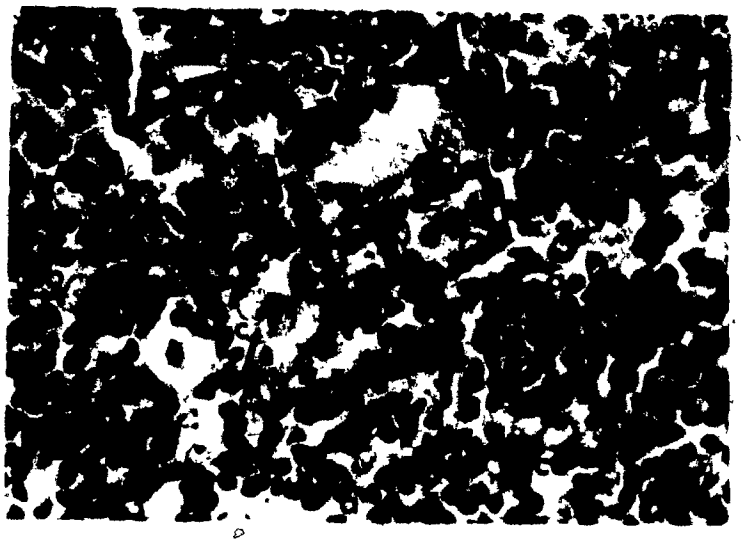


Figure 7.5b Photomicrograph of a thymus showing partial stage 2 of regeneration (HPS x400). Note the presence of severeal "small" dark irregular shaped individual epithelial cells (arrow,a), a few "large" pale individual epithelial cells (arrow,b), and two epithelial cell clusters (arrow,c). No Hassall's corpuscles were visible. The individual epithelial cells and the epithelial cell clusters were observed only in this particular area of the medulla.
The thymus was taken from B6AF1 mouse at day 75 after the injection of $20x10^6$A PLC.

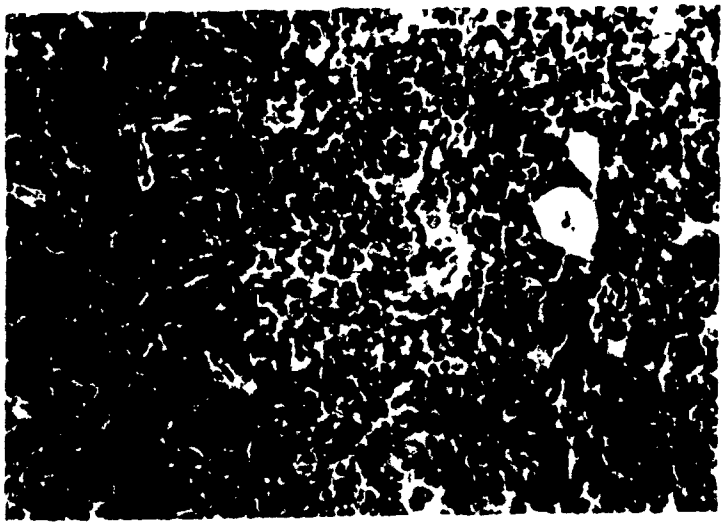


Figure 7.6a Photomicrograph of a thymus showing stage 3 of regeneration (HPS x200). Note the presence of a well defined cortex and a medulla. Also note the repopulation of the medulla with lymphocytes.
The thymus was taken from B6AF1 mouse at day 120 after the injection of $20x10^6$A PLC.

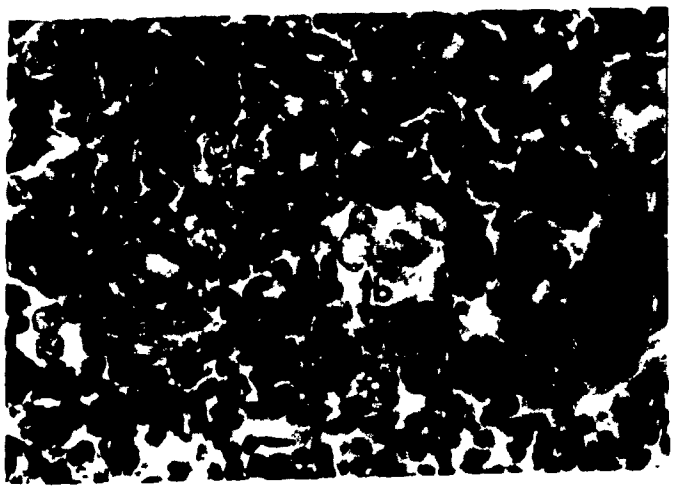


Figure 7.6b Photomicrograph of a thymus showing stage 3 of regeneration (HPS x400). Note the presence of several "large" pale rounded individual epithelial cells (arrow,a), and a large epithelial cell cluster (arrow,b). Also note that the "small" dark irregular shaped individual cells that were observed during stage 2 of regeneration (Fig.7.5b) are not visible. Numerous "large" pale individual epithelial cells and epithelial cell clusters were observed throughout the medulla.
The thymus was taken from B6AF1 mouse at day 120 after the injection of $20x10^6$A PLC.

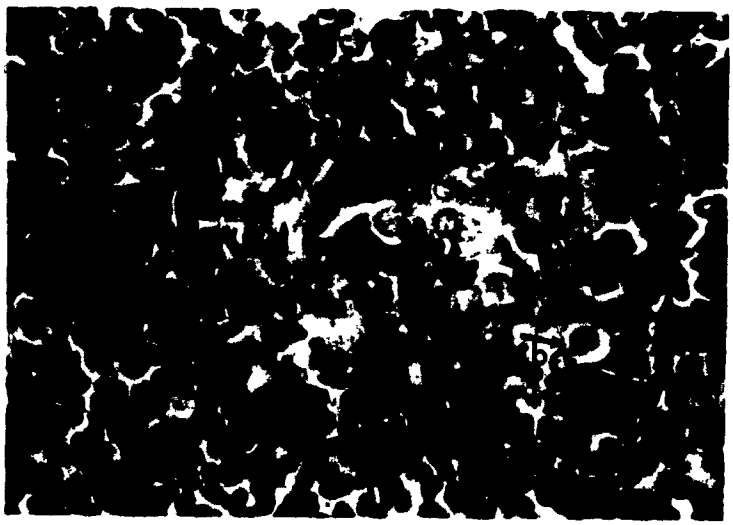


Figure 7.7 Photomicrograph of a thymus showing stage 4 of regeneration (a normal thymic medullary architecture) (HPS x400). Note the presence of a nicely-formed Hassall's corpuscle (arrow,a), Two epithelial cell clusters (arrow,b), and several "large" pale individual epithelial cells (arrow,c) in the medulla. Numerous such structures were dispersed throughout the medulla. The thymus was taken from B6AF1 mouse at day 160 after the injection of 20×10^6A PLC.

Table 7.8 Summary of the Characteristics of Various Stages of Thymic Regeneration

Stages of Thymic Regeneration	Characteristics of each stage of thymic regeneration
1st stage	- Increase in thymic size - Repopulation of cortex with lymphocytes - Reappearance of corticomedullary demarcation - Depleted medulla - No visible individual medullary epithelial cells or epithelial cell clusters - No visible Hassall's bodies
2nd stage	- Depleted medulla - Localized reappearance of several "small" dark irregular shaped individual epithelial cells - Rare epithelial cell clusters and "large" pale individual epithelial cells could be seen in localized areas of the medulla - No visible Hassall's bodies
3rd stage	- Repopulation of medulla - Abundant healthy "large" pale individual epithelial cells and epithelial cell clusters observed throughout the medulla - A few "small" dark irregular shaped individual epithelial cells were seen in the medulla - No visible Hassall's bodies
4th stage	- Normal medulla with abundant "large" pale individual epithelial cells, epithelial cell clusters and Hassall's bodies

Table 7.9 PFC response to SRBC of normal B6AF1 mice that were used as controls on different days after GVH induction

Days post GVH induction	No of mice	PFC response to SRBC [a]	
		PFC/spleen x 10^3	PFC/10^6 spleen cells
4	3	63 9, 95 1, 62 1	364 1; 880 6, 475 9
		$\bar{X}$ ± S.E = 73.7 ± 10.7	$\bar{X}$ ± S.E = 573.5 ± 157.1
8	3	123 9, 115 8; 105 0	1,197 7, 1,308 5, 1,272 7
		$\bar{X}$ ± S.E = 114.3 ± 4.6	$\bar{X}$ ± S.E = 1,259.6 ± 32.7
12	3	107 7, 123 0; 112 2	735 4; 891 3, 831 1
		$\bar{X}$ ± S.E = 114.3 ± 4.6	$\bar{X}$ ± S.E = 819.3 ± 45.5
30	3	83 0, 99 0; 77 1	748 7, 471 4; 535 4
		$\bar{X}$ ± S.E = 86.4 ± 6.5	$\bar{X}$ ± S.E = 585.2 ± 83.9
60	8	31 5; 120 0; 191 4; 97 0 295 8, 171 6; 232 8; 340 8	488 4; 1,818 2; 2,058 1; 1,630 0 1,972 0; 1,069 2; 1,437 0; 2,581.8
		$\bar{X}$ ± S.E = 185.0 ± 36.4	$\bar{X}$ ± S.E = 1,631.7 ± 227.2
90	7	83 1; 99 0; 77 1; 33 0 117 0; 102 0	748 7, 814 8, 535 4; 602 2 247 2; 1,130 4; 715 8
		$\bar{X}$ ± S.E = 85.5 ± 10.1	$\bar{X}$ ± S.E = 656.4 ± 124.3
120-130	6	132 9, 86 4, 120 9, 119 4 106 2; 111 3	1,230 6; 1,028 6; 1,439 3; 1,396 5 1,123 8; 1,016 4
		$\bar{X}$ ± S.E = 112.9 ± 6.5	$\bar{X}$ ± S.E = 1,205.9 ± 74.3
150-160	6	120 0, 120 3, 100 5, 106 2 73 8; 95 1	650 4; 662 8; 563 0; 687 9 502 1, 689 1
		$\bar{X}$ ± S.E = 102.7 ± 7.1	$\bar{X}$ ± S.E = 625.8 ± 31.1
180	5	109.8, 83 1; 96 0; 87 9 92 4	903 7; 791 4; 1,600 0, 915 6 724 7
		$\bar{X}$ ± S.E = 93.8 ± 4.5	$\bar{X}$ ± S.E = 987.1 ± 157.0

a The mean ± S.E PFC response to SRBC of all the normal mice is: PFC/spleen= 113.9±8.6 and PFC/10^6spleen cells= 976.9±77.1.

Table 7.10: PFC response to SRBC of B6AF1 mice on different days after the injection of $30x10^6$ A PLC.

Days post GVH induction	No. of mice	PFC response to SRBC	
		PFC/spleen x 10^{-3}	PFC/10^6 spleen cells
4	6	3.9; 6.0; 2.1; 6.6 2.7; 9.9	36.1; 64.4; 17.9; 60.4 21.1; 84.3
		$\bar{X} \pm$ S.E = 5.2 $\pm$ 1.2	$\bar{X} \pm$ S.E = 47.4 $\pm$ 10.8
8	3	0.0; 0.0; 0.0	0.0; 0.0; 0.0
		$\bar{X} \pm$ S.E = 0.0	$\bar{X} \pm$ S.E = 0.0
12	6	0.0; 0.0; 0.0; 0.0 0.0; 0.0	0.0; 0.0; 0.0; 0.0 0.0; 0.0
		$\bar{X} \pm$ S.E = 0.0	$\bar{X} \pm$ S.E = 0.0
30	6	0.0; 0.0; 0.0; 0.0 0.0; 0.0	0.0; 0.0; 0.0; 0.0 0.0; 0.0
		$\bar{X} \pm$ S.E = 0.0	$\bar{X} \pm$ S.E = 0.0

Table 7.11: PFC response to SRBC of B6AF1 mice on different days after the injection of 20×10^6 A PLC

Days post GVH induction	No. of mice	PFC response to SRBC	
		PFC/spleen x 10^3	PFC/10^6 spleen cells
4	6	24.6; 23.7; 29.7; 24.6 31.2; 16.5 $\bar{X} \pm$ S.E = 25.1 ± 2.4	235.1; 344.2; 349 2, 205 0 221 3; 148.1 $\bar{X} \pm$ S.E = 253.5 ± 32.6
8	3	0.0; 0.0; 0.0 $\bar{X} \pm$ S.E = 0.0	0.0; 0.0; 0.0 $\bar{X} \pm$ S.E = 0.0
12	6	0.0; 0.0; 0.0; 0 0 0.0; 0.0 $\bar{X} \pm$ S.E = 0.0	0.0; 0 0; 0.0; 0 0 0.0; 0.0 $\bar{X} \pm$ S.E = 0.0
30	5	0.0; 0.0; 0 0; 0 0 0.0 $\bar{X} \pm$ S.E = 0.0	0.0; 0.0; 0.0; 0.0 0 0 $\bar{X} \pm$ S.E = 0.0
60	5	0 0; 0.0; 0.0; 0 0 0.0 $\bar{X} \pm$ S.E = 0.0	0.0; 0.0; 0.0; 0 0 $\bar{X} \pm$ S.E = 0.0
90	6	0 0; 0.0; 0 6; 0 0 0.3; 0 0 $\bar{X} \pm$ S.E = 0.2 ± 0.1	0.0; 0.0; 6 6, 4.4; 0 0 $\bar{X} \pm$ S.E = 1.8 ± 1.2
120	9	1.2; 0 0; 0 0; 0 0 0.0; 0.0; 0.0; 0 0 0.0 $\bar{X} \pm$ S.E = 0.1 ± 0.1	11 1; 0 0, 0 0, 0 0 0 0; 0.0; 0 0; 0 0 0.0 $\bar{X} \pm$ S.E = 1.3 ± 1.3
150-160	11	1 2; 3.9; 0 0; 1 2 0.6; 0 3; 0 0; 0 0 0.0; 0.0; 0 0 $\bar{X} \pm$ S.E = 0.7 ± 0.3	11.7; 40 6; 0 0; 26 7 16.7; 9 1, 0.0; 0 0 0.0; 0.0; 0.0 $\bar{X} \pm$ S.E = 20.9 ± 5.8
180	6	37.5; 0.6; 25 5; 6 3 34 2; 36.9 $\bar{X} \pm$ S.E = 23.5 ± 6.6	342 5; 3.8; 200 7, 85.7 285 0; 482.4 $\bar{X} \pm$ S.E = 233.3 ± 71.2

Table 7.12: PFC response to SRBC of B6AF1 mice on different days after the injection of $10x10^6$ A PLC.

Days post GVH induction	No. of mice	PFC response to SRBC	
		PFC/spleen x 10^{-3}	PFC/10^6 spleen cells
4	6	32.7; 60.3; 87.3; 56.7 41.4; 43.8	465.8; 687.2; 1,134.5; 724.1 333.3; 363.2
		$\bar{X} \pm$ S.E = 53.7 ± 7.9	$\bar{X} \pm$ S.E = 618.0 ± 122.7
8	3	8.4; 2.1; 5.9	71.5; 22.9; 82.5
		$\bar{X} \pm$ S.E = 5.5 ± 1.8	$\bar{X} \pm$ S.E = 58.9 ± 18.3
12	6	0.0; 1.2; 0.0; 0.0 0.0; 0.0	0.0; 12.4; 0.0; 0.0 0.0; 0.0
		$\bar{X} \pm$ S.E = 0.2 ± 0.2	$\bar{X} \pm$ S.E = 2.1 ± 2.1
30	3	0.0; 0.0; 0.0	0.0; 0.0; 0.0
		$\bar{X} \pm$ S.E = 0.0	$\bar{X} \pm$ S.E = 0.0
60	7	89.7; 24.9; 22.8; 16.5 73.8; 1.5; 46.5	776.6; 164.4; 180.9; 171.9 540.7; 18.5; 476.9
		$\bar{X} \pm$ S.E = 39.4 ± 12.1	$\bar{X} \pm$ S.E = 332.8 ± 101.9

Table 7 13 PFC response to SRBC of B6AF1 mice on different days after the injection of 30×10^6 B6 PLC

Days post GVH induction	No of mice	PFC response to SRBC	
		PFC/spleen x 10^3	PFC/10^6 spleen cells
4	6	40.5; 59 7, 73.5, 48.6 68 7, 43.2	361 5, 421 2, 585 4, 363 6 417 1; 268 9
		X ± S.E = 55.7 + 5.6	X ± S.E = 402.6 ± 42.8
8	5	2 1, 8 7, 5.7, 3 9 8.4	22 5, 89 5, 38 7, 35 6 81 2
		X ± S.E = 5.8 + 1.3	X ± S.E = 53.5 ± 13 3
12	6	0 0, 0.0, 0.0; 0 0 0 0, 0.0	0 0, 0.0, 0 0, 0 0 0 0, 0 0
		X ± S.E = 0.0	X ± S.E = 0.0
30	6	0 0, 0.0, 0.0, 0.0 0 0, 0.0	0 0, 0.0, 0.0, 0 0 0 0, 0 0
		X ± S.E = 0.0	X ± S.E = 0.0
55	7	0 0, 0.0; 0.0, 0 0 0.0, 0 0, 0.0	0 0, 0 0, 0.0, 0 0 0 0, 0 0, 0.0
		X ± S.E = 0.0	X ± S.E = 0.0
90	6	0.0, 0 0; 0.0, 0.0 0.0; 0 0	0 0, 0.0, 0.0, 0 0 0 0, 0 0
		X ± S.E = 0.0	X ± S.E = 0.0
120	7	0 3, 0.6, 0.9, 0.0 0 0; 0 0; 0.0	4 0; 4.4, 7.4; 0 0 0 0, 0.0, 0 0
		X ± S.E = 0.3 + 0.1	X ± S.E = 2.3 ± 1.1
130	7	4 2, 2 1; 0 9, 0.0 0 0; 0.0, 0.0	35 4, 24 1; 9.7, 0.0 0 0, 0.0, 0 0
		X ± S.E = 1.0 + 0.6	X ± S.E = 9.9 ± 5.4
150	9	77 1, 46 8, 6 6, 9 0 0.9; 77 4, 0 6; 9 6 15 0	642 5; 528.8, 95 7; 107 1 11 5; 713 0, 8 2, 76 2 163.9
		X ± S.E = 27.0 ± 10.6	X ± S.E = 260.8 ± 94.5

Table 7.14: PFC response to SRBC of B6AF1 mice on different days after the injection of 20×10^6 B6 PLC.

Days post GVH induction	No. of mice	PFC response to SRBC	
		PFC/spleen x 10^{-3}	PFC/10^6 spleen cells
4	6	60.6; 50.7; 51.9; 75.3 55.5; 60.3	569.0; 344.5; 468.8; 774.7 483.7; 480.3
		$\bar{X} \pm$ S.E = 59.1 ± 3.6	$\bar{X} \pm$ S.E = 520.1 ± 58.7
8	5	105.0; 88.8; 96.4; 83.4 74.7	750.3; 682.8; 902.9; 907.0 690.7
		$\bar{X} \pm$ S.E = 89.6 ± 5.2	$\bar{X} \pm$ S.E = 786.8 ± 49.6
12	6	87.3; 69.3; 51.6; 153.6 137.1; 147.3	567.3; 508.1; 516.5; 1,602.5 846.3; 1,075.6
		$\bar{X} \pm$ S.E = 107.7 ± 17.9	$\bar{X} \pm$ S.E = 852.7 ± 175.6
30	3	78.3; 66.6; 90.3	542.1; 542.1; 586.7
		$\bar{X} \pm$ S.E = 78.4 ± 6.9	$\bar{X} \pm$ S.E = 566.9 ± 14.9
60	4	127.5; 141.8; 94.2; 114.9	817.3; 1,132.2; 713.6; 847.4
		$\bar{X} \pm$ S.E = 119.5 ± 10.0	$\bar{X} \pm$ S.E = 877.6 ± 89.6

Table 7.15: PFC response to SRBC of B6AF1 mice on different days after the injection of $10x10^6$ B6 PLC.

Days post GVH induction	No. of mice	PFC response to SRBC	
		PFC/spleen x 10^{-3}	PFC/10^6 spleen cells
4	6	87.6; 45.6; 54.3; 72.9 88.2; 81.3	983.2; 337.8; 402.2; 566.7 768.6; 763.4
		$\bar{X} \pm$ S.E = 71.7 ± 7.3	$\bar{X} \pm$ S.E = 636.9 ± 100.0
8	4	83.4; 74.4; 62.7; 73.5	611.6; 658.2; 504.8; 668.5
		$\bar{X} \pm$ S.E = 73.5 ± 4.4	$\bar{X} \pm$ S.E = 588.3 ± 34.6
12	7	112.2; 167.1; 125.1; 107.4 131.7; 117.0; 124.8	1,038.9; 1,194.0; 1,029.6; 688.5 936.8; 787.9; 821.3
		$\bar{X} \pm$ S.E = 126.4 ± 7.4	$\bar{X} \pm$ S.E = 923.1 ± 65.7
30	8	102.9; 15.3; 55.8; 51.9 217.8; 114.6; 87.6; 83.4	1,361.1; 179.9; 366.1; 436.9 2,304.8; 1,248.4; 842.7; 702.0
		$\bar{X} \pm$ S.E = 73.0 ± 7.4	$\bar{X} \pm$ S.E = 930.2 ± 244.8
60	4	88.7; 88.8; 111.9; 76.8	631.8; 730.9; 816.2; 474.1
		$\bar{X} \pm$ S.E = 91.6 ± 7.4	$\bar{X} \pm$ S.E = 633.2 ± 73.5

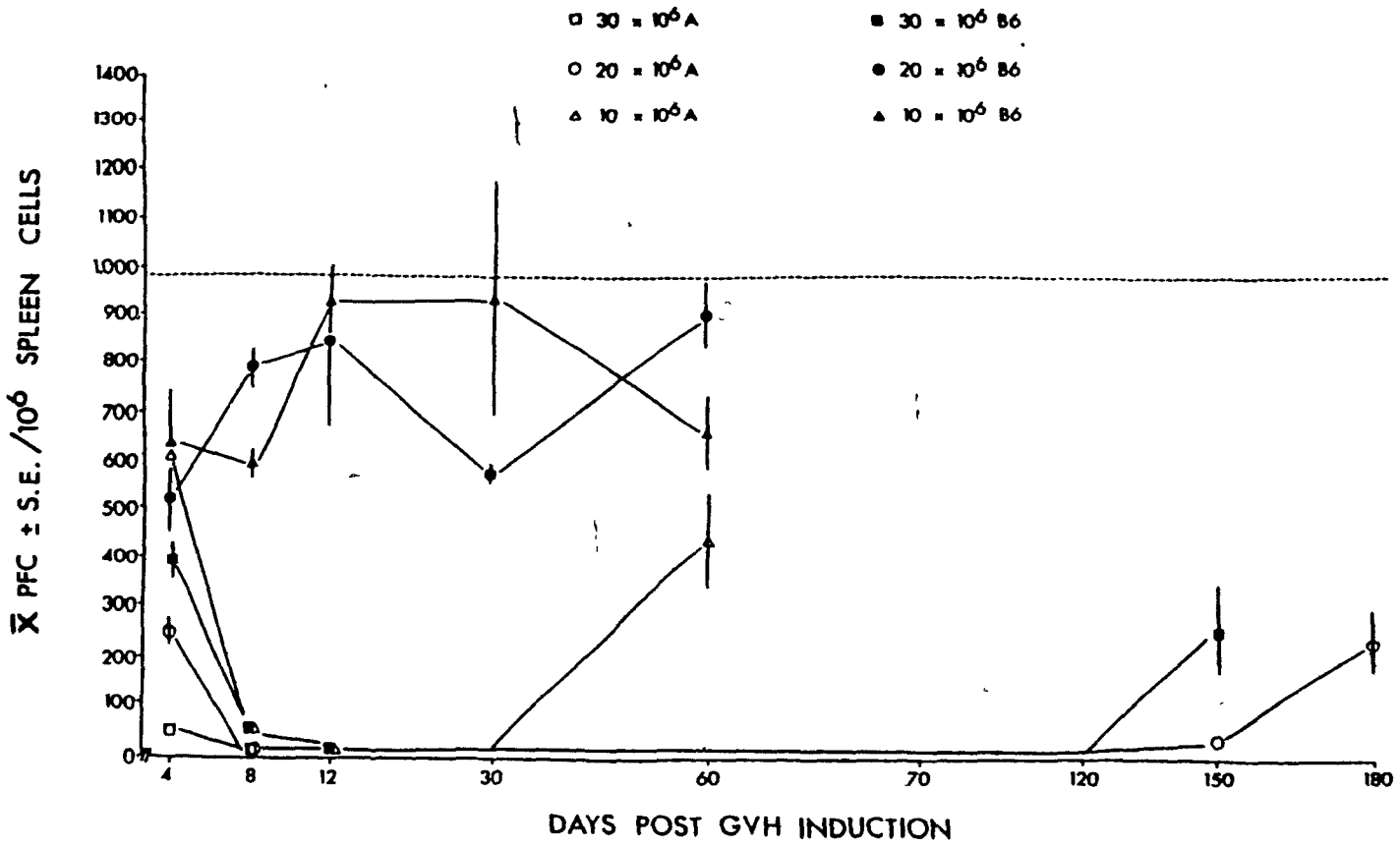


Figure 7.8 Summary of the PFC/10^6 spleen cell response to SRBC of B6AF1 mice on different days after the injection of different doses of either A or B6 PLC. The horizontal line is the mean PFC/10^6spleen cell response of normal B6AF1 mice that were used as controls on different days after the injections of PLC (see Table 7.9).

Table 7.16 Splenic nucleated cell numbers of normal B6AF1 mice that were used as controls on different days after GVH induction.

Days post GVH induction	No of mice	Splenic cellularity x 10^6 individual animals	Mean ± S.E.
8	3	97.2; 117.45; 157.9	124.2 ± 17.8
12	3	93.2, 79.7; 74.3	82.4 ± 5.6
16	3	130.9; 124.2; 121.5	125.6 ± 2.8
34	3	99.9; 111.0; 129.6	113.5 ± 8.7
64	8	64.5; 66.0; 93.0; 60.0 120.0; 128.4; 129.6; 105.6	95.9 ± 10.4
94	7	88.8; 97.2; 115.2; 115.2 106.8; 82.8; 114.0	102.9 ± 5.1
124	6	97.2; 75.6; 75.6; 76.9 85.1; 98.6	84.8 ± 4.3
164	6	147.6; 145.2; 142.8; 123.6 117.6; 110.4	131.2 ± 6.5
184	5	97.2; 84.0; 48.0; 76.8 102.0	81.6 ± 9.5

Table 7.17 Splenic nucleated cell numbers of B6AF1 mice on different days after the injection of $30x10^6$ A PLC.

Days post GVH induction	No of mice	Splenic cellularity x 10^6 individual animals	Mean ± S.E.
8	6	108.0; 93.2; 117.5; 109.4 128.3; 117.5	112.3 ± 4.8
12	3	68.4; 78.3; 56.7	67.9 ± 6.3
16	6	52.7; 28.4; 64.3; 44.6 28.4; 59.4	46.3 ± 6.3
34	6	29.7; 18.9; 37.8; 27.0 36.5; 25.7	29.3 ± 2.9

Table 7.18 Splenic nucleated cell numbers of B6AF1 mice on different days after the injection of $20x10^6$ A PLC.

Days post GVH induction	No of mice	Splenic cellularity x 10^6 individual animals	Mean ± S.E
8	6	97 2, 68 9, 85 1; 120 0 141.0; 111.5	103.9 ± 10.5
12	3	79.6; 55 4; 70.2	68.4 ± 7.1
16	6	41 9, 40 5, 63 5, 58 5 40 5; 41.9	47.7 ± 4.9
34	5	28 4, 39 2; 27 0, 30.0 31 5	34.2 ± 2.1
64	5	62 4, 70 8, 28 0, 25 5 22 5	41.8 ± 10.2
94	6	61 0, 73.5, 91 5, 52 5 69 0, 58 5	67.7 ± 5.7
124	9	102 0, 61 5, 73 5, 78 0 51.0; 75 0; 67 5, 72 0 49.5	70.0 ± 5.3
164	11	108 0, 96 0, 85 5, 45 0 36.0; 33 0; 78 2; 84 5 64 0; 60 5, 79 0	69.9 ± 7.4
184	6	109.5; 157.5; 127 5; 73 5 120 0; 76.5	110.8 ± 13.1

Table 7.19 Splenic nucleated cell numbers of B6AF1 mice on different days after the injection of $10x10^6$ A PLC.

Days post GVH induction	No of mice	Splenic cellularity x 10^6 individual animals	Mean ± S.E.
8	6	70.2; 87.5; 76 9, 78.3 124.2; 120.6	93.0 ± 9.6
12	3	117.5; 91.8, 71.6	93.6 ± 13.3
16	6	145.8; 97.2; 124 2; 132 3 99.9; 120.2	119.9 ± 7.7
34	3	60.8; 68.9; 54.0	61.2 ± 4,3
64	7	118 5, 151 5; 126 0; 96.0 136.5, 81 0, 97.5	115.1 ± 9.4

Table 7.20 Splenic nucleated cell numbers of B6AF1 mice on different days after the injection of $30x10^6$ B6 PLC.

Days post GVH induction	No of mice	Splenic cellularity x 10^6 individual animals	Mean ± S.E.
8	6	112.1; 141.7; 125.5; 133.7 164.7; 160.7	139.7 ± 8.3
12	5	93.2; 97.2; 147.2; 109.5 103.5	110.1 ± 9.7
16	6	84.0; 57.5; 51 0; 35.5 30.6; 42.0	57.3 ± 9.5
34	6	59.5; 21.0; 16.5; 23.5 28.5; 18.0	27.8 ± 6.6
59	7	55.4; 52 7; 63 5; 57 5 83.7; 47.5; 46.5	58.1 ± 4.8
94	6	67 5; 109 5; 63 0; 57.0 98 5, 125 2	86.8 ± 11.5
124	7	75 0; 109.5; 121 5; 78 8 87 0, 73.5; 73 5	88.4 ± 7.3
134	7	118.5, 87.0; 93 0; 72.5 108.0; 97.5; 93.0	95.6 ± 5.6
154	9	120.0, 88.5, 69 0; 84 0 78.0; 109.5; 73.5; 126.0 91.5	93.3 ± 6.9

Table 7.21 Splenic nucleated cell numbers of B6AF1 mice on different days after the injection of $20x10^6$ B6 PLC.

Days post GVH induction	No of mice	Splenic cellularity x 10^6 individual animals	Mean ± S.E.
8	6	106.5, 147.2; 110 7; 97.2 114.8; 125.3	116.9 ± 7.1
12	5	140.0; 130.0; 106.7; 92.0 108.2	115.4 ± 8.6
16	6	153.9; 136 4, 99.9; 95.9 162.0; 137.0	130.8 ± 11.2
34	3	144.5; 122.9; 153.9	140.4 ± 9.2
64	4	156.0; 124.8; 132.0; 135 6	137.1 ± 6.7

Table 7.22 Splenic nucleated cell numbers of B6AF1 mice on different days after the injection of $10x10^6$ B6 PLC.

Days post GVH induction	No of mice	Splenic cellularity x 10^6 individual animals	Mean ± S.E.
8	6	89.1; 135.0; 135.0; 130.9 114.8; 106.6	118.6 ± 7.6
12	4	136.4; 130.9; 124.2; 110.0	125.3 ± 5.7
16	7	108.0. 130.9; 121.5; 156.0 140.0; 148.5; 152.0	123.6 ± 14.1
34	8	75.6; 85.1; 152.4; 118.3 94.5; 91.8; 103.9; 118.8	105.1 ± 9.3
64	4	140.4; 121.5; 137.7; 162.0	140.4 ± 8.3

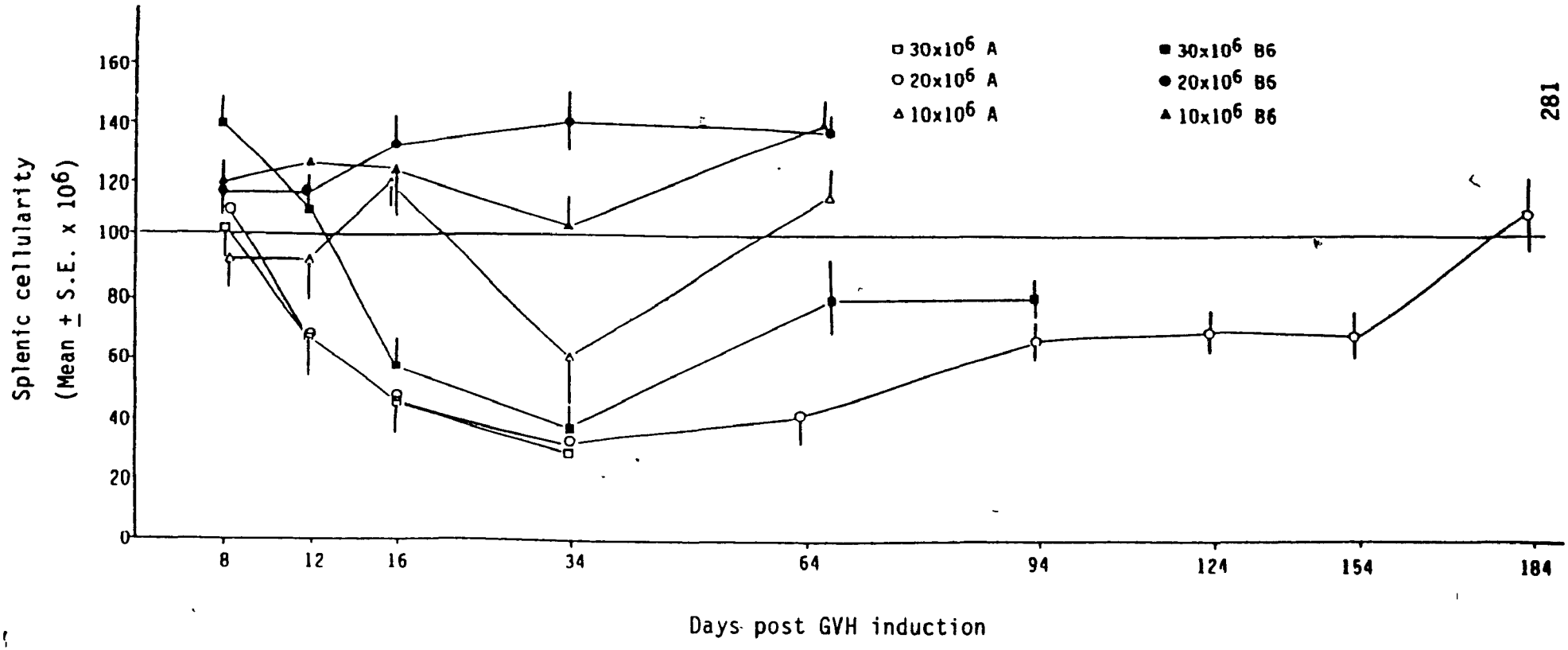


Figure 7.9: Summary of splenic cellularity of B6AF1 mice on different days after the injection of different doses of either A or B6 PLC. The horizontal line is the mean cellularity of normal B6AF1 mice that were used as controls on different days after GVH induction (see table 7.16).

Table 7.23: Splenic cellularity and PFC response to SRBC of B_6AF_1 mice used as controls in different experiments.

Experiment number	Splenic cellularity $x10^6$	PFC response to SRBC[a]	
		PFC/spleen $\pm$ S.E. $\times 10^{-3}$	PFC/10^6 $\pm$ S.E. spleen cells
1	156.65 $\pm$ 7.23	119.70 $\pm$ 22.99	635.42 $\pm$ 128.32
2	67.50 $\pm$ 5.27	72.20 $\pm$ 2.78	1,082.00 $\pm$ 95.54
3	89.13 $\pm$ 2.70	150.75 $\pm$ 26.45	1,668.60 $\pm$ 246.19
4	123.30 $\pm$ 14.75	89.93 $\pm$ 8.81	917.45 $\pm$ 164.00
5	88.50 $\pm$ 1.50	69.80 $\pm$ 2.09	789.82 $\pm$ 35.83
6	100.50 $\pm$ 7.10	76.40 $\pm$ 10.08	754.07 $\pm$ 49.95
7	138.63 $\pm$ 19.31	50.33 $\pm$ 6.47	402.39 $\pm$ 98.14
8	95.25 $\pm$ 3.9	250.80 $\pm$ 14.75	2,645.50 $\pm$ 191.72
9	119.00 $\pm$ 10.45	107.90 $\pm$ 9.7	906.67 $\pm$ 36.21

a 3 - 5 normal B_6AF_1 mice were used as control/experiment. The data presented is the mean $\pm$ S.E.

Table 7.24: Splenic Con , PHA and LPS responses of B_6AF_1 mice used as controls in experiments.

Experiment number	Mitogen responses cpm ± S.E.			
	0	Con A	PHA	LPS
1	11,652 ± 1,076	57,882 ± 1,488	72,588 ± 4,780	51,141 ± 3,109
2	5,185 ± 225	31,868 ± 1,328	56,492 ± 3,403	54,230 ± 5,419
3	4,084 ± 1,002	140,064 ± 12,452	90,304 ± 11,709	62,406 ± 5,623
4	9,699 ± 565	58,579 ± 7,979	72,578 ± 8,666	69,760 ± 2,716
5	7,886 ± 367	136,711 ± 6,998	138,731 ± 2,821	56,384 ± 3,228
6	15,038 ± 2,492	115,763 ± 11,509	125,748 ± 9,776	66,786 ± 2,412
7	14,980 ± 1,225	67,028 ± 5,160	62,030 ± 4,663	45,739 ± 1,980
8	2,596 ± 243	133,241 ± 243	152,090 ± 11,681	31,308 ± 1,128
9	6,978 ± 1,443	98,046 ± 3,421	80,495 ± 4,926	49,806 ± 3,310

Table 7.25 Percent of normal splenic cellularity, and % of normal splenic ConA, PHA, and LPS responses of GVH reactive B6AF1 mice with no PFC during the immune recovery phase of the GVH reaction.[a]

Experiment No. and (animal no.)		% of normal splenic cellularity[b,c]	% of normal mitogen responses		
			Con A	PHA	LPS
1	(1)	34	18	13	43
1	(5)	52	21	20	62
2	(8)	107	35	-	-
2	(9)	72	3	-	-
4	(2)	60	27	26	69
4	(6)	52	4	1	32
7	(1)	16	4	2	35
	(2)	49	101	12	60
	(3)	75	24	2	65
	(4)	79	7	7	1
	(5)	15	3	2	6
	(6)	34	59	9	0
	(7)	45	115	31	49
	(8)	19	8	4	132
8	(1)	88	6	4	75
	(2)	77	6	12	170
	(3)	181	0	3	115
	(4)	41	0	0	121
	(5)	44	17	13	235
	(6)	91	11	11	234
	(7)	33	18	16	219
Mean ± S.E.		60.29 ± 8.22	23.19 ± 6.88	9 89 ± 2.00	90.68 ± 17.47

a: % of normal splenic cellularity and splenic mitogen responses were calculated by using the splenic cellularity and mitogen responses of normal B6AF1 mice in a given experiment as 100%

b: The normal splenic cellularity for each experiment are shown in table 7.23 Normal splenic mitogen responses are shown in table 7.24

c: GVH reactions were induced in B6AF1 mice by injecting 30×10^6 B6 PLC. The animals were sacrificed between days 100-150 after GVH induction.

Table 7 26 Percent of normal splenic cellularity, PFC/10^6 spleen cell response to SRBC, and % of normal splenic ConA, PHA, and LPS responses of GVH reactive B6AF1 with 0.30 - 15.00 x 10^{-3} PFC/spleen response to SRBC during the immune recovery phase of the GVH reaction.[a]

Experiment no. and (animal no.)	% of normal splenic cellularity[b]	% of normal PFC/10^6 spleen cell response	% of normal mitogen responses[c]		
			Con A	PHA	LPS
1 (2)	91	2	95	67	135
1 (4)	43	2	51	41	118
2 (1)	138	3	92	58	105
(4)	156	4	80	31	118
(5)	165	5	70	26	115
(6)	198	4	116	32	103
(7)	196	0 21	55	30	118
3 (3)	76	13	75	52	129
(5)	42	2	81	22	78
(6)	114	1	62	63	80
(7)	116	7	79	40	112
(8)	57	10	85	52	98
(9)	114	8	85	43	82
4 (4)	83	2	123	46	82
(9)	114	8	81	26	108
5 (1)	117	10	56	54	105
(2)	136	2	42	43	135
(6)	85	2	51	49	58
6 (5)	88	4	85	56	121
(6)	106	5	76	58	134
(7)	103	18	81	45	156
(8)	101	13	128	49	128
(9)	139	9	66	35	138
7 (4)	93	5	125	27	127
(5)	129	5	98	94	94
(6)	86	12	82	34	128
Mean ± S.E.	110 35 ± 7.82	5.93 ± 0.88	81.54 ± 4.50	43.96 ± 3.47	111.77 ± 4.46

a, b, c see table 7.25

Table 7.27 Percent of normal splenic cellularity, PFC/10^6 spleen cell response to SRBC, and % of normal splenic ConA, PHA, and LPS responses of GVH reactive B6AF1 mice with 15.30 - 30.00 x 10^{-3} PFC/spleen response to SRBC during the immune recovery phase of the GVH reaction.[a]

Experiment no. and (animal no.)		% of normal splenic cellularity	% of normal PFC/10^6 spleen cell response	% of normal mitogen responses[c]		
				Con A	PHA	LPS
3	(4)	155	9	92	78	138
	(7)	150	10	74	40	88
4	(1)	125	15	116	107	73
	(6)	77	33	99	69	82
5	(4)	136	30	74	63	112
	(7)	132	20	65	58	133
6	(3)	87	25	69	53	146
	(4)	91	34	86	64	128
7	(8)	132	16	105	44	82
	(9)	159	16	89	116	103
	(10)	112	13	97	75	103
Mean ± S.E.		123.27 ± 8.49	20.09 ± 2.72	87.82 ± 4.84	69.73 ± 7.17	108.00 ± 7.63

a, b, c: see table 7.25

Table 7.28 Percent of normal splenic cellularity, PFC/10^6 spleen cell response to SRBC, and % of normal splenic ConA, PHA, and LPS responses of GVH reactive B6AF1 mice with 30.00 - more x 10^{-3} PFC/ spleen to SRBC during the immune recovery phase of the GVH reaction.[a]

Experiment no. and (animal no.)		% of normal splenic cellularity[b]	% of normal PFC/10^6 spleen response[b]	% of normal mitogen responses[c]		
				Con A	PHA	LPS
1	(3)	68	133	132	104	115
	(6)	99	115	141	158	119
2	(2)	213	59	91	90	91
	(3)	198	125	69	80	92
3	(1)	146	24	115	77	67
	(2)	133	22	100	76	102
4	(3)	61	342	109	79	79
5	(3)	169	51	76	68	125
	(5)	127	61	69	57	128
6	(1)	130	46	86	82	93
	(2)	120	45	111	79	113
Mean ± S.E.		133.09 ± 14.40	93.00 ± 27.50	99.91 ± 7.32	86.36 ± 7.98	102.18 ± 5.90

a, b, c: see table 7.25

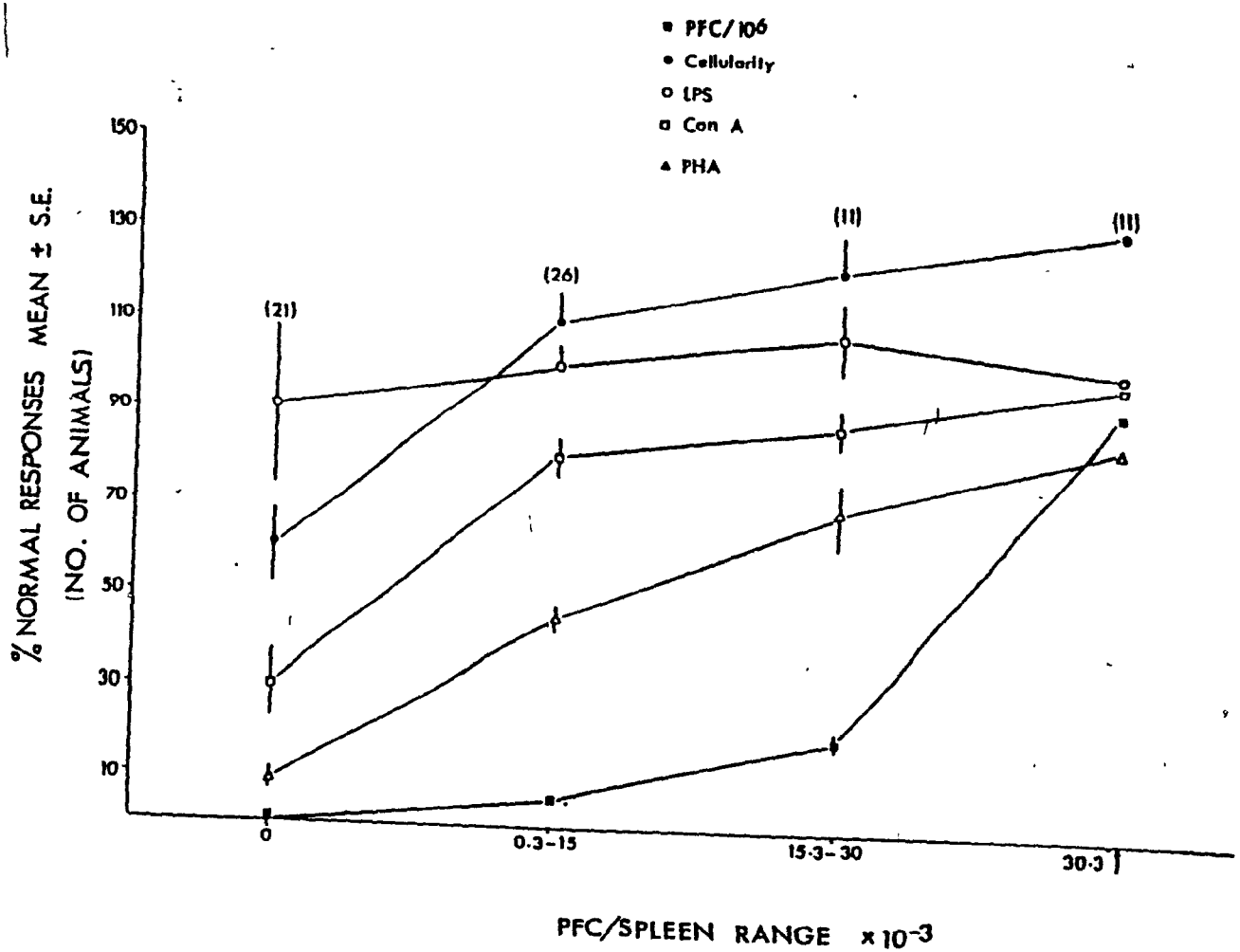


Figure 7.10 Summary of the relationship between splenic cellularity, PFC/10^6 spleen cell response to SRBC, ConA, PHA, and LPS responses in GVH reactive B6AF1 mice with different numbers of PFC/spleen to SRBC during the immune recovery phase of the GVH reaction.

Table 7.29: ^{3}H-Thymidine incorporation by IL-2 dependent CTLL cells in the presence of IL-2 containing supernatants from normal B6AF1 splenocytes, following stimulation with either Con A or PHA.

Experiment no.	^{3}H-Thymidine incorporation by CTLL cells in the presence of IL-2 containing supernatants (cpm±S.E.)			
	Mitogens used to produce IL-2			
	0	Con A	PHA	LPS
1	5,442 ± 623	24,811 ± 654	87,776 ± 2,609	
2	3,474 ± 233	37,946 ± 1,445	69,048 ± 3,369	
3	2,652 ± 696	52,940 ± 7,593	80,232 ± 8,579	3,128 ± 265
4	1,436 ± 37	63,410 ± 3,220	79,846 ± 4,987	1,996 ± 97
5	490 ± 17	76,444 ±	72,273 ± 3,007	
6	3,278 ± 246	53,454 ± 2,403	79,427 ± 2,191	2,998 ± 101
7	925 ± 85	44,090 ± 5,515	66,177 ± 8,286	

Table 7.30 Percent of normal ^{3}H-Thymidine incorporation by CTLL cells in the presence of IL-2 containing supernatants obtained following stimulation with either Con A or PHA of splenocytes of GVH reactive B6AF1 mice with no PFC/spleen to SRBC during the immune recovery phase of the GVH reaction.[a]

Experiment no. and (animal no.)		% of normal ^{3}H-Thymidine incorporation by CTLL cells in the presence of IL-2 containing supernatants.[a,b,c]	
		Mitogen employed to produce IL-2	
		Con A	PHA
1	(1)	30	16
	(5)	99	49
2	(8)	41	19
	(9)	49	19
4	(2)	61	24
	(6)	74	40
7	(1)	6	3
	(2)	5	3
	(3)	0	0
	(4)	4	1
	(5)	31	4
	(6)	33	4
	(7)	92	31
	(8)	76	26
Mean ± S.E.		42.93 ± 8.94	17.07 ± 4.17

a: % of normal ^{3}H-Thymidine incorporation was calculated by using ^{3}H-Thymidine incorporation by CTLL cells in the presence of IL-2 containing supernatants derived from normal B6AF1 splenocytes in a given experiment as 100%.

b: ^{3}H-Thymidine incorporation by CTLL cells in the presence of IL-2 containing supernatants derived from normal B6AF1 splenocytes following stimulation with either Con A or PHA for each experiment is shown in table 7.29.

c: GVH reactions were induced in B6AF1 mice by injecting $30x10^6$B6 PLC. Animals were sacrificed between days 100-150 after GVH induction.

Table 7.31 Percent of normal ^{3}H-Thymidine incorporation by CTLL cells in the presence of IL-2 containing supernatants obtained following stimulation with either Con A or PHA of splenocytes of GVH reactive B6AF1 mice with 0.03 - 15.00 x 10^{-3} PFC/spleen to SRBC during the immune recovery phase of the GVH reaction.

Experiment no. and (animal no.)		% of normal ^{3}H-Thymidine incorporation by CTLL cells in the presence of IL-2 containing supernatants[a,b,c]	
		Mitogen employed to produce IL-2	
		Con A	PHA
1	(2)	30	16
	(4)	46	37
2	(1)	87	57
	(4)	51	32
	(5)	66	44
	(6)	53	37
	(7)	34	42
3	(3)	37	18
	(5)	49	7
	(6)	62	19
	(7)	51	28
	(8)	21	17
5	(1)	52	18
	(2)	51	27
	(6)	70	34
6	(5)	56	19
	(6)	85	35
	(7)	73	40
	(8)	84	102
4	(4)	60	25
Mean ± S.E.		55.70 ± 3.98	32.75 ± 4.53

a, b, c: see table 7.30.

Table 7.32 Percent of normal ^{3}H-Thymidine incorporation by CTLL cells in the presence of IL-2 containing supernatants obtained following stimulation with either Con A or PHA of splenocytes of GVH reactive B6AF1 mice with 15.30 - more x 10^{-3} PFC/spleen to SRBC during the immune recovery phase of the GVH reaction.[a]

Experiment no. and (animal no.)		% of normal ^{3}H-Thymidine incorporation by CTLL cells in the presence of IL-2 containing supernatants.[b,c]	
		Mitogen employed to produce IL-2	
		Con A	PHA
1	(3)	125	67
	(6)	145	91
2	(2)	93	104
	(3)	111	91
3	(1)	78	33
	(2)	57	18
	(4)	37	7
4	(1)	78	73
	(3)	86	71
	(6)	94	79
5	(3)	59	60
	(4)	31	20
	(5)	70	78
	(7)	39	9
6	(1)	58	35
	(2)	81	46
	(3)	74	39
	(4)	79	48
Mean ± S.E.		77.39 ± 7.05	53.83 ± 7.03

a, b, c: see table 7.30

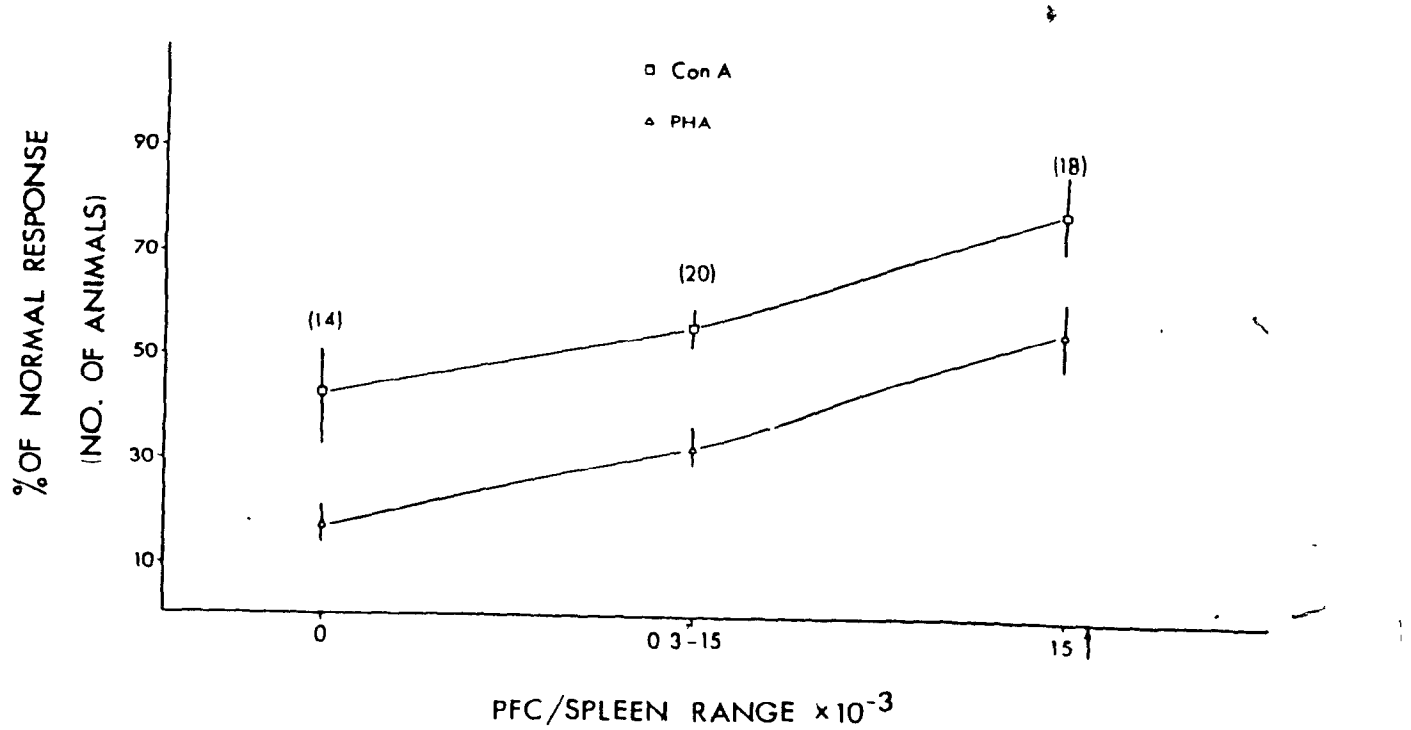


Figure 7.11 Summary of the %of normal IL-2 produced by splenocytes from GVH reactive B6AF1 mice with different numbers of PFC/spleen to SRBC, following stimulation with either ConA or PHA, during the immune recovery phase of the GVH reaction.

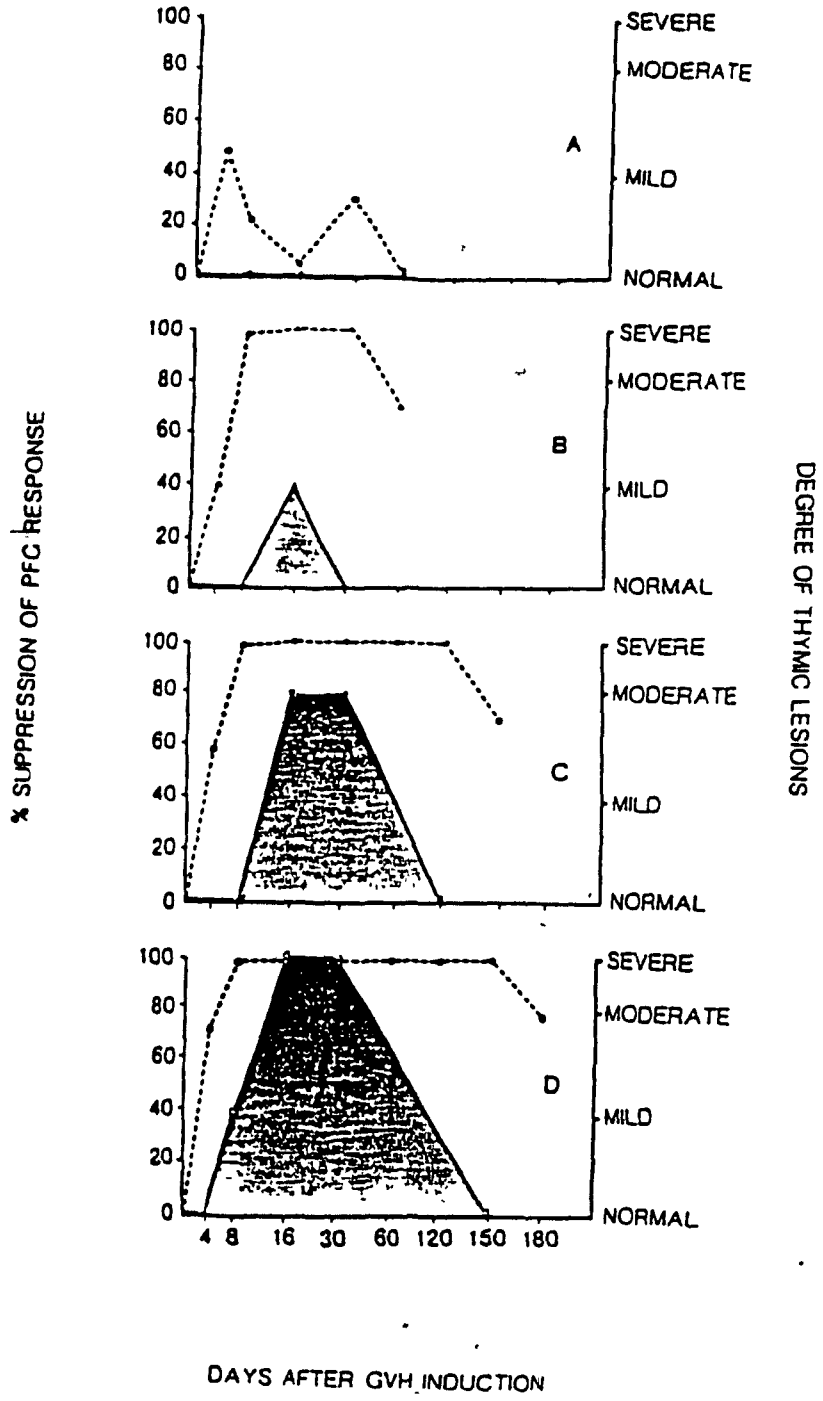


Figure 7.12 The relationship between the degree of thymic dysplasia and the duration of immunosuppression of the PFC response to SRBC. A, B6AF1 mice injected with $20x10^6$B6 PLC; B, B6AF1 mice injected with $10x10^6$A PLC; C, B6AF1 mice injected with $30x10^6$B6 PLC. D, B6AF1 mice injected with $20x10^6$A PLC.

CHAPTER EIGHT

ATTEMPTS TO RESTORE T-CELL PROLIFERATIVE FUNCTIONS *IN VITRO* OF GVH IMMUNOSUPPRESSED SPLENOCYTES DURING DIFFERENT STAGES OF THYMIC REGENERATION.

8.1 INTRODUCTION

In the previous chapter it was demonstrated that the thymuses as well as the immune functions of GVH reactive animals gradually recover from thymic injury and severe immunosuppression. The data presented in chapter seven also suggested that the thymic architectural recovery precedes the immune functional recovery. Moreover, it was noted that although the thymus may appear architecturally normal the animal may not regain immunocompetence (as assessed by the PFC response to SRBC). It was suggested that the time lag in the immune functional recovery after the thymus has regained its normal architecture may be due to the possibility that although the thymus appeared architecturally normal it had not yet recovered functionally (i.e. resumed its hormonal production). Experiments were therefore, designed to investigate the effects of agents which either mimic and/or mediate the actions of thymic hormones for example PGs of the E series (Bach and Bach, 1974; Garaci et al, 1981; Garaci et al, 1983). , on T-cell proliferative functions of immunosuppressed GVH T-cells during various stages of thymic regeneration.

8.2 EXPERIMENTAL DESIGN

The experimental protocol is outlined in figure 8.1. GVH reactions were induced in B6AF1 mice by injecting either $30x10^6$ B6 or $20x10^6$ A parental strain lymphoid cells. On different days post-GVH induction, animals from each GVH group

and normal B6AF1 mice were randomly selected from a pool of mice. The immune status of GVH reactive mice was assessed by measuring the PFC response to SRBC. Spleen cells from animals that were totally suppressed for the PFC response to SRBC (no PFC) were pooled, since earlier studies (chapter 7) showed that such animals were still severely suppressed for the Con A and PHA mitogen responses. The cells were divided into aliquots ($25-30 \times 10^6$ spleen cells/aliquot) and each aliquot was pretreated with either PGE1 or PGE2 and then tested for mitogen responsiveness, as described in section 2.19. Sub-optimum, optimum, and supra- optimun of both Con A and PHA were used in the mitogen assay The data are presented as cpm $\pm$ S E of triplicate cultures at a given mitogen concentration

The thymuses from all experimental GVH mice in the above studies were examined histologically.

8.3 RESULTS

8.3.1 EFFECT OF PGE1 OR PGE2 PRETREATMENT ON T-CELL PROLIFERATIVE RESPONSES OF NORMAL B6AF1 SPLENOCYTES TO THE OPTIMUMMITOGENCONCENTRATION.

Table 8.1 demonstrates the T-cell proliferative responses of PGE1 or PGE2 pretreated normal B6AF1 splenocytes following stimulation with the optimum concentration of either Con A or PHA. The data (Table 8.1) show that PGE1 or PGE2 pretreatment either had no significant effect or slightly inhibited T-cell proliferative responses of normal splenocytes.

8.3.2 EFFECT OF PGE1 AND PGE2 PRETREATMENT ON THE T-CELL PROLIFERATIVE RESPONSES OF NORMAL SPLENOCYTES TO INCREASING CONCENTRATIONS OF CON A AND PHA.

Figure 8.2 demonstrates the responsiveness of PGE1 and PGE2 pretreated normal B6AF1 splenocytes to increasing concentrations of Con A and PHA. The results show that at the supra-optimum mitogen concentration a decline in the proliferative response of PGE pretreated as well as non-PGE pretreated normal splenocytes is observed (bell shaped dose response curve).

8.3.3 EFFECTS OF PGE1 AND PGE2 PRETREATMENT ON CONA AND PHA RESPONSES OF GVH SPLENOCYTES TAKEN DURING DIFFERENT STAGES OF THYMIC REGENERATION.

On different days after GVH reaction induction, splenocytes from mice that expressed no PFC response (immunosuppressed GVH splenocytes) were pooled and tested for T-cell mitogen responses following pretreatment with PGE1 or PGE2.

8.3.3.1 EFFECT OF PGE1 OR PGE2 PRETREATMENT ON T-CELL PROLIFERATIVE RESPONSES OF GVH SPLENOCYTES TO THE OPTIMUM MITOGEN CONCENTRATION

Table 8.2 shows the number of B6AF1 mice that were totally immunosuppressed on different days after the injection of 20×10^6 A PLC.

Table 8.3 shows the T-cell proliferative responses to the optimum concentration of Con A and PHA of PGE1 or PGE2 pretreated immunosuppressed splenocytes On day 30 after the injection of $20x10^6$ A PLC, no significant differences were observed between the mitogen responses of PGE1 and PGE2 pretreated and non-PGE1 and PGE2 pretreated GVH splenocytes On day 30 after GVH induction, thymuses from the animals whose splenocytes were pooled displayed severe thymic dysplasia (Table 8.3). Pretreatment of day 75 post-GVH splenocytes with PGE1 or PGE2 again showed no significant differences between the net cpm responses to the optimum concentration of Con A or PHA of PGE1 pretreated and non-PGE pretreated splenocytes. However, only slight enhancement of Con A and PHA responses was observed following PGE2 pretreatment. The thymuses on day 75 after GVH induction showed various degrees of cortical regeneration only.

Splenocytes taken at day 120 post-GVH induction showed moderate increases in their Con A and PHA responses after PGE1 or PGE2 pretreatment when compared to non-PGE pretreated splenocytes. PGE2 pretreatment had a greater enhancing effect on Con A and PHA responses than PGE1 pretreatment. The enhancement of mitogen responsiveness following PGE1 and PGE2 pretreatment varied between 2-17 fold. On day 120 post-GVH induction, the thymuses showed various degrees of medullary epithelial cell regeneration.

The most dramatic enhancement of both Con A and PHA responses was observed when splenocytes were taken at day 160

after GVH induction and were pretreated with PGE1 or PGE2. At 160 days after GVH induction, thymuses displayed complete medullary regeneration with abundant visible epithelial cells and Hassall corpuscles, however, T-cell function had not recovered.

Table 8.4 shows the number of B6AF1 mice that were totally immunosuppressed on different days after the injection of $30x10^6$ B6 PLC. The data presented in table 8.5 show the T-cell proliferative responses to the optimum concentration of Con A and PHA of PGE1 and PGE2 pretreated immunosuppressed splenocytes taken from B6AF1 mice at different days after the injection of $30x10^6$ B6 PLC. The data demonstrate that PGE1 or PGE2 has a greater enhancing effect on both Con A and PHA responsiveness as the thymuses recover from the GVH induced medullary injury (Table 8.5, days 120 and 130 post-GVH), than the effect of PGE1 and PGE2 pretreatment on mitogen responses of GVH splenocytes when the thymuses displayed either moderate lesions (Table 8.5, day 30), or when only cortical regeneration was evident (Table 8.5, day 55).

Thus, in the two GVH combinations employed in this study, a similar pattern of enhancement of Con A and PHA responsiveness of GVH splenocytes following PGE1 or PGE2 pretreatment is observed. The enhancing effects of PGE1 and PGE2 on T-cell proliferative responses appear to be dependent upon thymic medullary regeneration at the time of PG pretreatment. These results suggest that T-cells that respond to Con A and PHA, may

require two signals to acquire immunocompetence: contact with thymic medullary epithelial cells and thymic factor(s) (or agents that mimic the effects of thymic factors).

8.3.3.2 EFFECT OF PGE1 AND PGE2 PRETREATMENT ON THE T-CELL PROLIFERATIVE RESPONSES OF GVH SPLENOCYTES TO INCREASING CONCENTRATIONS OF CON A AND PHA.

Figures 8.3-8.6 demonstrates the effects of PGE1 and PGE2 pretreatment on the proliferative responses to increasing concentrations of Con A and PHA of GVH splenocytes ($20x10^6$ A PLC --> B6AF1), taken during different stages of thymic regeneration. The data demonstrate that PGE1 and PGE2 pretreatment at the time of severe thymic lesions (Figure 8 3) or at the time of cortical regeneration (Figure 8.4) had no significant effect on T-cell mitogen responses. However, when the GVH splenocytes were taken at a time when medullary epithelial cells, but not Hassall corpuscles, had regenerated (Figure 8.5) PGE1 and PGE2 pretreatment slightly enhanced proliferative responses to Con A and PHA. The most pronounced enhancement of Con A and PHA responses of GVH splenocytes following PGE1 or PGE2 pretreatment was observed at the time when both the epithelial cells and Hassall corpuscles were visible in the thymic medulla (Figure 8.6). Moreover, the data presented in figure 8.6 clearly show that PGE1 and PGE2 pretreatment of GVH splenocytes results in a bell-shaped response pattern when increasing concentrations of Con A and PHA were used. This bell-shaped response pattern of GVH splenocytes (Fig.8.6) is similar to

that observed with normal splenocytes when increasing concentrations of Con A and PHA are employed (Figure 8.2).

Similarly, in a different GVH combination ($30x10^6$B6-->B6AF1), it was noted that PGE1 and PGE2 pretreatment had no siginficant effect on the responsiveness of GVH splenocytes to increasing concentrations of Con A and PHA when a moderate degree of thymic injury was visible (Figure 8.7) or when thymic cortical regeneration had taken place (figure 8.8). In this combination ($30x10^6$ B6-->B6AF1) also PGE1 and PGE2 pretreatment of GVH splenocytes enhanced the proliferative responses at all Con A and PHA concentrations tested at the time when thymic medullary regeneration was evident (Figures 8.9 and 8.10). Furthermore, with increasing mitogen concentrations a bell-shaped response was observed (Figures 8.9 and 8.10). These results ,collectively, suggest that PGE1 and PGE2 may be acting on a cell population that is essential for the initiation of the proliferative response.

8.4 DISCUSSION

The data presented in this chapter demonstrate the effects of PGE1 and PGE2 pretreatment on T-cell mitogen responses of GVH immunosuppressed splenocytes taken during different stages of thymic regeneration. The results show that PGE1 and PGE2 pretreatment had no significant enhancing effect on mitogen responses at the time when the thymuses displayed either moderate-severe lesions or when cortical regeneration was evident. The enhancement (restoration) of GVH immuno-

suppressed splenocyte mitogen response following PGE1 or PGE2 pretreatment was observed only when the thymuses displayed medullary regeneration. These results suggest that the immunocompetent T-cells that respond to T-cell mitogens may require two signals for functional maturation and that PGE1 and PGE2 may be acting on a cell population that plays an important role in the initiation of proliferative responses.

The pronounced enhancement of mitogen responses following PGE1 or PGE2 pretreatment of GVH splenocytes , at the time when the GVH-reactive thymuses show medullary regeneration, suggests that new post-thymic T-cell precursors (Stutman, 1975, 1977, 1978; Stutman and Shen, 1977, 1979) had repopulated peripheral lymphoid organs after passing through the recovered thymuses (architecturally normal thymuses). However, these post-thymic T-cell precursors were unable to respond to Con A and PHA, although the thymuses had recovered structurally. Possibly such thymuses have not yet resumed their hormone(s) production. Therefore, pretreatment of GVH splenocytes that have come in contact with "regenerated" thymic epithelium with either PGE1 or PGE2 results in greater restoration of T-cell mitogen responsiveness. In the previous chapter (chapter 7) we had suggested that the functional recovery (hormonal productions) of the thymus may take a longer time to recover than its architectural recovery. The data presented in this chapter demonstrating that the maximum restoration of T-cell proliferative function following PGE1 or PGE2 pretreatment is observed only when the thymus has recovered architecturally support our

earlier proposal. Several workers (Bach and Bach (1974; Garaci et al., 1981; Garaci et al., 1983) have shown that PGE1 and PGE2 mimic and/or mediate the effects of thymic hormones. Thus, the data presented in this chapter strongly suggest that the regeneration of thymic epithelium and Hassall's corpuscles may not be sufficient to induce T-cell immunocompetance and that thymic factor(s) may be required for complete T-cell functional maturation

Previous work from this laboratory (Seddik et al, 1984) and others (Stutman, 1975, 1977, 1978) supports the hypothesis that GVH immunosuppressed splenocytes which respond to T-cell mitogens following PGE1 and PGE2 treatment have been influenced by the "regenerated" thymuses. We have previously demonstrated that GVH dysplatic thymuses continued to recruit pre-T-cells from the periphery, however, these thymuses were unable to induce terminal maturation in the newly recruited pre-T-cells (Seddik et al., 1984). We have also reported that the GVH BM continues to produce pre-T-cells throughout the course of GVH reactions (Seddik et al., 1984). The pre-T-cells produced by the GVH BM become functional under the influence of a normal thymus (Seddik et al., 1984). Since it is well documented that immunocompetent T-cells which initiates Con A and PHA induced proliferation require a functional thymus (i.e. thymic epithelial cell contact as well as thymic factor(s) (Stutman, 1977, 1978; Bach and Carnaud, 1976), the data in the present study would therefore, suggest that PGE1 and PGE2 treated GVH splenocytes that respond to Con A and PHA may have "homed" to

the spleens after being processed by the regenerated thymuses

The data presented in this chapter further demonstrate that when increasing concentrations of mitogens are employed to induce proliferation of PGE1 or PGE2 pretreated splenocytes of GVH- reactive mice whose thymuses show medullary regeneration a bell-shaped curve is obtained This bell-shaped curve in response to increasing concentrations of mitogen is observed following PGE1 and PGE2 pretreatment of splenocytes obtained from both the GVH combinations ($20x10^6$ A PLC-->B6AF1 and $30x10^6$ B6 PLC-->B6AF1) This response pattern of PGE pretreated GVH splenocytes is identical to that observed when normal B6AF1 splenocytes are stimulated in the presense of increasing concentrations of mitogens

The fact that the PGE1 and PGE2 pretreatment of GVH immunosuppressed splenocytes restores mitogenic responses suggests that PGE1 and PGE2 are acting on a cell population which is required for the initiation of proliferative responses. If PGE1 and PGE2 are in fact acting on a cell population which is required for the initiation of the proliferative responses then it would appear from the present data that this cell population is present in the GVH spleens at the time of thymic medullary regeneration, but is not yet functional.

It has been reported that the cellular response to mitogens/antigens is determined by two factors; (i) the intrinsic resting state of the cell that initiates the immune

response at the time of the stimulus, and, (ii) the stimulus strength (Kook and Trainin, 1975). Since PGE1 and PGE2 have different effects on the mitogen responsiveness of normal and GVH immunosuppressed splenocytes, the data presented in this chapter suggest that normal and GVH immunosuppressed splenocytes may possess different intrinsic resting states It has been suggested that one of the roles of the thymus is to maintain T-cells in a responding state via thymic hormones (Kook and Trainin, 1975). If, as proposed in the previous chapter (chapter 7), thymic functions (hormone production) resume gradually following the gradual regeneration of thymic architecture during the recovery process, then it may be plausible that the cells which are required for the initiation of the mitogen responses are present in the spleens of the GVH immunosuppressed mice whose thymuses had regenerated structurally, but due to the lack/deficiency of thymic hormones may not be at an optimum intrinsic state to respond to the stimulus.

It should be remembered that in these studies splenocytes from several mice/day/GVH combination, which were totally immunosuppressed for the PFC response to SRBC, were pooled (Table 8.2 and 8.4). Although the thymuses from these mice were at a similar stage of regeneration on a given day post-GVH induction, it is possible that the degree of regeneration may not be the same in each ,i.e., the number of medullary epithelial cells and Hassall's corpuscles or the degree of functional recovery. It is important to take these data limita-

tions into consideration because variations in the degree of thymic regeneration may influence the resting state of the splenocytes These differences in the restimg state of splenocytes may also determine the degree of enhancement of proliferative responses following PGE1 and PGE2 pretreatment

In this chapter we have argued that PGE1 and PGE2 may be acting upon a cell population which initiates the immune response, but we have not characterized the cell. However, data reported from this laboratory as well as by other workers suggest that PGE1 and PGE2 may be acting upon a IL-2 producing T-helper cell population, which may be under the direct influence of thymic epithelial cells and/or their products Our earlier work suggested that the prolonged GVH T-cell immunodeficiency was due to a T-helper cell maturation arrest (Seddik et al., 1980, 1984) associated with thymic epithelial cell injury. Our recent data demonstrate that this GVH induced thymic injury results in marked depletion/reduction in IL-2 producing, but not IL-2 responding cells (Mendez et al , 1985a,b). Studies by Beardsley et al. (1983) provide direct evidence that the maturation of IL-2 producing cells is under the influence of thymic medullary epithelial cells and/or their products. The data presented in this chapter show that PGE1 and PGE2, which mimic the effect/action of thymic hormones (Bach and Bach, 1974; Garaci et al., 1981; Garaci et al., 1983), are most effective in initiating and inducing T-cell mitogen responses only when the GVH thymuses have regained normal architecture. These data suggest that the T-cell sub-

population, probably T-helper cells responsible for IL-2 production and thus initiating T-cell proliferation in response to T-cell mitogens (and possibly allo-antigens), requires both an intact thymic epithelium as well as thymic factor(s) (or agents that mimic the actions of thymic factor(s)).

In brief, the data presented in this chapter suggest that the status of the thymus, particularly its epithelium, may play a critical role in determining the degree and extent of T-cell immunodeficiecy (and probably the functional capability of Th lyl+ cells).

In the following chapter studies are reported on the restoration of GVH depressed splenic NK cell cytolytic activity and its relationship to thymic medullary dysplasia.

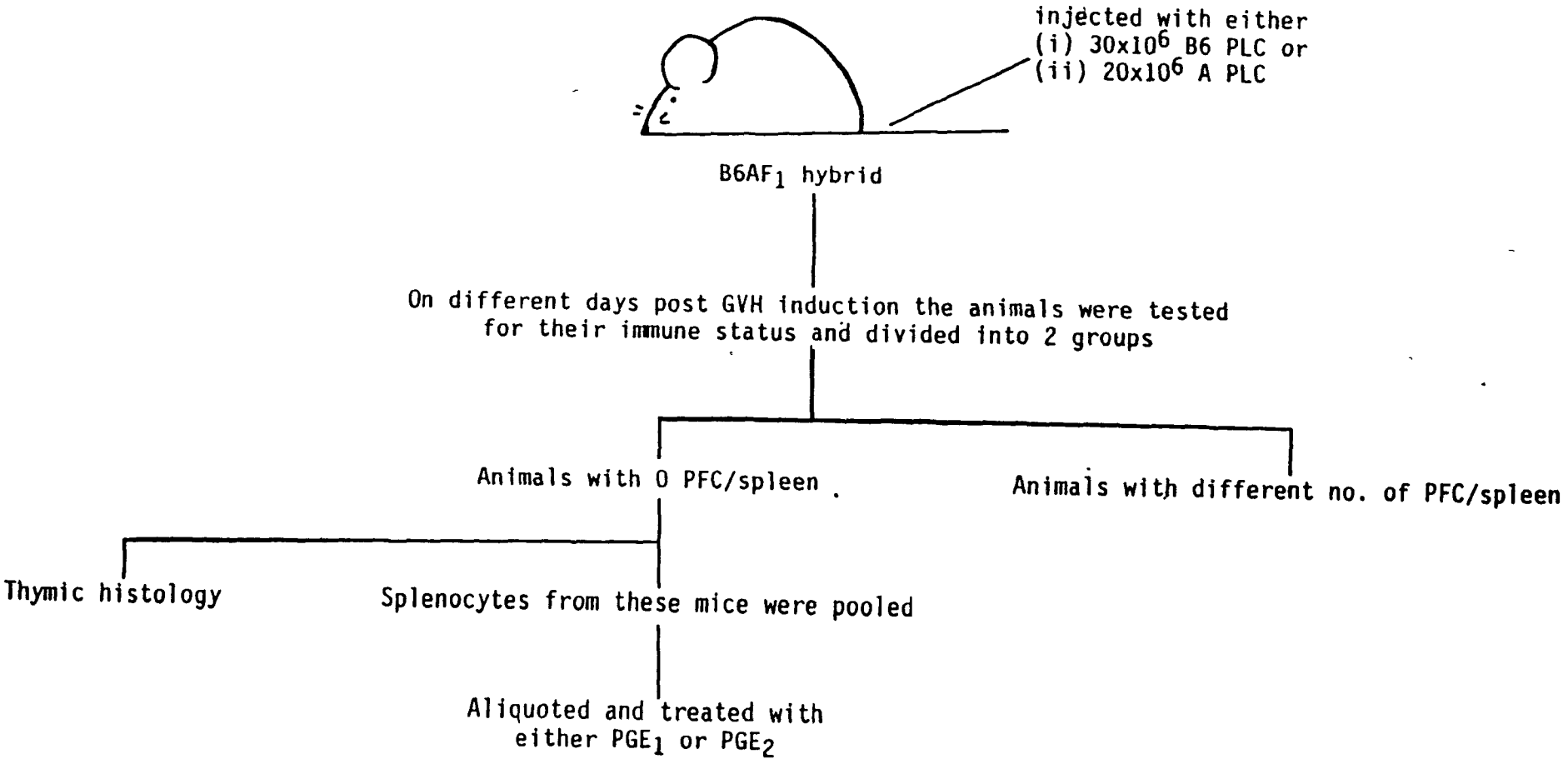


Figure 8.1 Experimental design used to investigate the role of thymic medullary epithelial cells in T-cell immunodeficiency during the course of GVH reactions.

Table 8.1 Effects of PGE1 and PGE2 pretreatment of normal B6AF1 splenocyte responses to optimum concentrations of Con A and PHA.

PG [moles] Pretreatment	mitogen responses net cpm ± S.E. x 10^{-3}[a]	
	ConA	PHA
-	178.6 ± 10.5	129.3 ± 13.5
PGE1 10^{-6}	150.7 ± 15.1	111.3 ± 11.1
PGE1 10^{-9}	165.5 ± 12.2	108.6 ± 15.1
PGE2 10^{-6}	136.2 ± 15.0	111.6 ± 16.4
PGE2 10^{-9}	151.8 ± 11.9	126.6 ± 17.8

a Pooled data of nine individual experiments

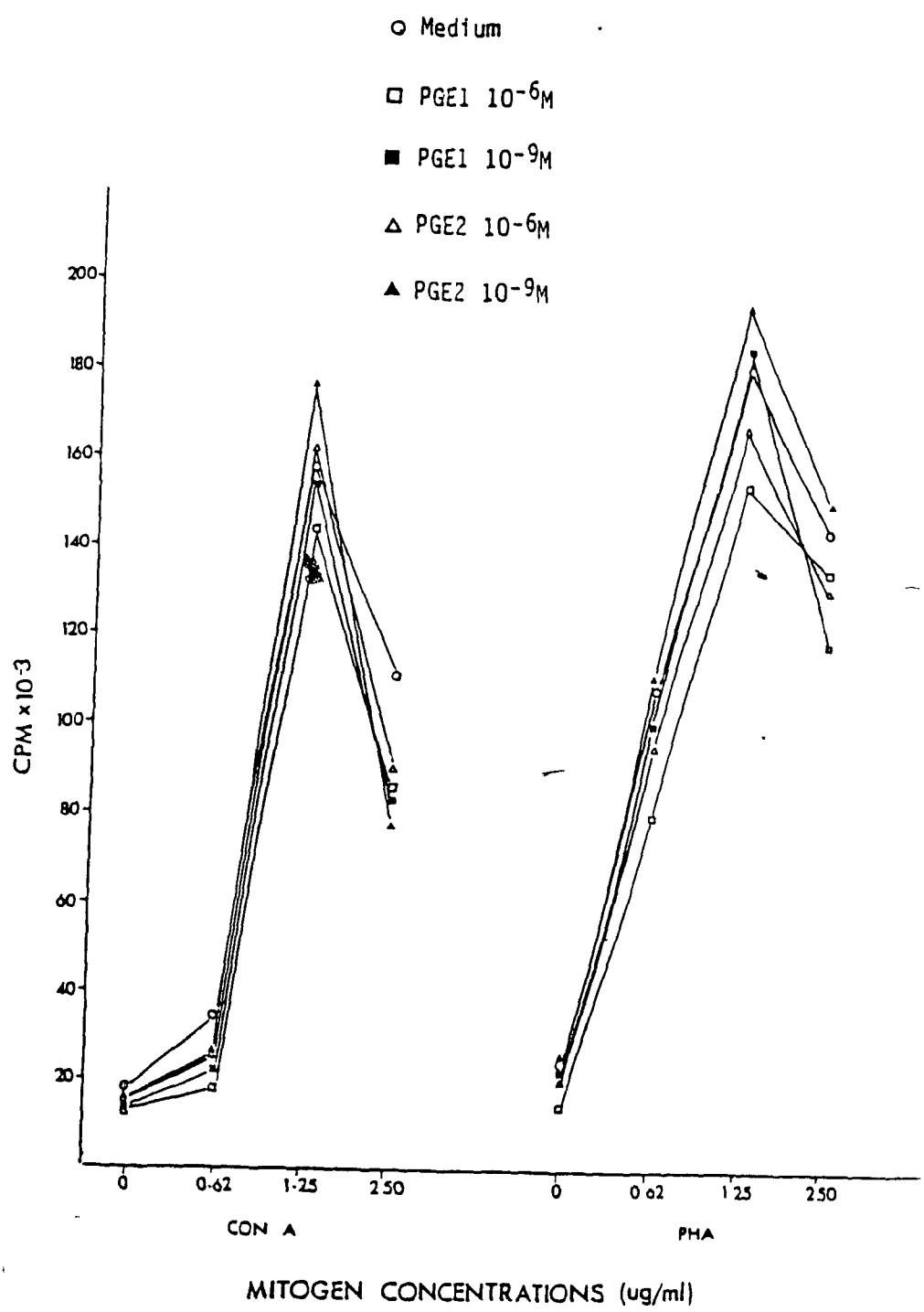


Figure 8.2 Effects of PGE1 and PGE2 pretreatment on normal B6AF1 splenocyte responses to different concentrations of ConA and PHA.

Table 8.2 Number of mice with zero PFC/Spleen on different days after the injection of $20x10^6$A PLC.

Days after GVH Induction	Number of mice injected with SRBC/day	Number of mice with zero PFC[a]
30	5	5
75	5	5
120	9	8
160	6	4

a Splenocytes from GVH mice with zero PFC were pooled for PGE1 and PGE2 Pretreatment

Table 8.3 Effects of PGE1 and PGE2 pretreatment on GVH immunosuppressed splenocyte responses to the optimum concentrations of ConA and PHA during different stages of thymic regeneration.

Day after GVH Induction[a]	PG[moles] Pretreatment	MITOGEN RESPONSES NET cpm ± S.E.x10^{-3} ConA (1.25 ugs)	PHA (1.25 ugs)	DEGREE OF THYMIC REGENERATION Cortical	Medullary HEC[c]	Medullary HC[d]
30	-	30.4 ± 5.2	0.7 ± 0.3	-	-	-
	PGE1 10^{-6}	22 9 ± 2.0	0.8 ± 6.4			
	PGE1 10^{-9}	32.9 ± 1.4	0.3 ± 0.4			
	PGE2 10^{-6}	28.3 ± 2.1	1.9 ± 1.4			
	PGE2 10^{-9}	26.1 ± 1.9	0.4 ± 0.2			
75	-	24.8 ± 2.3	1.4 ± 1.1	+	-	-
	PGE1 10^{-6}	22.5 ± 1.1	2.2 ± 1.5			
	PGE1 10^{-9}	25.2 ± 2.1	4.8 ± 0.8			
	PGE2 10^{-6}	36.7 ± 2.3	1.9 ± 0.3			
	PGE2 10^{-9}	36.4 ± 3.4	2.9 ± 0.6			
120	-	3.5 ± 0.2	-[b]	+	+	-
	PGE1 10^{-6}	5.6 ± 0.7				
	PGE1 10^{-9}	12.4 ± 2.9				
	PGE2 10^{-6}	14.3 ± 1.5				
	PGE2 10^{-9}	36.8 ± 1.8				
160	-	23.9 ± 0.6	41.2 ± 1.1	+	+	+
	PGE1 10^{-6}	88.6 ± 5.5	113.9 ± 10.4			
	PGE1 10^{-9}	106.9 ± 3.8	135.6 ± 7.9			
	PGE2 10^{-9}	111.6 ± 7.0	121.7 ± 10.8			
	-[b]	-[b]	-[b]			

a GVH reactions were induced in B6AF1 mice by injecting $20x10^6$A PLC: b not done:

c medullary epithelial cells: d Hassall corpuscles

Table 8.4 Number of mice with zero PFC/Spleen on different days after the injection of $30x10^6$B PLC.

Days after GVH induction	Number of mice injected with SRBC/day	Number of mice with Zero PFC[a]
30	6	6
55	7	7
120	7	4
130	7	4

a Splenocytes from GVH mice with zero PFC were pooled for PGE1 and PGE2 Pretreatment.

Table 8.5 Effects of PGE1 and PGE2 pretreatment on GVH immunosuppressed splenocyte responses to the optimum concentrations of ConA and PHA during different stages of thymic regeneration.

Day after GVH Induction[a]	PG [moles] Pretreatment	MITOGEN RESPONSES MET cpm ± S.E.x10^{-3} ConA (1.25 ugs)	PHA (1.25 ugs)	DEGREE OF THYMIC REGENERATION Cortical	Medullary MEC[c]	Medullary HC[d]
	-	21.7 ± 1.6	0.9 ± 0.4			
	PGE1 10^{-6}	22.9 ± 0.3	1.5 ± 0.2			
30	PGE1 10^{-9}	28.7 ± 0.6	2.4 ± 0.8	-	-	-
	PGE2 10^{-6}	24.5 ± 0.5	1.8 ± 0.8			
	PGE2 10^{-9}	32.6 ± 0.6	0.9 ± 1.1			
	-	16.5 ± 1.3				
	PGE1 10^{-6}	17.9 ± 1.5				
55	PGE1 10^{-9}	21.0 ± 1.7	b	+	-	-
	PGE2 10^{-6}	40.9 ± 1.6				
	PGE2 10^{-9}	25.4 ± 1.8				
	-	2.5 ± 0.2	1.8 ± 0.4			
	PGE1 10^{-6}	15.5 ± 0.2	5.3 ± 0.5			
120	PGE1 10^{-9}	35.9 ± 4.4	19.9 ± 2.0	+	+	+
	PGE2 10^{-6}	65.9 ± 4.3	27.1 ± 0.4			
	PGE2 10^{-9}	61.1 ± 4.5	32.2 ± 2.3			
	-	17.5 ± 0.1	2.9 ± 1.4			
	PGE1 10^{-6}	67.5 ± 4.4	23.5 ± 1.1			
130	PGE1 10^{-9}	70.2 ± 0.8	32.8 ± 1.3	+	+	+
	PGE2 10^{-6}	65.8 ± 2.4	21.4 ± 1.0			
	PGE2 10^{-9}	68.9 ± 1.4	32.9 ± 2.4			

a GVH reactions were induced in B6AF1 mice by injecting $30x10^6$ B6 PLC: b not done:
c Medullary epithelial cells: d Hassall corpuscles

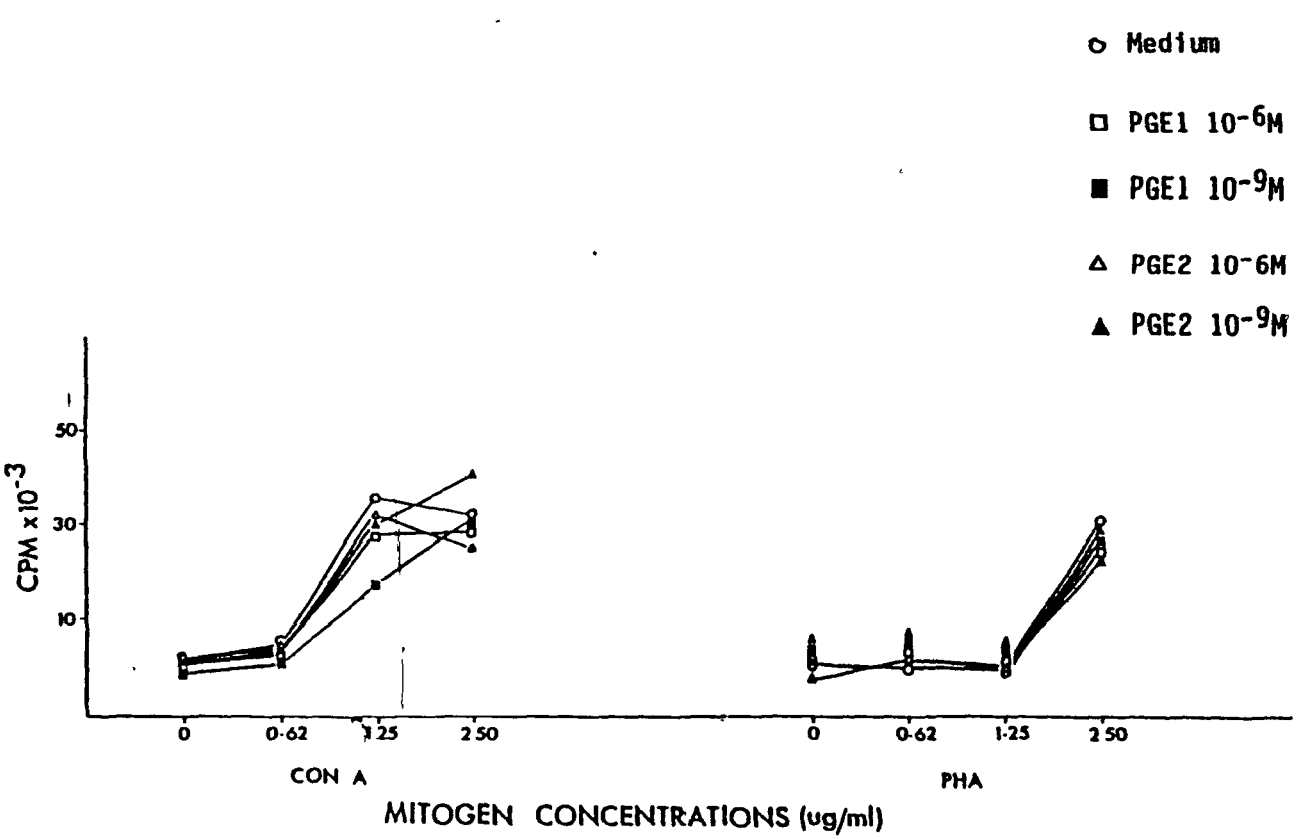


Figure 8.3 Effects of PGE1 and PGE2 pretreatment of GVH immunosuppressed splenocytes on their responses to different concentrations of ConA and PHA at the time of severe thymic dysplasia. GVH reactions were induced in B6AF1 mice by injecting $20x10^6$ A PLC. Splenocytes were taken at day 30 after GVH induction. Each point represents the mean cpm of triplicate cultures. The S.E.at each point did not exceed 12% of the mean.

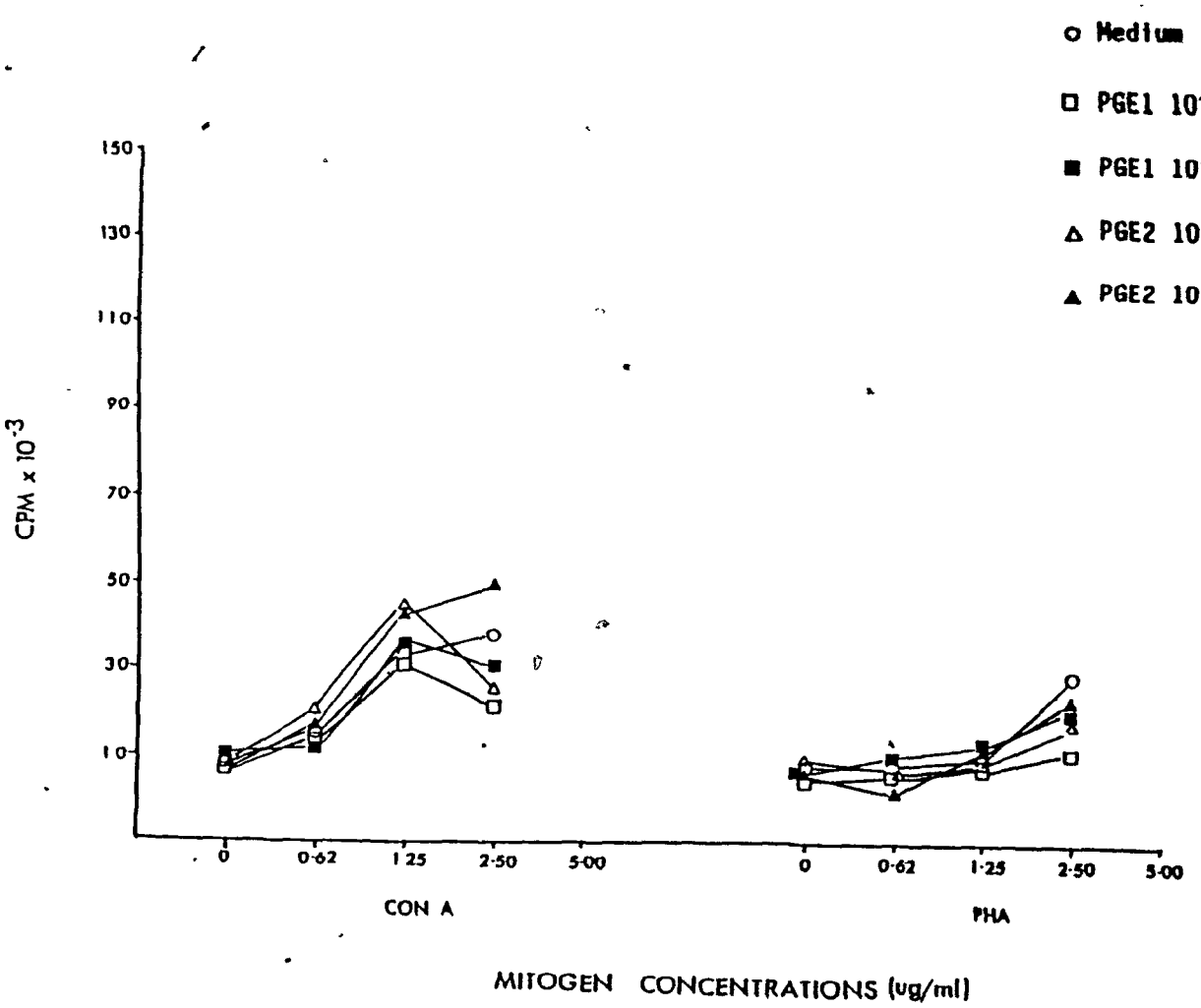


Figure 8.4 Effects of PGE1 and PGE2 pretreatment of GVH immunosuppressed splenocytes on their responses to different concentrations of ConA and PHA at the time of cortical regeneration. GVH reactions were induced in B6AF1 mice by injecting 20×10^6A PLC. The splenocytes were obtained at day 75 after GVH induction. Each point represents the mean cpm of triplicate cultures. The S.E. at each point did not exceed 12% of the mean.

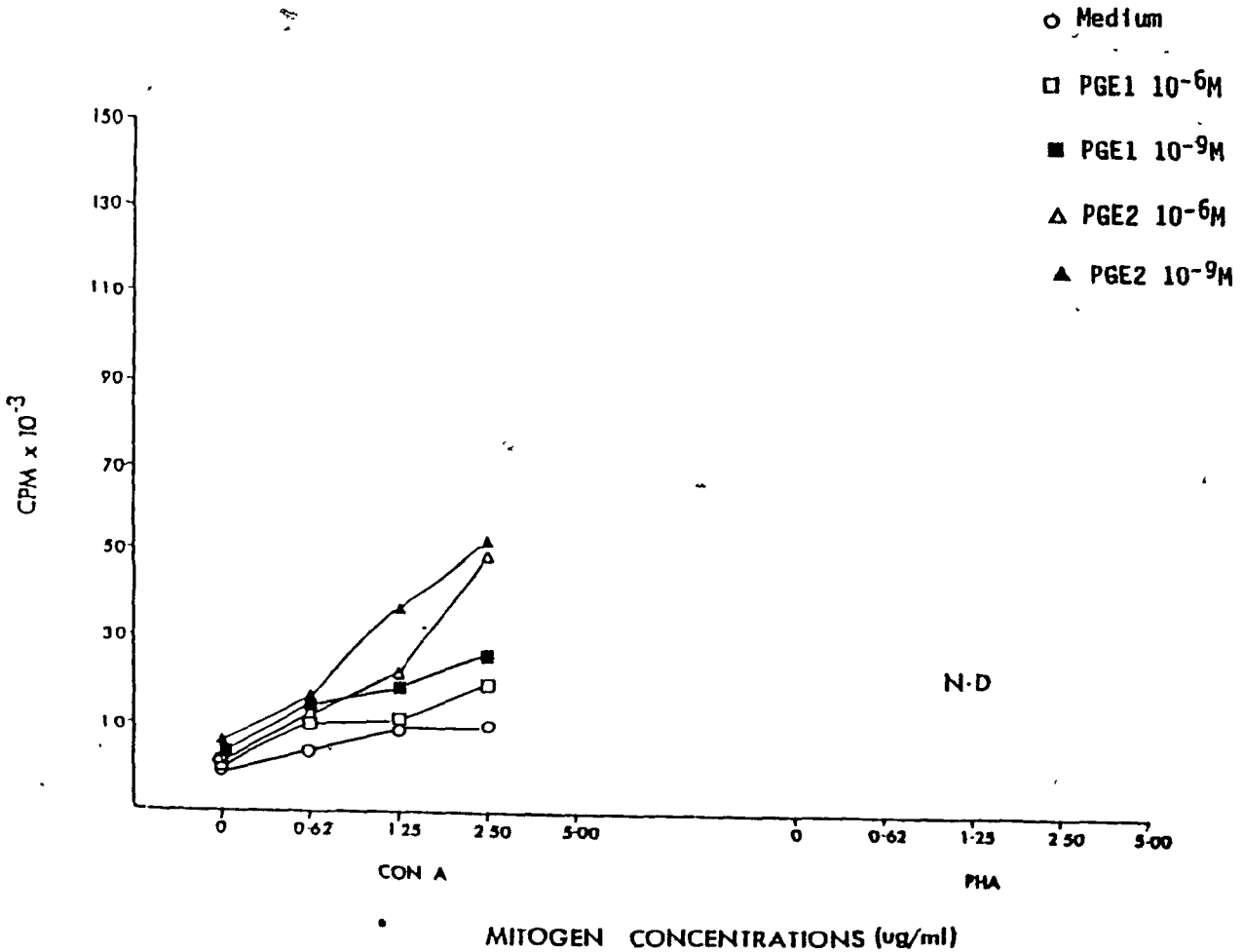


Figure 8.5 Effects of PGE1 and PGE2 pretreatment of GVH immunosuppressed splenocytes on their responses to different concentrations of ConA and PHA at the time of medullary epithelial cell regeneration. GVH reactions were induced in B6AF1 mice by injecting 20×10^6 A PLC. Splenocytes were obtained at day 120 after GVH induction. Each point represents the mean cpm of triplicate cultures. The S.E. at each point did not exceed 12% of the mean.

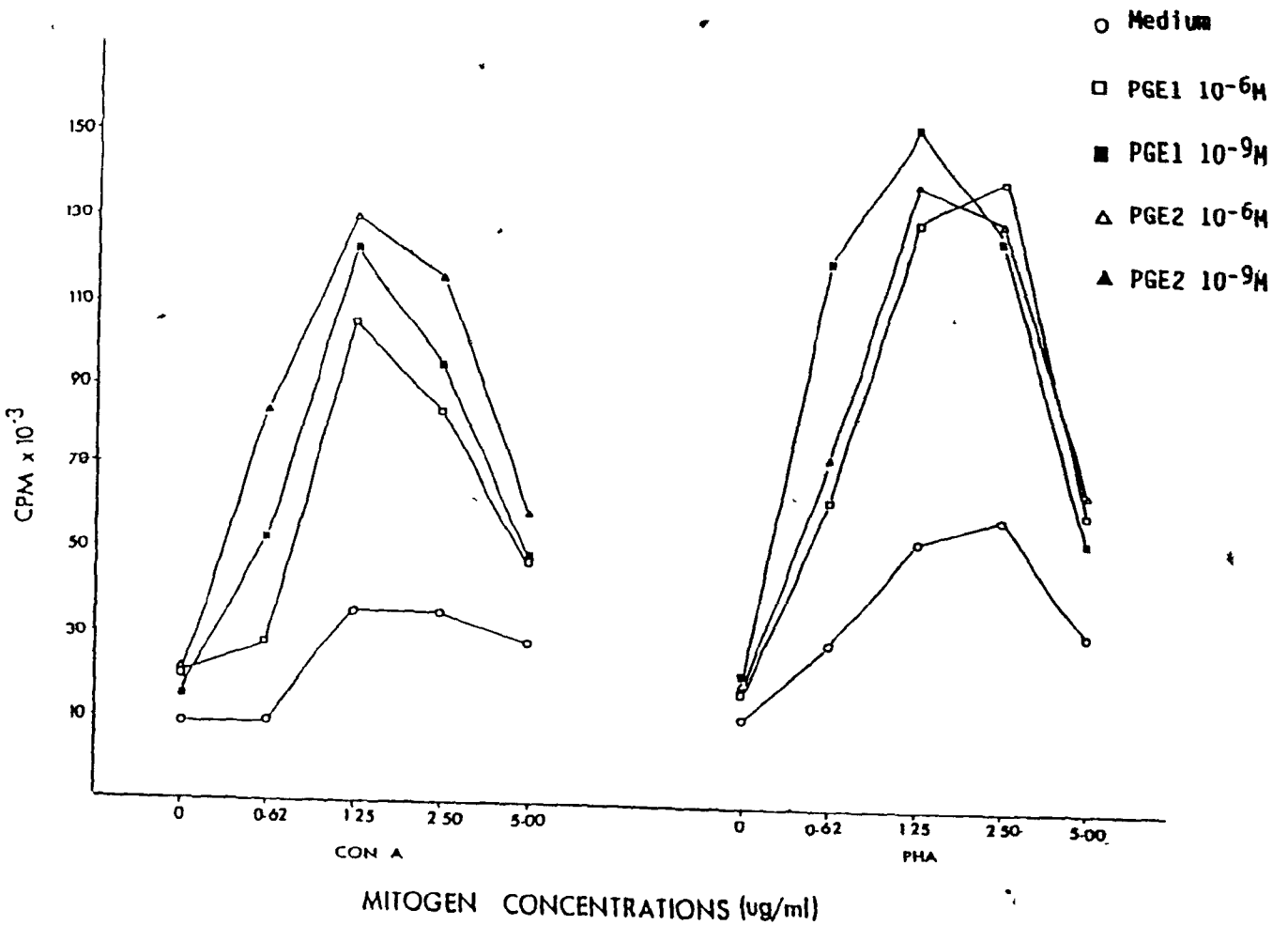


Figure 8.6 Effects of PGE1 and PGE2 pretreatment of GVH immunosuppressed splenocytes on their responses to different concentrations of ConA and PHA at the time of medullary epithelial cell and Hassall's corpuscles regeneration. GVH reactions were induced in B6AF1 mice by injecting $20x10^6$A PLC. Splenocytes were obtained at day 160 after GVH induction. each point represents the mean cpm of triplicate cultures. The S.E. at each point did not exceed 12% of the mean.

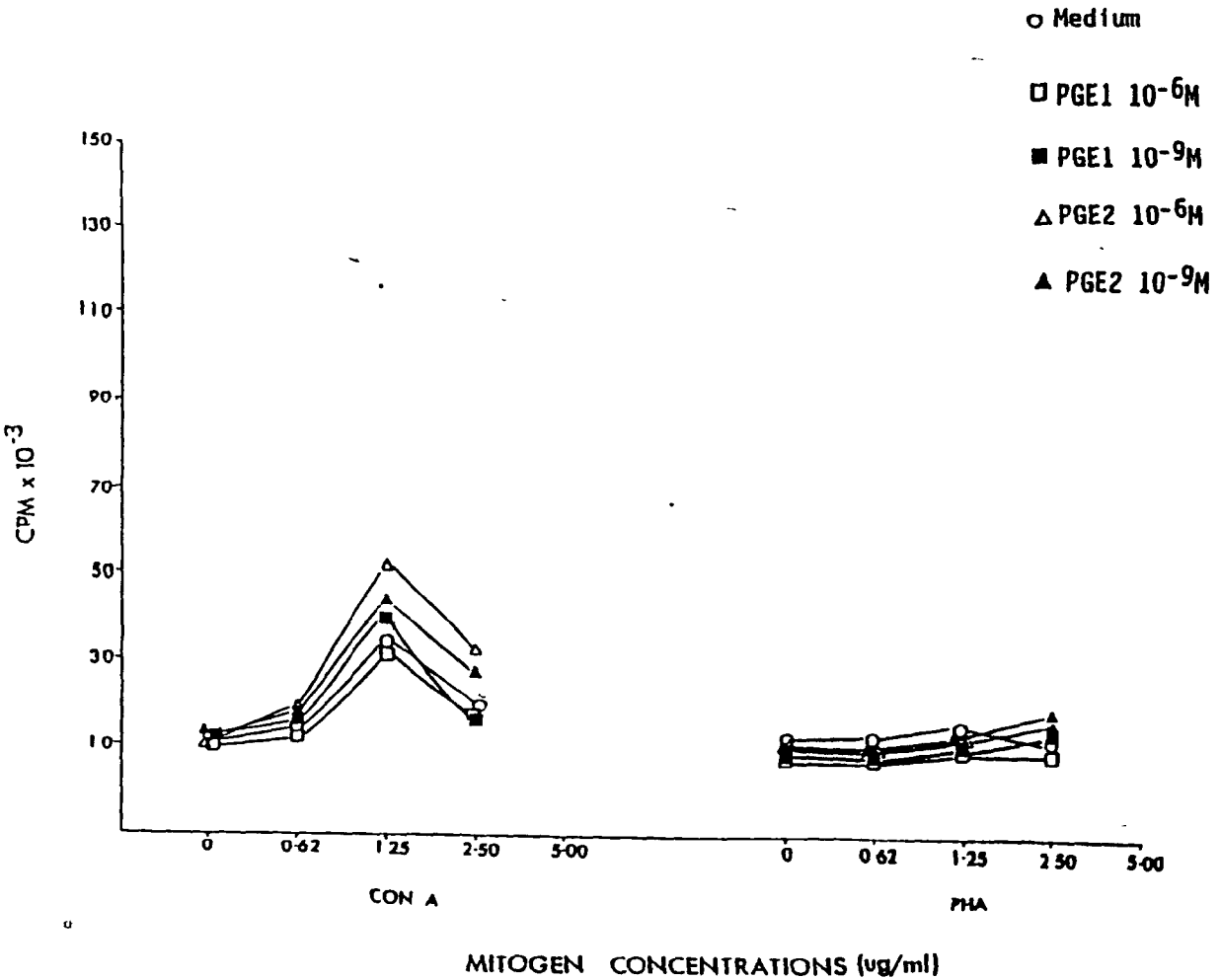


Figure 8.7 Effects of PGE1 and PGE2 pretreatment of GVH immunosuppressed splenocytes on their responses to different concentrations of ConA and PHA at the time of moderate thymic dysplasia. GVH reactions were induced in B6AF1 mice by injecting $30x10^6$ B6 PLC. Splenocytes were obtained at day 30 after GVH induction. Each point represents the mean cpm of triplicate cultures. The S.E. at each point did not exceed 12% of the mean.

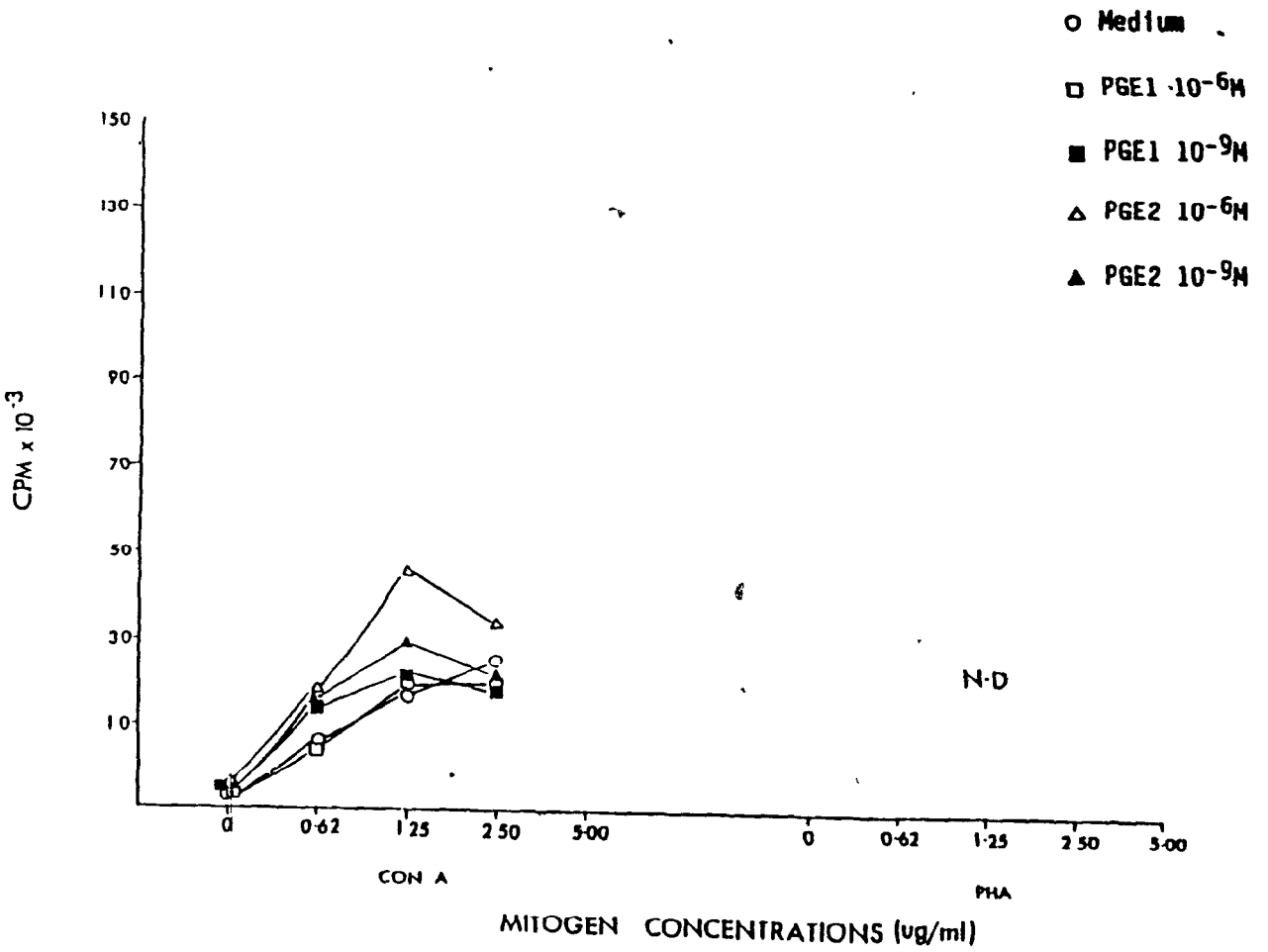


Figure 8.8 Effects of PGE1 and PGE2 pretreatment of GVH immunosuppressed splenocytes on their responses to different concentrations of ConA and PHA at the time of thymic cortical regeneration. GVH reactions were induced in B6AF1 mice by injecting $30x10^6$ B6 PLC. Splenocytes were obtained at day 55 after GVH induction. Each point represents the mean of triplicate cultures. The S.E. at each point did not exceed 12% of the mean.

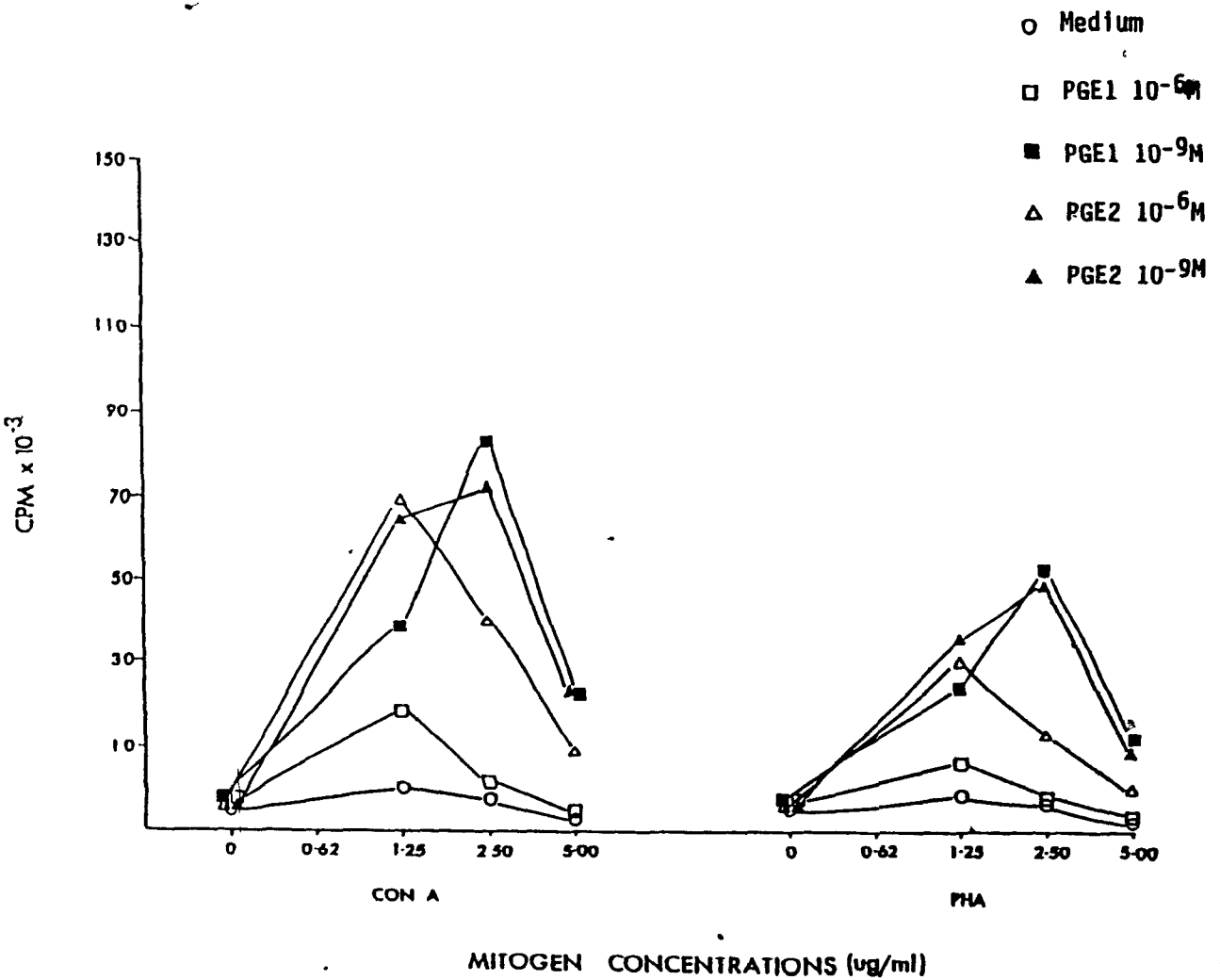


Figure 8.9 Effects of PGE1 and PGE2 pretreatment of GVH immunosuppressed splenocytes on their responses to different concentrations of ConA and PHA at the time of thymic medullary regeneration. GVH reactions were induced in B6AF1 mice by injecting $30x10^6$ B6 PLC. Splenocytes were obtained at day 120 after GVH induction. Each point represents the mean cpm of triplicate cultures. The S.E. at each point did not exceed 12% of the mean.

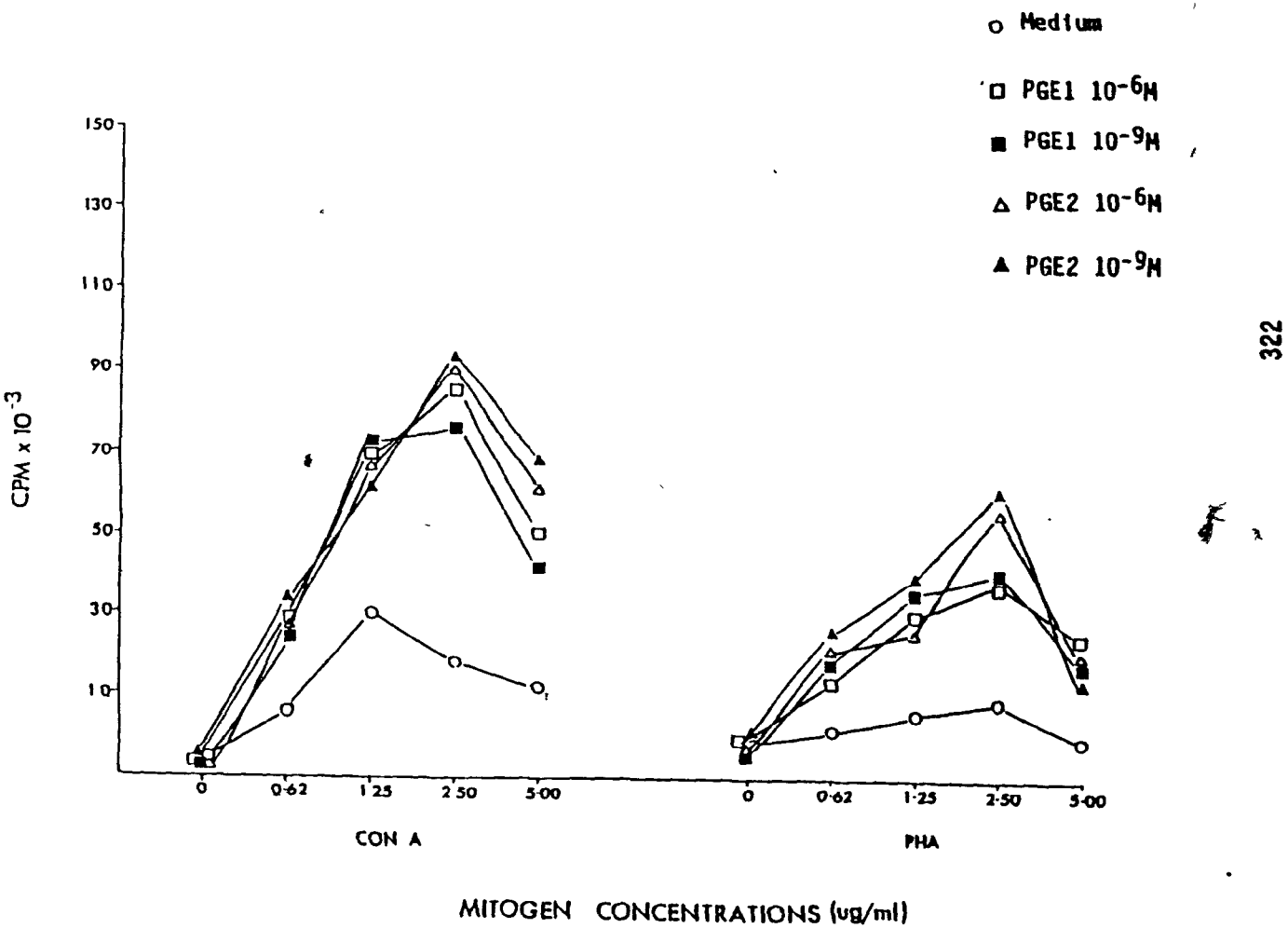


Figure 8.10 Effects of PGE1 and PGE2 pretreatment of GVH immunosuppressed splenocytes on their responses to different concentrations of ConA and PHA at the time of thymic medullary regeneration. GVH reactions were induced in B6AF1 mice by injecting $30x10^6$B6 PLC. Splenocytes were obtained at day 130 after GVH induction. Each point represents the mean cpm of triplicate cultures. The S.E. at each point did not exceed 12% of the mean.

CHAPTER NINE

AN INVESTIGATION INTO THE MECHANISM(S) OF DEPRESSED SPLENIC NK CELL ACTIVITY DURING GVH REACTIONS: RESTORATION OF DEPRESSED SPLENIC NK CELL ACTIVITY OF GVH MICE *IN VIVO* AND *IN VITRO*.

9.1 INTRODUCTION

In chapter 3 we demonstrated that by day 30 after GVH- induction splenic NK cell activity was severely depressed In chapters 6 and 7 data were presented suggesting that the GVH- induced severe prolonged T-cell immunodeficiency was closely associated with thymic medullary dysplasia In chapter 8 we showed that T-cell proliferative functions of GVH immunosuppressed splenocytes could be partially restored by PGE pretreatment only when the thymuses of the GVH-reactive mice displayed medullary regeneration. Since NK cell activity is believed to be thymus independent, (nude mice possess high NK cell activity and thymectomy increases NK cell activity), in this chapter we have therefore investigated the possible causes of depressed splenic NK cell activity during GVH reactions and whether the depressed splenic NK cell activity of GVH-reactive mice could be restored at the time when thymic medullary lesions were visible.

9.2 EXPERIMENTAL DESIGN

A diagramatic representation of the experimental design is shown in figure 9.1. GVH reactions were induced in B6AF1 mice by injecting $30x10$ $B6^6$ PLC. GVH reaction induction was confirmed by suppression of the PFC response to SRBC on days 8 and 12.

In the first series of experiments, GVH-reactive and normal mice were randomly selected. On different days after GVH

induction the animals were sacrificed under ether anesthesia. Blood from GVH and normal mice was collected by cardiac puncture and pooled for each group. The blood was allowed to clot for 45-60 minutes at room temperature, and serum was collected (see section 2.14 for details). The serum was then tested for interferon (IFN) levels (see section 2.15). Single cell suspensions were made of GVH and normal spleens. The cells of each suspension were partially purified to obtain NK cells (see section 2.5 for detail) and were tested for NK cell cytotoxicity. The thymuses from GVH animals were fixed in 5% formalin for histology.

In the second series of experiments, GVH reactions were induced in B6AF1 mice by injecting $30x10^6$ B6 PLC. Age and sex matched normal B6AF1 mice served as controls. These GVH-reactive mice were divided into two groups, G1 and G2. Similarly, normal B6AF1 mice were also divided into two groups, N1 and N2. GVH and normal B6AF1 mice from groups G1 and N1 were randomly selected and injected with Poly I:C (100ug/mouse) I.P. in a volume of 0.3 ml at the time when NK cell activity of GVH-reactive mice was severely depressed and IFN could not be detected in their serum. The GVH and normal B6AF1 mice of groups G2 and N2 were not injected with Poly I:C and served as controls. Eighteen to twenty four hours after the adminstration of Poly I:C, the mice (G1,G2, N1, and N2) were sacrificed. Serum was obtained, as described above, and tested for IFN titers.

The spleens from each group were pooled and single cell suspension were prepared. NK cell activity was then assessed Thymuses were removed, fixed in 5% formalin, and studied histologically.

The third series of experiments was designed to investigate whether NK cell activity in NK cell depressed GVH-reactive splenocytes could be restored by in vitro treatment with either PGs and Poly I:C alone or in combination Splenic and BM mononuclear cellularity and thymic histology were also studied.

9.3. RESULTS

9.3.1. CONFIRMATION OF THE INDUCTION OF GVH REACTION:

To confirm that a GVH reaction was induced mice were randomly selected from a pool of GVH-reactive animals and the PFC response to SRBC was assessed. Table 9.1 demonstrates that the GVH-reactive animals were totally suppressed by day 12 after GVH induction, thus confirming the induction of GVH reactions.

9.3.2. KINETICS OF SPLENIC NK CELL ACTIVITY AND SERUM INTERFERON TITERS AFTER THE INDUCTION OF GVH REACTIONS:

Table 9.2 demonstrates the kinetics of splenic NK cell activity and serum IFN titers (both measured in the same mice) during the course of GVH reactions. The data presented show that splenic NK cell activity and serum IFN titers were

augmented by day 4 after GVH-induction. NK activity peaked by day 8 and IFN levels were maximum by day 12, followed by depressed NK activity and IFN titers by day 30 after GVH induction. These results show a close association between augmented splenic NK cell activity and serum IFN titers during the course of GVH reactions.

9.3.3 EFFECTS OF IN VIVO ADMINISTRATION OF POLY I:C ON SPLENIC NK CELL ACTIVITY AND SERUM INTERFERON TITERS OF GVH IMMUNOSUPPRESSED MICE:

Table 9.3. shows the effects of in vivo adminstration of poly I:C, an IFN inducer, on NK cell activity and IFN production in GVH-reactive animals when both NK cell activity and IFN titers had declined. The results show that a single in vivo injection (I.P.) of Poly I:C (100ug/animal) increased NK cell activity to levels of normal non-Poly I:C treated mice, which was much less than the NK activity observed in normal mice treated with Poly I:C. IFN production and NK cell activity of GVH mice treated with Poly I:C was only about 25-30% of NK activity and IFN titers observed in normal mice treated with Poly I:C. These data show that IFN producing cells and NK cells are present in the GVH- reactive animals and that Poly I:C boosted NK cell activity and serum IFN titers show a close association, i.e., both NK cell activity and IFN levels of GVH mice were 25-30% of normal B6AF1 response following Poly I:C adminstration.

9.3.4. THYMIC HISTOLOGY:

Table 9.4. shows that on days 30 and 35 after GVH induction moderate thymic lesions were observed in both untreated and Poly I:C treated GVH mice, whereas normal B6AF1 mice that received Poly I:C possessed a normal thymic architecture, suggesting that Poly I:C did not have any effect on the thymus.

9.3.5. BM NUCLEATED CELLULARITY FOLLOWING GVH REACTION INDUCTION

Figure 9.2 shows the BM cellularity of B6AF1 mice on different days after the injection of $30x10^6$ B6 PLC. The results show that the BMs of GVH mice became severely depleted of nucleated cells by day 20 after GVH induction. Spontaneous repopulation of the BM starts by day 30 and by day 60 after GVH induction complete repopulation of the BM was observed.

9.3.6 SPLENIC NUCLEATED CELLULARITY FOLLOWING GVH REACTION INDUCTION:

Figure 9.3 shows the splenic cellularity of B6AF1 mice on different days after the injection of $30x10^6$ B6 PLC. The data show that the spleens of GVH mice become severely depleted of nucleated cells by day 30 post-GVH induction. Figure 9.3 further shows that by day 60 post-GVH induction only partial repopulation of the spleens of GVH animals with mononuclear cells was observed. The kinetics of splenic nuclear cell repopulation in different GVH combinations was also shown in chapter 7 (Figure 7.9).

9.3.7. EFFECTS OF PGS AND POLY I:C PRETREATMENT ON NORMAL

AND GVH IMMUNODEPRESSED SPLENIC NK CELL ACTIVITY:

Table 9.5 shows the effect of different concentrations of PGE1, PGE2 and PGF2 on normal B6AF1 splenocytes. PGE1 and PGE2 pretreatment suppressed splenic NK cell activity of normal F1 cells in a dose-dependent manner, whereas PGF2 had no significant effect.

Table 9.6. demonstrates the effect of pretreatment with different PGs, either alone or in combination with Poly I:C, on the NK cell activity against YAC targets of normal and GVH splenocytes. When splenic NK cells from GVH mice were treated with either PGs or Poly I:C prior to mixing with targets, a slight enhancement of NK cell activity was observed. When splenic cells from GVH-reactive mice were pretreated with both PGs and Poly I:C higher NK cytotoxicity was induced than NK cytotoxicity induced in GVH splenocytes by PG or Poly I:C treatment alone. In contrast, PGE1 or PGE2 pretreatment inhibited endogenous as well as in vitro Poly I:C activated normal splenic NK cell activity.

9.4 DISCUSSION

The results presented in this chapter show that the early enhancement (up to day 12 after GVH induction) and the latter depression (by day 30 after GVH induction) of splenic NK cell activity parallels serum IFN titers during the course of GVH reactions. By day 30 after GVH induction, both the splenic NK cell activity as well as serum IFN titers could only be

partially boosted by *in vivo* administration of Poly I·C. In addition, NK cell activity of GVH immunosuppressed splenocytes could be boosted following *in vitro* treatment with PGs and Poly I:C, however, unlike the splenic T-cell proliferative function (chapter 8), splenic NK cell activity of GVH immunodepressed mice could be restored by PGs and Poly I:C treatment at the time when the thymic medullary injury was still evident The results suggest that NK cells are present in the spleens of GVH mice but are not able to express fully their cytolytic potential. Furthermore, the restoration of splenic NK cell activity *in vitro* of GVH mice does not require complete regeneration of the thymic medulla.

In chapters 4, 5, and 6 we have shown that the early appearance of NK cell activity, probably of donor origin, may play an important role in the development of GVH induced tissue damage. Therefore, an understanding of the mechanism(s) responsible for enhanced NK cell activity following GVH induction may be useful for manipulating and possibly preventing the development of tissue damage associated with GVH reactions. In chapter 3, data were presented (Figures 3.8 and 3.9) showing that supernatants from GVH-reactive lymphoid cells were able to induce NK cell activity (YAC killing) in normal lymph node and bone marrow cells. Although, the active component of the GVH supernatants that induced NK cell activity in normal lymphoid cells was not characterized, it is possible that the supernatants contained IFN. We have previously reported high titers of IFN in supernatants obtained from *in*

vitro cultured GVH splenocytes (Zawatsky et al., 1979). The data presented in this chapter (Table 9.2) show a close association between augmented serum IFN titers and augmented splenic NK cell activity early after GVH reaction induction. Thus, the data presented in this chapter would strongly suggest that during the early phase of GVH reactions augmented NK cell activity may be due, at least in part, to augmented IFN production. This augmented IFN production may play an important role in recruiting and/or activating NK cells of both host and donor origin. The activated donor NK cells may then inflict tissue damage (chapter 6).

The data presented in table 9.3 show that on days 30 and 35 after GVH induction, splenic NK cell activity as well as serum IFN titers of GVH immunodepressed mice could be partially boosted by in vivo administration of Poly I:C. The data in tables 9 3 show that, in absolute terms, the Poly I:C induced augmentation of NK cell activity in GVH mice was significantly lower than Poly I:C induced augmentation of NK cell activity in normal mice (15% and 13% in GVH mice versus 56% and 49% in normal mice). However, the ratio of Poly I:C boosted NK cell activity to endogenous NK cell activity in both the GVH immunosuppressed and normal mice was the same, i.e., between 3-4 fold increase (Table 9.3). Table 9.3 further shows that the endogenous GVH splenic NK cell activity was approximately 30% of the endogenous normal NK cell activity and that the Poly I:C boosted GVH splenic NK cell activity and serum IFN titers were also approximately 30% of the Poly I:C boosted normal NK cell

activity and serum IFN titers. Furthermore, the data presented in figure 9.3 (also see chapter 7; Figure 7.9) show that by day 30 after GVH induction the splenic cellularity of GVH mice is also approximately 30% of the normal cellularity The data thus show that NK cells and IFN- producing cells are present in the lymphoid tissues of GVH induced immunodepressed mice and that the function of the cells could be boosted by Poly I C These results suggest that depressed splenic NK cell activity and IFN production may be due, at least in part, to depletion of splenic mononuclear cells.

The data presented in table 9 6 demonstrate that GVH depressed splenic NK cell activity can also be boosted by in vitro treatment of GVH splenocytes with PGs and Poly I C at the time of splenic cell depletion. In contrast, PGE1 and PGE2 suppressed both the endogenous and Poly I:C boosted normal splenic NK cell activity. The boosting of GVH depressed splenic NK cell activity by PGs and Poly I:C treatment at the time of splenic cell depletion (day 30-45 after GVH induction) suggests that some other mechanism(s) beside splenic mononuclear cell depletion may be involved in the depression of splenic NK cell activity. One mechanism responsible for the depressed GVH splenic NK cell activity at the time of splenic mononuclear cell depletion may be a continuous production of lymphokines during the early phase of the GVH reaction. The continuous production of lymphokines may deplete the substrates necessary for lymphokine production. The depressed production of these lymphokines may result in decreased NK cytolytic activity. It

has been reported that mice infected persistently with virus or injected continuously with IFN inducers become hyporeactive, i.e., either produce very low amounts of IFN or fail to produce any IFN in response to subsequent stimuli (Stringfellow, 1978). During GVH reactions there is a continuous production of IFN up to day 12, followed by a decline reaching background levels by day 30 after GVH induction. After day 30, IFN can only be partially boosted by Poly I:C (Table 9.3). Lapp et al. (1980) have also reported that GVH splenocytes produce copious amounts of PGE up to day 10, followed by a rapid decline to undectable levels by day 30 after GVH induction. PGE production could not be induced by C.parvum after day 30 of GVH induction (Lapp unpublished observations). Since in vitro pretreatment of GVH splenocytes with PGs and Poly I:C boosts splenic NK cell activity, it is plausible that decreased production of IFN and/or PG by cells in GVH-reactive animals may also, at least in part, contribute to the depressed splenic NK cell activity of GVH mice, in addition to the splenic mononuclear cell depletion.

The data presented in this chapter (Figure 9.3) further show that by day 60 after GVH induction splenic cellularity increases and reaches about 65% of normal splenic cellularity, yet the splenic NK cell activity remains severely depressed (Table 9.6). On day 60 after GVH induction, the depressed splenic NK cell activity can also be boosted by in vitro pretreatment of GVH splenocytes with PGs and Poly I:C (Table 9.6). The boosting (restoration) of GVH depressed splenic NK

9.6). The boosting (restoration) of GVH depressed splenic NK cell activity by PG and Poly I:C pretreatment at the time of splenic repopulation suggests that some other mechanism(s) besides splenic mononuclear cell depletion and hyporeactivity of the surviving cells may be involved in the depression of splenic NK cell activity (since "newly" formed mononuclear cells should be present in the repopulated spleens) The data presented in figure 9.2 show that by day 20 after GVH induction, the BM of GVH mice becomes severely hypocellular (BM mononuclear cellularity may reach approximately 17% of normal) However, by day 60 post-GVH induction, BM cellularity spontaneously increases and reaches normal levels (97% of normal) (Figure 9.2). It was shown in chapter 3 (Figure 3 7) that the BM NK cell activity not only reappeared but was dramatically augmented by day 60 after GVH induction, a time when BM cellularity reaches normal levels (Figure 9.2). In contrast, splenic NK cell activity remained severely depressed up to day 150 after GVH induction (chapter 3, Figure 3.5). The fact that the NK cell activity recovers in the BM but not the spleen (chapter 3), by day 60 after GVH induction, suggests that NK cells produced in the BM are exported to the spleen, however, these splenic NK cells do not undergo spleen "dependent" maturation and are unable to express their full cytolytic potential.

Several studies have recently reported heterogeneity within NK cells (Tai et al., 1980; Lust et al., 1981). It has been suggested that certain sub-populations of NK cells are BM

dependent while others are spleen dependent for their complete maturation (Lust et al., 1981). The spleen dependent NK cell populations, although produced in the BM (Haller, 1977; Haller et al., 1977), perhaps undergo further maturation and differentiation in the splenic microenvironment (Lust et al., 1981). It is possible that the splenic microenvironment of GVH-reactive animals may not be conducive to further maturation of NK cells (expression of cytolytic potential). In fact, Pattengale et al. (1983) have reported that the spleens of GVH-reactive animals have altered histology. The data presented in this chapter (Table 9.6) do not show whether boostable splenic NK cells at the time of splenic repopulation are "new" BM derived NK cells or not. However, the data presented in table 9.6 showing that PGs and Poly I:C pretreatment can boost GVH depressed splenic NK cell activity at the time of splenic repopulation (Figure 9.3) suggest that NK cells are present in the repopulated spleens but fail to express their cytolytic potential.

In brief, the data presented in this chapter (and also in chapter 3) suggest that the continued depression of splenic NK cell activity in GVH mice may be due to at least four mechanisms : (i) splenic mononuclear cell depletion; (ii) hyporeactivity of lymphokine producing cells; (iii) a PG sensitive suppressor mechanism in the spleen but not in the bone marrow; and, finally, (iv) lack of expression of cytolytic potential by splenic NK cells, i.e., a defect in spleen

dependent NK cell maturation. These four possibilities may not be mutually exclusive

The association between thymic dysplasia and NK cell activity (Tables 9.4 and 9.6) does not concur with the relationship between thymic dysplasia and T-cell function as described in the previous chapter (chapter. 8) The data presented in this chapter showing that NK cell activity can be restored even when thymic medullary dysplasia is evident suggest that splenic NK cell activity can be restored earlier than splenic T-cell proliferative function and that the restoration of splenic NK cell activity is independent of thymic medullary regeneration . It is believed that NK cells and T-cells belong to the same lineage, and represent different stages in maturation/differentiation (Herberban et al., 1979; Herberman and Holden, 1978; Shellam, 1977; Kaplan, 1985) . It has been proposed that NK cells are pre-T-cells and the thymic hormones (or the presense of a normal thymus) induced depression of NK cell activity reflects a shift in pre-T-cells (NK cells) into more mature T-cells (Bardos et al., 1982) Similarly, thymectomy (or the absense of the thymus as in nu/nu mice) increases the pre-T-cell pool and therefore, increases NK cell activity (Shellam, 1977; Herberman et al., 1979). If NK cells are pre-T-cells as is believed, then it is possible that the presense of boostable NK cells in GVH spleens may represent immature pre-T-cells which do not require an architecturally normal thymic medulla. In fact, we have observed that GVH BM continues to produce pre-T-cells (Seddik et al., 1984b)

regardless of the severity of the thymic medullary lesions.

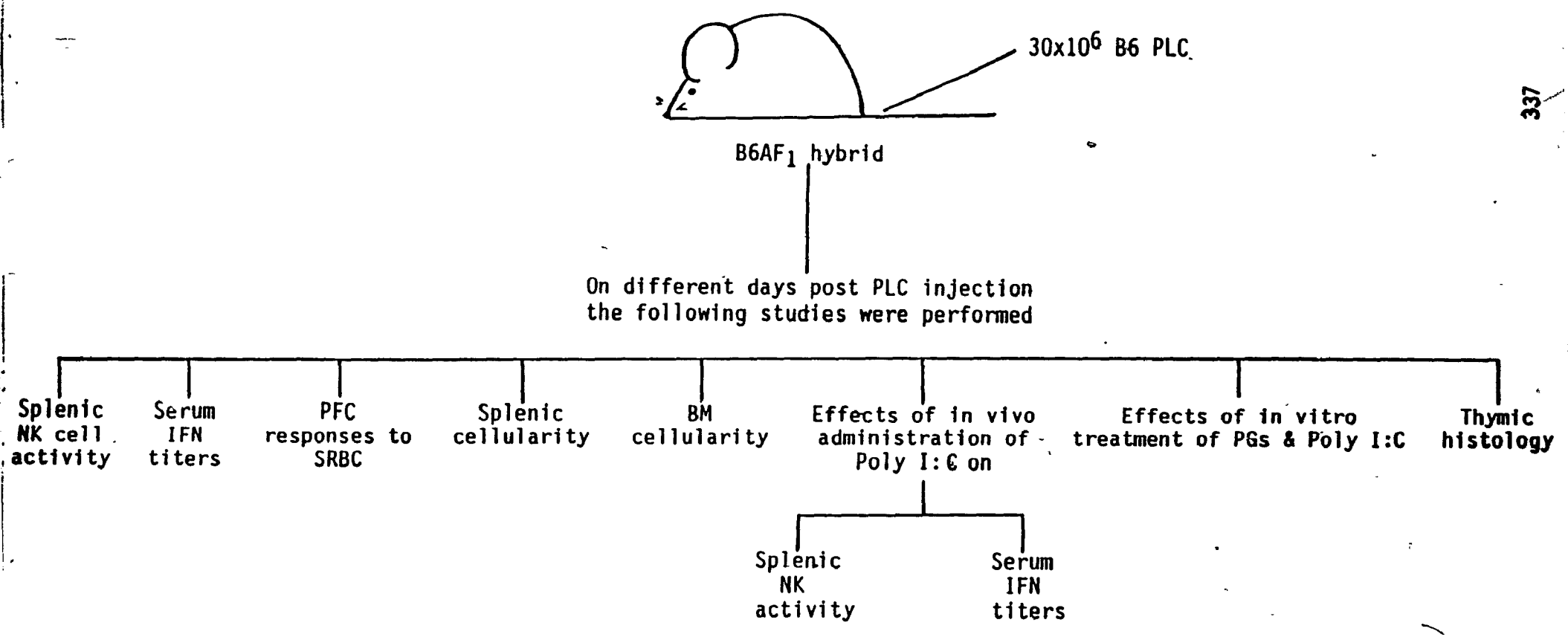


Figure 9.1 Experimental design used to investigate the possible mechanism(s) of depression of splenic NK cell activity during the course of GVH reactions.

Table 9.1: PFC/spleen response to SRBC of B6AF1 mice after the injection of $30x10^6$ B6 PLC.

Days after GVH induction [a]	PFC/spleen ± S.E X 10^{-3}
0	117.3 ± 11.7
8	9.7 ± 3.3
12	0.1 ± 0.1

a Three animals/day were tested for the PFC response.

Table 9.2: Splenic NK cell activity and serum IFN titers of B6AF1 mice on different days after the injection of $30x10^6$B6 PLC.

Days after GVH Induction	% Specific Lysis[b] YAC-1	P815	Serum IFN titers (u/ml)[c]
-	12	1	0
4	18	1	40
8	44	8	80
12	32	9	120
30	2	-	0

a 4 animals/day were randomly selected from a pool of mice

b The effector : target cell ratio used was 50:1

c On each day IFN titers were determined in pooled serum of 4 animals

Table 9.3 Effect of in vivo administration of poly I:C on NK cell activity and IFN production in normal and GVH reactive B6AF1 hybrids.

Groups	Poly I:C Treatment in vivo	Days Post GVH Induction	% Specific Lysis YAC	% Specific Lysis P815	IFN Titer (u/ml)
Expt.1					
B6AF1	-	-	15.41	0.89	0
B6AF1	+	-	56.00	4.55	560
B6AF1 (GVH)	-	30	4.10	-0.46	0
B6AF1 (GVH)	+	30	15.48	1.46	150
Expt.2					
B6AF1	-	-	10.62	-1.11	0
B6AF1	+	-	49.22	3.19	480
B6AF1 (GVH)	-	35	3.25	ND	0
B6AF1 (GVH)	+	35	12.99	ND	140

Table 9.4: Degree of thymic injury observed in GVH mice treated with Poly I:C.

Days after GVH induction	Poly I:C treatment (100 um/mouse)[a]	Thymic Injury[b]	
		Cortex	Medulla
0	-	-	-
	+	-	-
30	-	+	+
	+	+	+
35	-	+	+
	+	+	+

a (-) no poly I:C treatment; (+) poly I:C treatment

b (-) no lesions were observed; (+) lesions were visible

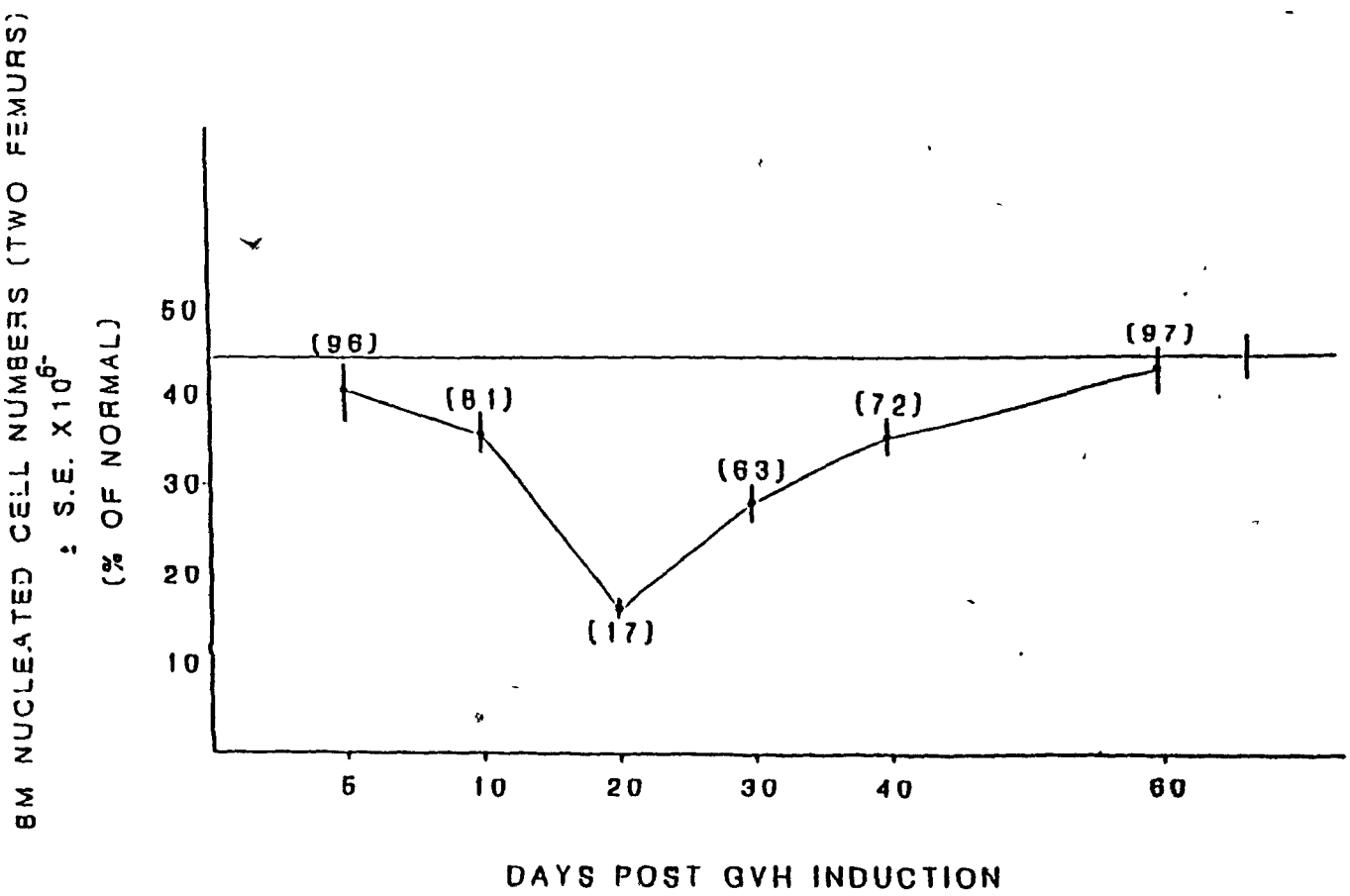


Figure 9.2 Kinetics of BM nucleated cell depletion and repopulation in B6AF1 mice after the injection of $30x10^6$ B6 PLC. On each day after GVH induction 4 animals were tested. The data is presented as mean ± S.E. The experiment was performed two times. Each experiment showed similar kinetics. Results of one experiment are shown.

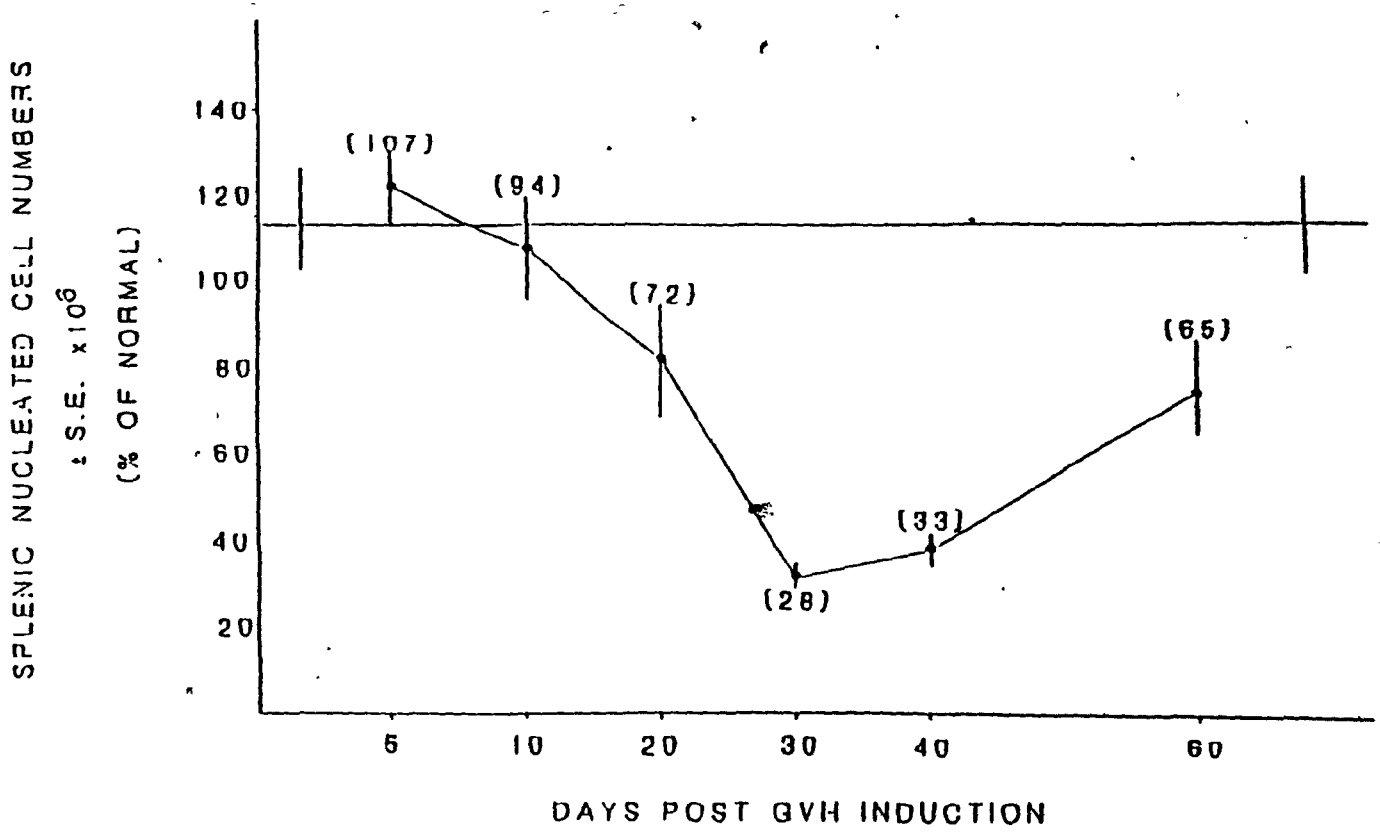


Figure 9.3 Kinetics of splenic nucleated cell depletion and repopulation in B6AF1 mice after the injection of $30x10^6$ B6 PLC. On each day after GVH induction 4 animals were tested. The data is presented as mean ± S.E. The experiment was performed two times. Each experiment showed similar kinetics. Results of one experiment are shown.

Table 9.5 Effects of pretreatment of splenocytes with different concentrations of PGE1, PGE2, and F2α on splenic NK cell activity of normal B6AF1 mice.

PG (moles) Treatment	% Cytotoxicity against YAC Targets[a] E:T 50:1
-	13.7 ± 2.1
PGE1 10^{-4}	7.2 ± 1.5
PGE1 10^{-6}	10.3 ± 2.5
PGE1 10^{-9}	13.5 ± 1.1
PGE1 10^{-12}	14.8 ± 1.4
PGE2 10^{-4}	8.8 ± 1.5
PGE2 10^{-6}	9.0 ± 2.0
PGE2 10^{-9}	11.3 ± 2.3
PGE2 10^{-12}	14.7 ± 1.6
PGF2α 10^{-4}	13.2 ± 1.1
PGF2α 10^{-6}	14.9 ± 2.2
PGF2α 10^{-9}	16.0 ± 2.5
PGF2α 10^{-12}	12.6 ± 1.3

a The data is presented as mean ± S.E. of three seperate experiments

Table 9.6 Effects of in vitro pretreatment of splenocytes with either PGs and Poly I:C alone or in combination on splenic NK cell activity of normal and GVH reactive B6AF1 mice.[a]

Treatment		% Specific lysis of YAC-1 (E:T = 50:1)			
		Days Post GVH Induction			
PGs (moles)	Poly I:C (100ug/ml)	0	30	45	60
-	-	10.0	0	2.0	3.0
E1 10^{-6}	-	6.0	-	3.5	4.0
E1 10^{-9}	-	9.0	1.0	5.0	7.0
E2 10^{-6}	-	7.0	-	4.5	9.0
E2 10^{-9}	-	7.5	4.0	5.5	8.0
F2α 10^{-6}	-	9.0	-	6.5	9.0
F2α 10^{-9}	-	8.5	2.0	5.0	7.0
-	+	17.5	2.5	5.5	7.0
E1 10^{-6}	+	9.5	-	7.5	13.0
E1 10^{-9}	+	11.0	8.0	6.0	14.5
E2 10^{-6}	+	12.5	-	13.0	19.5
E2 10^{-9}	+	15.0	7.0	21.0	21.0
F2α 10^{-6}	+	14.0	-	13.0	20.5
F2α 10^{-9}	+	16.5	10.5	9.5	16.5

a GVH reactions were induced in B6AF1 mice by injecting $30x10^6$ B6 PLC.

CHAPTER TEN

GENERAL DISCUSSION AND SUMMARY OF THE RESULTS

GENERAL DISCUSSION

The results presented in this thesis demonstrate the relationship between the immune functional, morphological, and histopathological changes associated with the GVH reaction. We have investigated the relationship between these three features of the GVH reaction by: (i) inducing GVH reactions of various intensities in different GVH genetic combinations (A-->B6AF1; B6-->B6AF1); and, (ii) employing parent into F1 GVH combinations with or without an NK cell functional defect.

In brief, on the basis of the data presented in this thesis, the GVH reaction (at least in the parent into non-X-irradiated F1 hybrid) can be divided into 3 phases: (1) The early active phase (between days 1-16 after GVH induction) during which both the host (as measured by splenomegaly) as well as donor (as measured by development of tissue damage) cells are active. During this phase of the GVH reaction severe immunosuppression of the PFC response to SRBC and/or T-cell proliferative responses are also observed. (2) The "quiescent" phase (between days 17-50 after GVH induction) during which the T-cell, B-cell, and NK cell functions are severely deficient and GVH-associated tissue lesions are clearly visible. (3) The late active phase (beyond day 50 after GVH induction) during which regeneration of tissue architecture and immune function takes place.

The observations presented in this thesis demonstrate that the intensity of events (related to donor NK cell function) during the early active phase of the GVH reaction may determine both the duration of the quiesent phase as well as the time required for the tissue architectural regeneration and immune function recovery during the late active phase of the GVH reaction. Thus, the significance of the events during the early active phase of the GVH reaction is obvious. We have investigated various GVH-associated events during the early active phase of the GVH reaction by performing correlative studies. Such correlative studies of the various GVH-associated parameters during the early active phase are important in determining those parameters that can predict later events during the course of a GVH reaction. In the following section a brief discussion is presented on the relationship between the various immune functional, morphological, and histopathological changes observed during the early active phase of the GVH reaction.

The data presented in this thesis show a clear dissociation between the GVH-induced splenomegaly and the development of GVH-induced histopathological lesions in lymphoid and non-lymphoid organs (chapter 6 and 7). The two main assay systems routinely employed to assess the induction and intensity of GVH reactions are splenomegaly and mortality/weight loss assays. Splenomegaly measures the proliferative phase, whereas mortality/weight loss measures the

effector phase of the GVH reactions (for details of the two phases of the GVH reaction see chapter 1). Splenomegaly is a consequence of donor T-cell activation which results in the recruitment of host cells, mainly macrophages and macrophage-like cells. In contrast, mortality and weight loss are a consequence of GVH-induced tissue damage. Most workers have employed either splenomegaly or mortality and weight loss assays to assess the induction and intensity of GVH reactions However, the few workers who have employed the two assays under similar conditions have observed a lack of correlation between the two.

No studies are available on the relationship between splenomegaly and the development of histopathological lesions. Such studies are important since it has been suggested that mortality and weight loss may be due to tissue damage. It is, however, not essential that tissue damage would always lead to mortality. It is, therefore, possible that although splenomegaly may not correlate with mortality, it may still correlate with the intensity of tissue damage (the possiblity, therefore exists that tissue damage is more sensitive than mortality). The data presented in chapters 6 and 7 clearly demonstrate a lack of correlation between the degree of splenomegaly and the intensity of GVH-associated histopathological lesions. These data strongly suggest that the splenomegaly and GVH-associated tissue damage are two distinct features of the GVH reaction and may be mediated by separate mechanisms.

Previous data from this laboratory have demonstrated two distinct phases of GVH-induced immunosuppression, namely, an early phase and a late phase each caused by different mechanisms (Lapp et al., 1985). During the early phase, at least two mechanisms were found to be operative. First, GVH-reactions resulted in a quantitative increase in splenic macrophages (Elie and Lapp, 1976,1977) which produced copious amounts of PGE (Lapp et al,1980) and suppressed T-helper cell function. Second, the early GVH induced immunosuppression was also found to be, at least in part, associated with the depletion of thymic and/or T-cell derived factor(s) (Grushka and Lapp,1971, 1974; Lapp et al, 1974; Elie et al., 1974). The results presented in chapter 6 showing that the bg/bg PLC are able to induce splenomegaly and early severe immunosuppression of the PFC response but only partial suppression of T-cell mitogen responses suggest that the bg/bg PLC can activate mechanisms responsible for the recruitment of host cells (splenomegaly) as well as early severe immunosuppression of the PFC response. Whether both the excess PGE production as well as the depletion of thymic and/or T-cell derived factor(s) are essential for the severe suppression of T-cell proliferative responses is not clear at the moment. Our results show: (i) a dissociation between the severe suppression of the PFC response to SRBC and the severe suppression of the T-cell mitogen responses early after GVH induction; and, (ii) a dissociation between the degree of splenomegaly and the severe suppression

of T-cell mitogen responses (chapter 6).

The dissociation of the PFC response to SRBC and the T-cell mitogen responses suggest that T-cell dependent B-cell responses (PFC response) are more susceptible to the early suppressive effects of the GVH reaction than the T-cell proliferative responses. The suppression of the PFC response, but not the T-cell proliferative response, can be observed in the absence of visible thymic alterations (chapter 6; see bg/bg---->+/bg GVH group). This dissociation between the suppression of the T-cell cooperative responses and the T-cell and B-cell proliferative responses early after GVH induction has also been observed in our more recent studies. In these studies (Ghayur, Seemayer, and Lapp, unpublished observations) attempts were made to deplete donor B6 (non-bg/bg mutant) mice of their endogenous as well as recruitable NK cell precursors (the protocol for NK cell depletion in donor mice is discussed in detail below). Lymphoid cells from these NK depleted donor mice were then used to induce GVH reactions in normal B6AF1 mice. On day 22 after GVH induction the animals were assessed for the PFC response to SRBC and T-cell and B-cell proliferative responses. The results obtained from these studies showed that the GVH mice were severely suppressed for the PFC response to SRBC (15-20% of the normal response), however both the T-cell and B-cell proliferative responses were normal/near-normal (85-110% of normal response). Moreover, splenomegaly was observed in these GVH mice but no histopathological lesions developed in the thymus and non-

lymphoid organs (Ghayur, Seemayer, and Lapp; unpublished observations).

The precise reason for the differences in susceptibilities to suppression of the PFC response and the mitogen responses early after GVH induction is not yet clear. However, since T-cell proliferative functions were only partially suppressed but the PFC responses were severely suppressed, the data presented in chapter 6 (GVH group bg/bg-->+/bg) would suggest that T-cell functions that are required for cooperation with B-cells in the PFC assay may be more sensitive to thymic and/or T-cell derived factor(s) depletion than T-cell proliferative functions. On the other hand, it is also possible that T-helper cell functions that are involved in helping B-cells in the PFC assay are more sensitive to the suppressive effects of PGE than T-cell functions involved in the T-cell proliferative responses. However, the extreme dependence of T-cell cooperative functions on the presence of "functional" thymic epithelial cells and Hassall's corpuscles is strengthened by results showing that during the immune functional recovery in GVH mice the PFC response to SRBC is observed only after the thymic epithelial cell clusters and Hassall's corpuscles have regenerated (chapter 7).

The early severe suppression of both the T-B-cell cooperative responses (the PFC response to SRBC) and T-cell proliferative responses was observed consistently in only those

groups of GVH mice in which moderate-severe thymic lesions developed (chapter 5, and 6). If the depletion of thymic and/or T-cell derived factor(s) is (are) critical for inducing severe suppression of T-cell function then our data would suggest that the depletion of thymic and/or T-cell derived factor(s) might result earlier than the visible moderate-severe thymic lesions. The arguement that the depletion of thymic and/or T-cell derived factor occurs earlier than the visible moderate-severe thymic lesions is based upon observations showing that the T-cell proliferative responses were severely suppressed by day 8 and the visible thymic alterations (mild thymic lesions, see chapter 5) also started to appear on the same day. However, moderate-severe thymic lesions were clearly observed on day 16 after GVH induction (chapter 5). The group of GVH mice that displayed mild thymic lesions (chapter 5; F1 mice injected with $10x10^6$A PLC) also became severely suppressed by day 16 after GVH induction. The mild thymic dysplasia in this GVH combination was characterized by partial depletion of cortical lymphocytes and disappearance of medullary epithelial cell clusters and Hassall's corpuscles. However, "small" individual medullary epithelial cells and cortico-medullary demarcation were clearly visible. The disappearance of "large" individual epithelial cells, medullary epithelial cell clusters and Hassall's corpuscles, but not the "small" individual epithelial cells, may represent thymic changes that are associated with thymic factor depletion. It is possible that the "small" individual thymic medullary epithelial cells may not be able to either produce enough thymic factor(s) or totally lack the

capacity to produce such factors. Factor production may be a property of "large" individual epithelial cells, epithelial cell clusters and Hassall's corpuscles present in the medulla.

In contrast to the early immunosuppression, severe-persistent GVH-induced immunosuppression was found to be, at least in part, due to thymic medullary dysplasia which resulted in a T-helper cell maturational arrest (Seddik et al., 1979, 1980). Our recent studies have further shown that thymic dysplasia resulted in a depletion of IL-2 producing, but not, IL-2 responding, cells (Mendez et al., 1985a,b). IL-2 production is essential for T-cell responses (both mitogenic and antigenic). The data presented in chapter 6 show that the +/bg PLC which induced severe prolonged immunosuppression of T-cell mitogenic responses also induced moderate-severe thymic dysplasia. In contrast, the bg/bg PLC which lacked the capacity to induce severe prolonged immunosuppression were also deficient in mechanism(s) that induce thymic dysplasia. Similarly, when 20 and $10x10^6$ B6 PLC were injected into B6AF1 mice only early partial immunosuppression was observed. These cell doses failed to induce both severe persistent immunosuppression as well as moderate-severe thymic dysplasia (chapters 4, 5, and 7). These results suggest a close association between the severe persistent suppression of T-cell proliferative responses and moderate-severe thymic lesions. Moreover, the data presented in chapter 7 clearly show that the duration of severe prolonged T-cell immunosuppression is

determined by the initial severity of thymic injury. T-cell functional recovery (as assessed by mitogen responsiveness, IL-2 production, and the PFC response) was gradual and coincided with the gradual regeneration of the thymic architecture (chapter 7). Furthermore, T-cell functions of immunosuppressed GVH mice could be restored in vitro only at the time when the thymic medullary regeneration was observed (chapter 8). These results strongly suggest an important role for the GVH induced thymic medullary dysplasia in maintaining the GVH-induced prolonged immunosuppression. The possible mechanisms involved in maintaing the thymus in a dysplastic state have been discussed in chapter 7 (section 7.4).

Since the intensity of the initial tissue damage determines the time required for the recovery of tissue lesions and immunosuppression (chapter 7) it is important to determine the cell type responsible for inflicting tissue damage. The data presented in this thesis on the relationship between the various immune functions and the development of moderate-severe tissue damage demonstrate that the tissue lesions started to appear at the time when T-cell functions were severely suppressed and NK cell activity was at its peak or highly augmented (chapters 4 and 5). Moreover, following GVH induction, the development of tissue injury correlated with the activation of donor NK cell activity but not host NK cell activity (chapter 6). The role for NK cells in GVH pathogenesis is further strengthened by recent findings showing that : (i) specific donor-anti-host CTLs may not be involved in GVH

induced pathogenesis (Hamilton,1984; Judas and Peck,1983; Mason, 1981); and, (ii) administration of anti-asialo GM1 (ASGM1) antibodies (which eliminate NK cells), to hosts 2 days before or just prior to GVH induction prevents GVH-associated mortality and the development of moderate-severe skin lesions (Charley et al., 1983; Varkila and Hurme, 1985b). However, the origin of NK cells (host, donor, or both host and donor) that plays an active role in GVH-associated tissue damage is still controversial.

In the studies cited above in which AsGM1 antibodies were administered to the recipients prior to GVH induction, it was suggested that NK cells of host origin were the effector cells inflicting GVH-associated tissue damage(Gharley et al., 1983; Varkila and Hurme, 1985b). The concept that NK cells of host origin induce tissue lesions and mortality was strengthened by data showing that treatment of donor cells or donor mice with AsGM1 antibodies could not prevent GVH-associated mortality (such treatment eliminates endogenous NK cells). However, in assigning an active role for host NK cells in GVH pathogenesis the following possibility should be considered: the endogenous NK cells may not be important, but rather NK cells and/or NK-like cells (as assessed by YAC killing) which are "activated" and recruited following alloantigenic stimulation may be the effectors responsible for the GVH- induced tissue lesions.

If the above possibility is true then the administration

of AsGM1 antibodies to the host 2 days before or just prior to the induction of a GVH reaction would eliminate alloantigen "activated" and recruited NK cells of donor origin as well as NK cells of host origin In contrast, in vivo administration of AsGM1 antibodies to the donor mice or in vitro treatment of donor cells with AsGM1 antibodies would only eliminate endogenous NK cells but not the recruitable precursors of NK and/or NK-like cells In support of the above argument we have recently observed that treatment of donor B6 mice with AsGM1 antibodies could not prevent either the GVH-induced moderate-severe tissue injury or the severe persistent immunosuppression of both T-and B-cell proliferative responses and the PFC response to SRBC (Ghayur, Seemayer, and Lapp unpublished observations) However, when the donor B6 mice were injected with $15x10^6$ B6AF1 lymphoid cells (spleen and lynph node) and AsGM1 antibodies and 36-40 hours later $50x10^6$ B6 lymphoid cells from these treated donor mice were injected into B6AF1 mice, neither GVH-associated histopathological lesions developed nor immunosuppression of T-cell proliferative function was observed (Ghayur, Seemayer, and Lapp unpublished observations). This treatment of donor mice with F1 cells and AsGM1 antibodies probably eliminated endogenous as well as "activated" and recruited NK cells in the donor mice The results described above regarding the prevention of GVH pathogenesis and immunosuppression by treating donor mice with F1 cells and anti-AsGM1 antibodies in fact may reflect the situation in which B6 bg/bg PLC (deficient NK cell function) were injected into +/bgF1 mice (normal NK cell functions)

(chapter 6) These results (chapter 6, and our unpublished observation) strongly suggest that activated NK cells of donor origin but not the activated NK cells of host origin may play an active role in GVH-associated tissue damage (chapter 6, compare GVH groups bg/bg-->+/bg and +/bg-->bg/bg)

The fact that moderate-severe tissue damage was only observed in the presence of activated donor but not activated host NK cells, suggests specificity for donor NK cells towards host tissue The possible mechanisms for the generation of donor-anti-host specific NK cells (or NK-like cells) during the GVH reactions have been discussed in detail in chapter 6 However, it is worth noting that NK cells are classically defined on a functional basis (ability to kill YAC targets) YAC killing can be induced *in vitro* during mixed lymphocyte reactions (MLR) (Nieminin and Seksela,1984, Morretta et al, 1984) MLRs are *in vitro* correlates of GVH reactions *in vivo* Thus, NK cells can be induced following allogeneic stimulation Dokhelar et al (1982) in humans, and Clancy et al (1983) in rats have suggested that specific alloantigen reactive T-cells can exert NK and/or NK-like functions at some stage of their maturation/differentiation following GVH induction and that these cells may serve as effectors of the GVH pathogenesis If allo-antigen activated T-cells at a certain stage of their maturation/differentiation can exert NK-like activity and can either directly or indirectly induce tissue damage, it then becomes important to investigate the relationship between T-

cells and NK cells early after the induction of the GVH reaction.

The data presented in this thesis demonstrate an inverse relationship between T-cell and NK cell function early after GVH induction (chapter 4, and 5) These data suggest that the T-cells and NK cells may have different sensitivities to suppression and/or activation Several workers have suggested that NK cells are pre-T-cells or prothymocytes (Herberman and Holden, 1978; Herberman et al., 1979, Kaplan, 1985). Our data show that NK cell or NK-like cell activity can be induced in the normal thymocyte population (chapter 5). Induction of NK activity in human thymocytes by supernatants derived from MLR cultures (Toribio et al,1983) also supports the notion that NK cells are either present as a resident population within the normal thymus or NK-like activity can be induced in thymocytes During thymic ontogeny cells with NK properties are the first to appear in the thymus (Habo et al., 1980; Koo et al., 1982) These cells with NK properties disappear in the fetal thymus at the time of appearance of a functional thymic medulla (Habo et al., 1980; Koo et al., 1982). The studies presented in this thesis show highly augmented NK cell activity in the thymus between days 60-120 after GVH induction (chapter 3). The studies presented in chapter 7 of this thesis show that between days 60-120 after GVH induction complete regeneration of the thymic cortex, but not the medulla, is observed. Collectively, these studies suggest that NK cells may be present in the thymus and may represent a stage in T-cell

maturation/differentiation.

Several workers have shown a decrease in NK cell cytolytic activity by thymic hormones (Bardos et al , 1982), which are produced by medullary epithelial cells and Hassall's corpuscles (Stutman,1977). These workers have suggested that the decrease in NK cell activity by thymic hormones may be due to a shift from NK cells (pre-T-cells) towards more mature T-cells (Bardos et al., 1982) In contrast, in thymectomized or nude mice (which lack a thymus) the augmented NK cell activity, may be due to the accumulation of pre-T-cells (Shellam, 1977, Herberman et al., 1975a; Herberman et al., 1979) We have previously reported (and as discussed above) that early after the induction of GVH reactions, depletion of thymic and/or T-cell derived factors are observed which result in the depression of T-cell functions (Grushka and Lapp,1971,1974, Lapp et al , 1974, Elie et al., 1974). A depletion of thymic factor(s) could result in an increase of NK cells of the pre-T-cell lineage. Furthermore, early after GVH induction, IFN (Zawatsky et al., 1979; also see chapter 9) and PGs (Lapp et al., 1980) are produced. Both of these lymphokines inhibit T-cell functions (proliferation). In contrast, IFN is a potent inducer of NK cell activity (Gidlund et al , 1978, Djeu et al , 1979; Trinchieri and Santoli, 1978; Senik et al , 1979) Enhancement of NK cell activity by IFN is due to (a) enhancement of the lytic potential of mature NK cells and, (b) recruitment and maturation of pre-NK cells (Seksela et al ,

1979; Heron et al., 1979; Minato et al., 1980; Senik et al., 1980). IFN in combination with PGs enhance the recycling of NK cell lytic ability (Targan,1981). Thus, early after GVH induction, a combination of factors that would exert a negative effect on T-cell functions would exert a positive influence on NK cell activity.

If NK cells or a subpopulation of NK cells are pre-T cells it would then appear that under the conditions of intense immune reactions, eg., a GVH reaction, in which the classical specific immune responses (more evolved responses) fail or cannot function, the more primitive effector mechanism(s), i.e., NK or NK-like cells would take over. Karre (1985) has recently suggested that in mammals NK cells constitute a more primitive defense mechanism as compared to the T-cell defence system. This concept of a functional shift from more evolved to primitive levels by the immune system during a GVH reaction, as suggested above, may have implications in understanding the functional aspects of the immune system. A shift in the immune system towards more primitive functions under adverse conditions may also provide some insight into the effector mechanisms during GVH reactions in animals and in human recipients who have been prepared for bone marrow transplantation with various regimens (eg., Cyclophosphamide, Cyclosporin A and x-irradiation).

The results presented in this thesis and as discussed above demonstrate that during the eafly active phase of the GVH

reaction a complex series of events are initiated which result in various immune functional, morphological, and histopathological alterations associated with the GVH reaction These various GVH-associated alterations may be mediated by different mechanisms (or a combination of mechanisms) and can be dissociated from one another on the basis of a lack of correlation. This lack of correlation between various GVH-associated parameters employed to assess GVH reactions raises important questions. (1) Can the use of one parameter at a particular time early after GVH induction provide information about the GVH reaction as a whole or does it only provide information about one particular aspect of the GVH reaction at a certain time?. (2) Can the results of one parameter at a certain time be used to extrapolate its effect on other GVH-associated parameters?. These questions are important especially when: (a) GVH reactions are induced after various forms of manipulations of the host and/or donor mice; (b) the host and/or donor mice are deficient in a particular cell function; or (c) GVH reactions of various intensities are induced in different genetic combinations The data presented in this thesis certainly suggest that the use of one parameter at a particular point after GVH induction tells little about the GVH reaction as a whole, but rather provides information regarding that specific parameter during a particular phase of the GVH reaction.

A surprising aspect of the results is that dysplastic

thymuses regenerate a normal architecture and eventually normal function. The time required for the regeneration of the tissue architecture and immune functional recovery during the late active phase of the GVH reaction is determined by the severity of events during the early active phase of the GVH reaction (chapter 7). In the following section we shall discuss the regeneration of the immune system (both structural and functional) during the late active phase of the GVH reaction. The emphasis in the following section shall be on the possible role of the regenerating thymus and the BM in providing a milieu in which normal immune functions of an adult animal can develop.

The thymic dysplasia (chapters 5,6, and 7), which results in the destruction of medullary epithelial cells and Hassall's corpuscles (Seemayer et al., 1977, 1978), results in a T-helper cell maturational defect (Seddik et al., 1979, 1980). This thymic destruction in GVH animals may serve as an equivalant to in vivo "thymectomy". Similarly, GVH reactions induce histopathological lesions (Githen et al., 1968; Ishihara and Shimanina, 1980; Hirabayashi, 1981) and severe depletion of nucleated cells in the BM (chapter 9, Figure 9.2). The depletion of BM has been shown to be associated with the cessation of B-cell genesis (Xenatostas et al., 1986). However, with time after GVH induction, complete regeneration of the thymic medullary architecture (chapter 7) as well as complete repopulation of the BM (chapter 9) are observed. The thymic regeneration takes place in the following order: cortical

regeneration and appearance of cortico-medullary demarcation; regeneration of individual medullary epithelial cells, and, finally, epithelial cell clusters and Hassall's corpuscles (chapter 7). Moreover, the regeneration of thymic architecture may occur earlier than thymic functional recovery (hormone production) (chapter 8). The regeneration of the thymus and the repopulation of the BM are associated with the recovery of T-cell and B-cell immunocompetance. The immune functional recovery is also gradual and appears in the following order B-cell proliferative responses; Con A responsive T-cells; PHA responsive T-cells; and, finally T-helper cell dependent B-cell responses (chapter 7). Thus, the gradual thymic medullary regeneration, the gradual BM repopulation, and the gradual immune functional recovery provide an in vivo model to study the role of thymic medullary components and the BM in the development of the immune system in an adult animal.

A comparison of the kinetics of BM repopulation and thymic regeneration (in B6AF1 mice injected with $30x10^6$ B6 PLC) shows that thymic cortical regeneration (cortical repopulation) (chapter 7) and the appearance of cortico-medullary demarcation occur approximately at the time of complete BM repopulation (about day 60 after GVH induction, chapter 9). In B6AF1 mice injected with $20x10^6$ A PLC thymic cortical regeneration was observed by day 75 after GVH induction (chapter 7; Figure 7.3). In this GVH combination complete BM repopulation was also observed by day 70-75 after GVH induction (Xenacostas, Ghayur, Osmond, and Lapp, unpublished obervations). Whether the

Cortico-medullary demarcation appears earlier than the cortical repopulation is not clear. Furthermore, at the time of cortical regeneration and the appearance of cortico-medullary demarcation, although the cortex is densely packed with lymphocytes, the medulla is still depleted of lymphocytes (Fig.7.4a). This observation raises interesting questions: (a) does the appearance of cortico-medullary demarcation "actively" exclude thymocytes from entry into the medulla and therefore allows the medullary cells (epithelial cells) to regenerate ?, (b) do the medullary cells (epithelial cells) provide a signal (factor(s)) which allows the cortical thymocytes (selected, more mature thymocytes) to enter the medulla ?; or, (c) do the cortical thymocytes in some way provide a signal for the regeneration of the medulla?. The later possibility is intriguing since during thymic ontogeny the first wave of thymocytes entering the thymic anlage provides a stimulus for the differentiation and development of the thymic medulla (epithelial cells) (LeDouarin and Jetereau, 1975; Owen and Ritter,1969; Moore and Owen,1967). However, during ontogeny it takes 2-3 days for the development of the medulla, yet in the adult GVH animal the medullary regeneration takes approximately 60-75 days after cortical regeneration (chapter 7). Whether this difference in the time of development of the medulla in the adult GVH animal and during ontogeny is either due to the different environmental conditions under which the thymus has to develop (as discussed in chapter 7) or the mechanism(s) of thymic development in an adult animal and that during ontogeny

are totally different is not clear.

It has been suggested that the main function of the thymus is to generate clonal diversity and delete self-reactive clones (Burnet, 1962; Jerne, 1971); impart H-2 restriction (Zinkernagal et al,1978a,b; Bevan,1977; Fink and Bevan,1978), and, finally induce the capacity to initiate and amplify the T-cell immune response (generation of IL-2 producing T-helper cells) (Hunig, 1983; Scollay,1984) . Studies from our laboratory have shown that the destruction of medullary epithelial cells results in the depletion of IL-2 producing T-helper cells (Mendez et al., 1985a,b). Beardslay et al. (1983) have shown that thymic medullary epithelial cells either directly or through the elaboration of factors are responsible for inducing IL-2 producing capacity in T-cells. The medullary-derived lymphocytes are more mature IL-2 producing T-helper cells which are essential in the H-2 (class 11) restricted initiation of immune responses (Lonai and McDevitt, 1977; Vadas et al, 1976; Nagy et al., 1978; Scollay, 1984; Zinkernagal et al., 1978b) On the other hand, the cortical thymocytes, which represent an immature population, lack the capacity to initiate T-cell immune responses by themselves, however, in the presense of IL-2 and antigen are capable of generating cytotoxic T-lymphocytes (CTLs) which are H-2K/D restricted (Nagy et al , 1976; Wagner et al., 1980a,b; Scollay, 1984; Zinkernagal, 1978b). It is therefore believed that the cortical lymphocytes contain precursors of CTLs.

In light of the above mentioned studies, the data presented in chapter 7 of this thesis showing that complete medullary regeneration takes approximately 60-75 days after cortical regeneration raises the following question. Are the lymphocytes released into the periphery by the regenerating thymus which is devoid of a functional medulla?. The studies presented in this thesis and other available studies on GVH dysplastic thymuses have not directly shown that the cortical lymphocytes are exported into the periphery. However, the evidence does suggest that : (a) the GVH BM does produce pre-T cells (Seddik et al., 1984b): (b) the GVH dysplastic thymuses (with severe medullary dysplasia) are capable of recruiting pre-T cells (Seddik et al., 1984b): (c) the spleens of GVH mice with severe medullary dysplasia contain IL-2 responsive cells (cortical cells?) (Mendez et al., 1985a,b): (d) splenocytes taken from mice with thymic medullary dysplasia, but with cortical regeneration, contain NK cells (which are believed to be immature T-cells) (Ghayur et al., 1981; also see chapter 9): (e) splenocytes taken from mice which show medullary regeneration can be induced to undergo T-cell mitogen dependent proliferative responses (Chapter 8), suggesting that these splenocytes may have been exported from the regenerating thymus. Thus, these observations, although indirect, suggest that the thymus may be recruiting and exporting cells to the periphery throughout its different stages of regeneration.

If the T-cells do leave the regenerating thymus which

lacks a functional medulla and if the thymic Ia restriction elements which enable the T-helper cells to initiate class II restricted immune responses are present in the medulla, then what is the functional status of the T-cells leaving a thymus without a well developed and a functional medulla?. Is it possible that these T-cells would recognize self-Ia in the periphery as "foreign" and react against it (since such T-cells would not encounter self-Ia in the thymus)?. In fact, evidence is available in the literature showing that under circumstances in which the thymic Ia+ medullary cells are selectively affected, self Ia+ reactive (auto-reactive) cells do arise. Cheney and Sprent (1985) have shown that chronic administration of Cyclosporin A (CsA) to mice selectively resulted in both a striking decrease in overall thymic Ia expression and the development of syngeneic GVH disease. The development of syngeneic and autologous GVH disease following the withdrawl of CsA treatment has also been reported by Glazier et al. (1983a,b) and Hess et al. (1985). Hess et al. (1985) reported a highly significant association of syngeneic GVH disease with the development of anti-Ia specific cytotoxic T-cells. These workers (Hess et al., 1985) suggested that the integrity of thymic tissue may be a critical factor in the development of syngeneic GVH disease. The importance of the thymus in the induction of syngeneic GVH disease is further supported by the results of Glazier et al. (1983a,b). Santos et al. (1985) have speculated that CsA may alter the intrathymic educational process or accentuate a maturational failure of T-lymphocytes. The development of T-lymphocytes in a thymic environment with

reduced Ia expression would result in altered T-cell maturation with the subsequent induction of Ia-specific cytotoxic T-cells (Glazier et al., 1983a,b; Hess et al., 1985; Santos et al., 1985). The maturation of T-lymphocytes in a thymic environment lacking class II determinants would likely result in a failure to recognize these Ia antigens as self (Hess et al., 1985; Santos et al., 1985). If the GVH splenocytes contain IL-2 responsive cortical cells (pCTL), cells with NK cytolytic potential, and cells which presumably lack self Ia+ restriction, then it is tempting to ask why these cells do not react against self Ia and initiate a second round (after the initial acute GVH reaction) of more generalized tissue injury (secondary disease) ? What role does the regenerating BM and the thymus play in providing a milieu for the proper development of the regenerating immune system in an adult animal?.

It has been reported that following allogeneic or semi-allogeneic BM transplantation, non-specific suppressor cells appear (Beschorner et al., 1983; Lum et al., 1982; Tsoi, 1982; Tutschka et al., 1982; Holda et al., 1985). Such non-specific suppressor cells are BM derived and appear during the regeneration of the immune system. Appearance of non-specific suppressor cells in the spleens of non-x-irradiated GVH mice have also been observed (Mendez and Lapp, 1986). These non-specific suppressor cells in the non-X-irradiated GVH mice inhibited the proliferation of as well as IL-2 production, by

parent, and F1 spleen cells (Mendez and Lapp, 1986). Moreover, such suppressor cells were most active by day 60 after GVH induction (Mendez and Lapp, 1986), a time when BM repopulation was observed (Xenacostas et al., 1986). Recent studies have shown that non-specific suppressor cells in the spleens of GVH mice have an NK cell-like morphology and following percol density seperation appear in the same fraction as NK cells but are non-cytolytic (Holda et al., 1985). These suppressor cells are termed as natural suppressor cells (Holda et al., 1985). It has been suggested that natural suppressor cells may play an important role in tolerance induction (Strober et al., 1984; Holda et al., 1985). It is possible that the non-cytolytic NK cells, as described in chapter 9 of this thesis in which NK-like activity (YAC killing) could be induced by in vitro PG and Poly I:C treatment, may represent such natural suppressor cells.

Studies from other laboratories have also demonstrated the appearance of antigen specific suppressor T-cells (Ts) in X-irradiated allogeneic BM reconstituted animals (Tutschka et al., 1981, 1982a,b; Ildstad and Sachs, 1985). The appearance of these antigen specific Ts-cells is observed following the "decline" in non-specific suppressor cells (Tutschka et al, 1982a; Santos et al., 1985). Thus, it appears that during the development of the immune system in an adult x-irradiated animal after allogeneic or semiallogeneic BM reconstitution, non-specific suppressor cells appear first, followed by antigen specific Ts cells. Stable chimeras or tolerant animals result

only when antigen-specific Ts-cells appear (Tutschka et al , 1982a,b, 1981, Santos et al , 1985) (and in some cases both non-specific and antigen specific cells have also been detected) In cases when the antigen-specific Ts-cell do not appear the animals fail to regain normal weight and die, suggesting that tolerance did not develop and the animals probably died of autoreactivity (secondary disease). Santos et al (1985) have recently suggested that the thymus gland may play a crucial role in the development of stable chimeras (tolerant mice) Lack of thymic functions (hypo-functions) would prevent the generation of stable chimeras (prevent tolerance) (Santos et al., 1985)

The above studies suggest the following (1) During the regeneration of the immune system BM derived non-specific suppressor cells appear first, followed by the appearance of antigen-specific Ts cells. Presence of both the non-specific suppressor cells and the antigen specific Ts cells are also reported in animals undergoing immune regeneration, suggesting that the shift from non-specific to specific cells may be gradual. (2) The thymus gland plays a central role in the generation of antigen-specific Ts cells (3) Stable chimeras and complete immunological recovery are observed only in cases when antigen-specific Ts-cells appear. On the basis of the above mentioned studies one can speculate that the non-specific and specific Ts-cells provide an inert mileau for the development of the regenerating immune system probably by

preventing the activation and/or proliferation of the "self-reactive" cells which could initiate a secondary disease

If the thymus gland plays a central role in providing the "switch" mechanism from non-specific to specific Ts-cells, as suggested by Santos et al (1985), than the data presented in this thesis on the gradual regeneration of the thymus following BM repopulation may provide some information regarding (i) the presence of non-specific and antigen specific Ts-cells, and, (ii) the crucial role of the thymus in providing the "switch" from non-specific to specific Ts-cells A hypothetical model for the role of the regenerating thymus and repopulated BM in providing a proper environment for the development of the regenerating immune system in an adult animal is shown in figure 10 1 and described briefly below

The model presented in figure 10 1 assumes that the repopulating BM would export pre-T-cells to the thymus which would repopulate the thymic cortex (TR-cells - with the potential to react against self in the periphery) and non-specific suppressor cells (Tns-cells) into the periphery (spleen). At this stage (stage 1) the antigen specific suppressor cells (Ts-cells) would not be present in the periphery. During thymic cortical regeneration but severe medullary dysplasia, the ratio of TR:Tns in the periphery would be in favor of the Tns-cells. With the gradual regeneration of the thymic medulla the release of TR from the thymus would decrease and, under the influence of regenerating thymic

medulla, T-helper cells which require thymic factors to initiate the proliferative responses (chapter 8) as well as Ts would increase As the Ts increase in the periphery the Tns would decrease, possibly again under the influence of the regenerating thymus. Thus, at the time when complete medullary regeneration (both structural and functional) is observed the cells present in the periphery would constitute T-helper cells which could initiate an immune responses and Ts cells, but only minimal, if any Tns cells Such a scheme of T-cell development would explain (i) the first appearance of non-specific suppressor cells followed by the appearance of antigen-specific cells, (ii) the gradual shift from non-specific to antigen-specific suppressor cells, and, (iii) the crucial role of the thymus in the generation of stable chimeras

Most of the studies cited above regarding the question of stable chimeras were conducted by injecting allogeneic or semiallogeneic BM cells into X-irradiated recipients. In contrast, the studies presented in this thesis were conducted by injecting parental lymphoid cells into non- X-irradiated F1 mice. However, it should be mentioned that in the X-irradiated recipients thymic dysplasia similar to that described in this thesis and as reported previously by our laboratory (Seemayer et al., 1977,1978), has been observed (Rappaport et al., 1979, Beschorner et al., 1982). Since the studies presented in this thesis clearly show that the recovery of T-cell immune function is closely associated with the regeneration of the thymic

architecture, it would appear that in X-irradiated allogeneic chimeras immune recovery would also be associated with the regeneration of thymic architecture In fact, normal thymic architecture is observed in animals and in humans who eventually become stable chimeras If in allogeneic radiation chimeras the immune recovery is dependent upon thymic regeneration, then all the animals should be able to recover completely from immunosuppression after the initial GVH-induced tissue damage and survive

It is, however, readily observed that in the allogeneic situation not all the animals become stable chimeras and mortality is still observed in long term survivors, probably due to secondary disease However, in the experiments reported in this thesis, a very high frequency of the animals survived The difference in the mortality of long term survivors of allogeneic chimeras and our studies using the parent into non-x-irradiated F1 mice may be due to the following reason in allogeneic chimeras the recipients are x-irradiated and then reconstituted with allogeneic or semiallogeneic BM cells This procedure presumably totally abrogates the host stem cells and the regenerating immune system arises from the donor BM stem cells. In contrast, in our studies the stem cell pool of the recipient is persumabaly not completely abolished, since the BM mononucleated cellularity drops to about 15% of normal and within 10-15 days after this depletion spontaneous BM repopulation begins (see figure 9.2). Furthermore, in our system the recipients are injected with spleen and lymph node

cells which possess much lower numbers of stem cells. Thus, in our system the ratio of donor:host ~~cells~~ during the regeneration of the immune system may be much lower than compared with the donor:host ratio in allogeneic radiation chimeras. It is possible that the ratio of donor:host cells in the regenerating immune system may be crucial in determining both the extent of immunological reconstitution and the long term survival of the animals.

An understanding of the development of the immune function in an adult animal may provide information which could help in understanding various normal and abnormal patterns of immune function. In the following section we would like to discuss, briefly, the issues which we feel can be better understood by studying the regeneration of the primary lymphoid organs (thymus and BM) and the immune functional recovery in an adult GVH-reactive animal.

The studies presented in this thesis on the regeneration of the immune function demonstrate the presence of highly cytolytic NK cells in the regenerating thymus It has been suggested that NK cells may play an important role in homeostasis (Cudkowicz and Hochman, 1979). Moreover, it has been shown recently that NK cells lyse a subpopulation of thymocytes (Hanson et al., 1980) and BM cells (Hansen et al., 1981). If NK cells play a role in homeostasis, these cells should be present in the BM and thymus, the two organs actively involved in the production of B cells and T cells respectively NK cells originate in the BM (Haller et al., 1977; Haller and

Wigzell, 1977) and low levels of their cytolytic activity can be detected in the adult BM. It is generally assumed that NK cells are not present in the adult thymus (Kiessling et al., 1975a). However, NK cytotoxic activity can be induced in normal thymocytes (Toribio et al., 1983; also see chapter 5) We have previously suggested that NK cells may be present in the adult thymus as a resident population (Roy et al , 1982) Zoller et al. (1981) have, in fact, isolated highly active NK cells from the adult murine thymus NK cells have been reported in the thymus during ontogeny (Habu et al., 1980; Koo et al , 1982). Our results showing the presence of highly active NK cells in the thymus and BM during the regeneration of immune function also suggest that NK cells do migrate to the thymus The presence of NK cells or NK-like cells in the regenerating thymus of an adult animal raise some interesting questions. (i) Are the NK cells that appear in the thymus different from the NK cells that appear in the BM in terms of their target specificity ?. If so, do these two NK populations regulate different cell populations (T-cells and other BM derived cells). (ii) Do the thymic NK cells possess different surface markers from the BM derived NK cells?. These questions may be important in understanding the functional and phenotypic heterogeneity observed within NK cells, eg., the function of NK cells with and without T-cell surface markers.

In contrast to the appearance of NK cell activity in the thymus and BM, NK cytolytic activity does not reappear in the

spleen and lymph nodes during immune functional regeneration (up to day 150 after GVH induction). However, NK cell cytolytic activity can be induced in GVH-splenocytes by *in vivo* administration of Poly I:C as well as by *in vitro* treatment of GVH-splenocytes with PGs and Poly I:C (chapter 9). This restoration of NK cell cytolytic activity suggests that NK cells are present in the spleens of GVH mice, but in a state where they do not express lytic potential. Whether the non-cytolytic NK cells in the spleens represent a population (or a certain stage of differentiation) which is different from the highly cytolytic NK cells present in the thymus and BM or whether the non-cytotoxic NK cells in the spleen perform some other function (eg., function as suppressor cells) during the regeneration of the immune function remains to be determined However, the fact that cytolytic activity can be induced in splenic non-cytolytic NK cells by *in vitro* treatment with PGs and Poly I:C may be of significance in understanding the functional heterogeneity of NK cells as well as the mechanism of NK cytolysis.

An important aspect of the GVH reaction is the increased susceptibility to development of spontaneous tumors later during the course of the GVH reaction (Armstrong et al., 1967,1970; Gleichmann et al., 1972,1975; Cornelius, 1972). The tumors that so often develop in these long term survivors of the GVH reactions are mostly of B-cell origin. An analysis of the data presented in this thesis on the regeneration of the immune function in an adult GVH animal clearly shows that the

B-cell proliferative function recovers earlier than the T-cell proliferative function and the T-helper cell function that cooperate with B-cells (chapter 7). In fact, the data presented in Table 7.25 show that in some mice which were severely suppressed for the T-cell proliferative functions, the B-cells were "hyperactive" to LPS . These observations suggest that for a considerable period of time, the B-cells may be the only "competent" cell type present in the spleens of GVH-reactive mice. However, such B-cells in the spleens of GVH mice may not be under the regulatory influence of either the T-cells (since spleens were severely deficient in T-cell functions) or NK cells [the data presented in chapter 3, as well as our previously published work (Roy et al, 1982), show severe depression in splenic NK cell activity] NK cells have been reported to play an important role in regulating B-cell functions (Storkus and Dawson, 1986; Brieva et al., 1984, Abruzzo and Rowley, 1983; Nabel et al., 1982). It is possible that the differences in the rate of regeneration of the functional B-cells and T-cells and the functional status of NK cells in the spleens and lymph nodes may be one of the reasons responsible for the susceptibility of B-cells to transformation B-cells in the spleens of GVH mice without regulatory influence and help (protection) from other immune cells would be susceptible to transformation.

If NK cells serve as cytolytic effectors in anti-viral immune responses (Welsh, 1978; Djeu et al., 1982; Santoli and

Koprowski, 1979), in surveillance against neoplastic cells (Oje,1979; Talmadge et al., 1980; Hanna and Burton, 1981), and in the regulation of B-cells then the lack of expression of lytic potential by the splenic NK cells of GVH mice may contribute to increased susceptibility to viral infections and in the development of spontaneous B-cell tumors during the later stages of the GVH reactions. Cornelius (1972) has reported that the spleens and lymph nodes are the primary site of tumor development in GVH mice. It is interesting to note that tumors have not been detected in the thymuses and BM of GVH animals. Both these organs are sites of highly augmented NK cell activity during the later stages of the GVH reaction Whether the augmented BM NK cell activity plays any role in preventing the development of B-cell lymphomas in the BM is not known. However, it is possible that although increased NK cell activity is observed in the thymuses and BM of GVH mice later during the course of the GVH reactions, these NK cells may not be effective in controlling infections that localize in the spleen and lymph nodes, in regulating B-cells, and in eliminating neoplastic cells developing in the spleen and lymph nodes.

The studies in this thesis showing different histological patterns of the thymuses during the induction of (chapter 5) and regeneration from (chapter 7) thymic injury may be important not only from the point of view of the role of thymus in the production of functional T-cells but also from a clinical perspective. Borzy et al. (1979) have described seven

different patterns of thymic histology in immunodeficiency diseases. These different histologic changes of the thymus provide evidence for the heterogeneity in combined immunodeficiency diseases (CID). A comparison of these different thymic histologic patterns in CID patients, as described by Borzy et al. (1979), and those reported in this thesis (chapters 5 and 7) demonstrates several similarities Moreover, Borzy et al (1979) reported that the degree of abnormality of immune function does not predict the degree of dysplasia seen in the thymus For example, two patients who had low E-rossette-forming cell numbers and very low proliferative response, displayed partial cortico-medullary differentiation On the other hand, one patient who had T-cell deficiency showed a normal thymic architecture (however, it is possible that thymic abnormalities, either structural or functional may give rise to peripheral suppressor mechanisms, as discussed above) Our results also show that the degree of thymic dysplasia and immune functional abnormalities do not correlate For example, F1 mice whose thymuses showed only partial cortical and medullary changes (mild lesions) were immunosuppressed early after GVH induction. On the other hand, during the regeneration phase, although the thymus displayed normal architecture the animals were still severely deficient in T-cell function. Borzy et al. (1979) suggested that the differences in the thymic histology and immune functions observed in the CID patients may represent different stages of the disease. This notion of Borzy et al. (1979) is of interest since recent studies have

suggested that the presence of maternal lyphocytes in CID patients may incite GVH disease in fetuses and thereby induce a variety of immune functional deficiencies

Studies on the structural/functional development of the T-cell system (as reported in chapters 7 and 8) may also provide information into the mechanism(s) of certain forms of immunodeficiency diseases and can help in devising means to restore immune competence in certain innumodeficiency states Incefy et al. (1981) have recently classified different paterns of severe combined immunodeficiency diseases (SCID) depending upon the presence or absence of cells which could be induced to proliferate in response to T-cell mitogens and/or express T-cell surface antigens following treatment with thymic hormones Willis-Carr et al. (1978) have reported that cells from some immunodeficient patients can be induced to express T-cell properties following treatment with thymic epithelial cell supernatants (TES) alone. However, cells from other patients required thymic epithelial cell contact in addition to TES. Furthermore, Gelfand et al. (1980) have shown that transplantation of thymic epithelium into SCID patients can restore immune function. The studies presented in this thesis also clearly show that T-cell proliferative functions of GVH-immunosuppressed mice can be restored by PGE1 and PGE2 treatment only at the time when thymic medullary regeneration has taken place (chapter 8). These studies collectively suggest that the status of the thymus, particularly its epithelium, may play a critical role in determining the degree and extent of T-

cell immunodeficiency. Knowledge of thymic morphology in relation to T-cell function in immunodeficiency syndromes may be beneficial in planning therapy for these patients.

THE END

Figure 10.1 A hypothetical model illustrating the role of repopulated BM and the regenerating thymus in preventing secondary diease/ chronic GVH reaction and providing a milieu for the proper regeneration of the immune system in an adult animal during the immune recovery phase of the GVH reaction.

EC; epithelial cells

Hc; Hassall's corpuscle

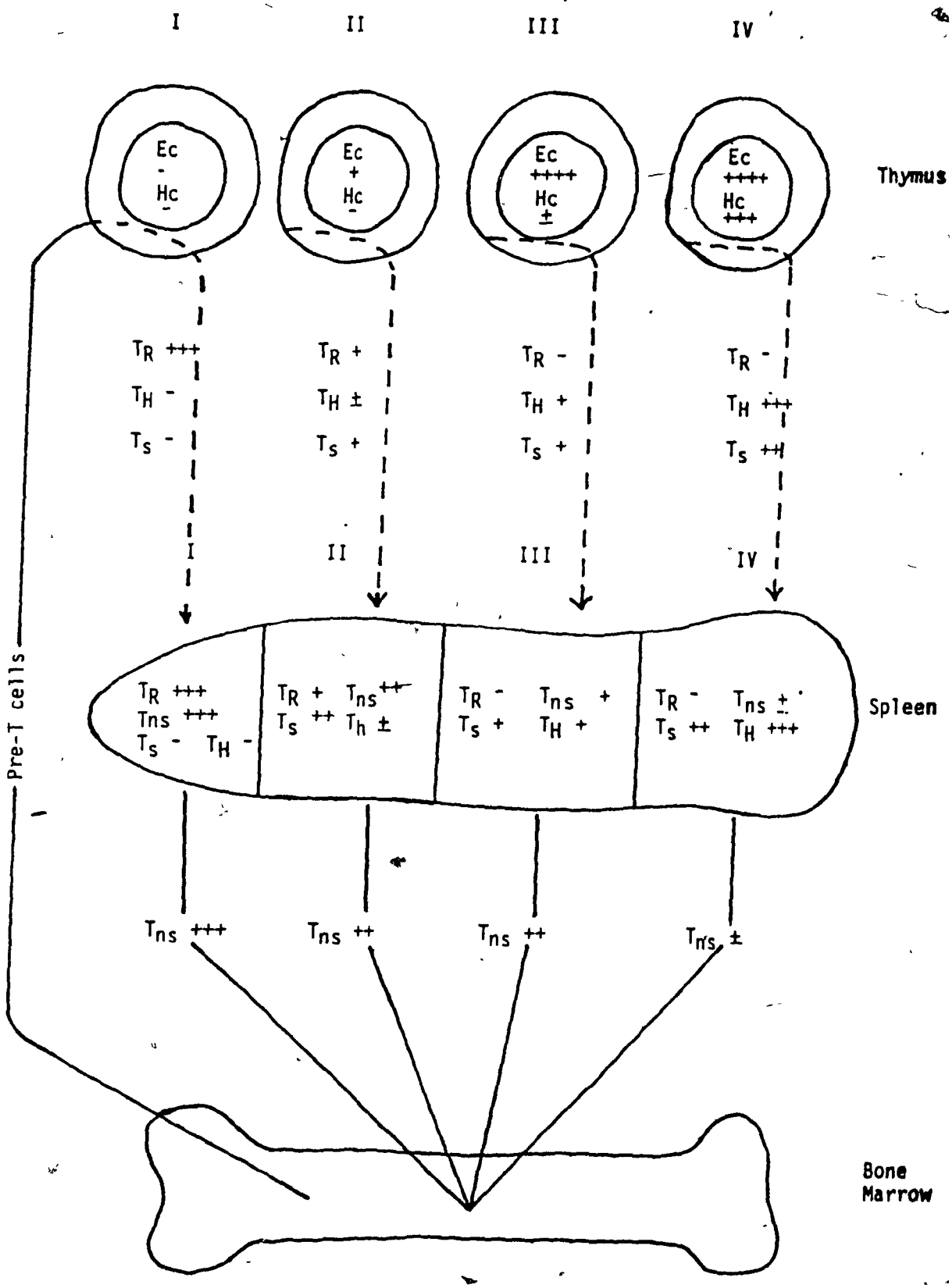
T_R; Self Ia reactive cell

T_H; T-helper cell

T_S; Antigen specific T-suppressor cell

Tns; Non-specific bone marrow derived suppressor cell

Stages of Thymic Regeneration



SUMMARY OF THE RESULTS

SUMMARY

When GVH reactions were induced in B6AF1 mice injecting different doses (10, 20, or $30x10^6$) of either parental strain A or B6 lymphoid cells the following results were obtained:

(1) On the basis of NK cell activity, the chronic GVH reaction can be divided into three phases.

(a) Early phase augmented NK cell activity is observed in spleen, lymph nodes, thymus, and BM

(b) Intermediate phase the NK cell activity becomes depressed in the spleen, lymph nodes, thymus, and BM

(c) Late phase. NK cell activity reappears in the thymus and BM, but remains depressed in the spleen and lymph nodes

(2) The splenocytes and the lymph node and BM cells taken from GVH mice during the early phase of the GVH reaction produce factor(s) which induce/augment NK cell activity in lymph node and BM cells of normal mice.

(3) The overall augmented NK cell activity observed in the spleen, lymph node, thymus, and BM of GVH mice is dependent upon the dose of PLC injected.

(4) The degree of augmented splenic NK cell activity of GVH - reactive mice is dependent upon the number and strain of PLC injected. B6 PLC induce greater overall splenic NK cell

activity than A strain PLC.

(5) The degree of augmented BM NK cell activity of GVH reactive mice is also dependent upon the number and strain of PLC injected . When B6 PLC are injected into B6AF1 mice, the magnitude of augmented BM NK cell activity is dependent upon the number of PLC injected In contrast, A PLC do not induce a dose-dependent augmentation of BM NK cell activity.

(6) The activation of splenic P-815 effector cells in GVH-reactive F1 mice is dependent upon the donor cell genotype A PLC, but not B6 PLC, are highly effective in activating splenic P-815 effector cells.

(7) Depending upon the number and strain of PLC injected into F1 mice, two distinct patterns of peak splenic NK cell activity are observed; namely, an early peak (day 8) and a late peak (day 16).

(8) The relationship between the two patterns of peak splenic NK cell activity and the severity of histopathological lesions that developed in the non-lymphoid organs of F1 mice after GVH induction showed that:

(i) When peak splenic NK cell activity is observed early moderate to severe lesions develop.

(ii) When peak splenic NK cell activity is appears later either mild lesions or no lesions are observed.

(9) The relationship between the splenic NK cell activity and T and B-cell function show that the peak and/or highly augmented splenic NK cell activity is observed at the time when both the T and B-cell function are severely suppressed

(10) Histopathological lesions appear at the time when NK cell activity is at its peak and/or highly augmented and both the T and B-cell function are severely suppressed

(11) Low doses of B6 PLC ($10x10^6$ and $20x10^6$), although induced splenic NK cell activity (a late peak), these cell doses neither induced severe immunosuppression nor histopathological lesions.

(12) $30x10^6$ B6 is the critical dose required to induce both severe immunosuppression as well as moderate degree of histopathological lesions in B6AF1 mice.

(13) Augmented thymic NK cell activity is observed irrespective of the number and strain of PLC injected to induce GVH reactions. However, the time of appearance of augmented thymic NK cell activity is dependent upon the number and strain of PLC injected. Two distinct patterns of the time of appearance of thymic NK cell activity are observed, namely, an early appearance (day 4) and a late appearance (day 8)

(14) A comparison of the two patterns of appearance of thymic

NK cell activity and the development of thymic histopathological changes shows that:

(i) When thymic NK cell activity appears early, moderate to severe thymic lesions develop.

(ii) When thymic NK cell activity appears late, either mild thymic lesions develop or no thymic lesions are observed

(15) Both the time of appearance and the later severity of tissue injury in the thymus and non-lymphoid organs, in a given GVH combination, are the same

(16) Thymocytes taken from GVH-reactive animals (at the time of augmented thymic NK cell activity) produce factor(s) that can induce NK activity in normal thymocytes.

(17) The GVH-induced thymic dysplasia is not an all or none event. Depending upon the number and strain of PLC injected, different degrees of thymic dysplasia can be characterized; for example, mild. moderate, and severe.

(18) On day 16 after GVH induction, the different degrees of thymic lesions were characterized as follows:

(A) Mild lesions:

(i) Intact cortico-medullary demarcation .

(ii) Presence of "small" individual epithelial cells in the medulla.

(iii) Total dissappearance of epithelial cell clusters and

Hassall's corpuscles in the medulla.

(B) Moderate lesions

(i) Total loss of cortico-medullary demarcation

(ii) Total disappearance of epithelial cell clusters and Hassall's corpuscles in the medulla.

(iii) Only rare, if any, small individual epithelial cells were visible in the medulla

(C) Severe lesions

(i) Total loss of cortico-medullary demarcation

(ii) Total loss of individual epithelial cells, epithelial cell clusters, and Hassall's corpuscles in the medulla

(iii) Presence of intense lymphocytic infiltrates in the medulla

(19) On day 30 after GVH-induction, severe atrophy and hypocellularity were observed in thymuses of those groups of F1 mice that displayed total loss of cortico-medullary demarcation on day 16 (moderate-severe lesions) after GVH induction In contrast, on day 30 normal thymic architecture was observed in those groups of F1 mice that displayed an intact cortico-medullary demarcation on day 16 (mild lesions) after GVH induction.

(20) With time after GVH induction, the dysplastic thymuses started to recover and regained a completely normal architecture.

(21) The time required by the dysplastic thymuses to regain a normal architecture was dependent upon the severity of initial thymic dysplasia on day 16 after GVH induction.

(22) The regeneration of the thymic architecture was gradual. During thymic regeneration 4 stages were characterized:

(1) Stage 1: repopulation of the cortex, but not the medulla, and reappearance of cortico-medullary demarcation.

(2) Stage 2: reappearance of abundant "small" individual epithelial cells, and a few "large" individual epithelial cells and epithelial cell clusters.

(3) Stage 3: repopulation of abundant "large" epithelial cells and epithelial cell clusters.

(4) Stage 4: reappearance of abundant epithelial cell clusters and Hassall's corpuscles.

(23) By day 16 after GVH induction, severe suppression of T-cell and B-cell proliferative responses as well as the PFC response to SRBC were observed in GVH-reactive mice that displayed mild, moderate, or severe thymic lesions. However, with time after GVH induction, recovery of all immune functions is observed.

(24) The immune functional recovery is gradual and takes place in the following order: LPS responsive B-cells, Con A responsive T-cells; PHA responsive T-cells; and, finally, the PFC response to SRBC

(25) During the immune recovery phase IL-2 production by splenocytes following Con A stimulation is greater than following PHA stimulation

(26) The recovery of the PFC response to SRBC is gradual, i e, various numbers of PFC to SRBC are observed in the spleens of the recovering mice

(27) The recovery of the PFC response is observed only after the thymus has regained a normal architecture

(28) A "lag" period is observed in the recovery of the PFC response to SRBC after the thymus has regained a normal architecture, suggesting that thymic architectural recovery preceded thymic functional recovery.

(29) GVH reactions also resulted in depletion of nucleated cells in the spleen of F1 mice by day 30 after GVH-induction The degree of splenic nucleated cell depletion was dependent upon the number and strain of PLC injected to induce GVH reactions.

(30) With time after GVH-induction, repopulation of the spleen of GVH-reactive mice with nucleated cells was observed. The time required for complete splenic repopulation was determined by the initial severity of the GVH reaction (on day 16 after GVH induction).

(31) When GVH reactions were induced in B6AF1 mice by injecting $30x10^6$ B6 PLC, the depletion and repopulation of BM with nucleated cells preceded the depletion and repopulation of the spleen, suggesting that the changes observed in the splenic nucleated cell numbers reflect the changes in the BM

(32) When splenocytes from GVH-reactive B6AF1 mice displaying different degrees of thymic regeneration were pretreated with PGE1 or PGE2 and then stimulated with Con A and PHA the following results were obtained:

(i) PGE1 and PGE2 pretreatment of GVH immunosuppressed splenocytes failed to restore T-cell proliferative responses when either moderate-severe thymic lesions were observed or when thymic cortical regeneration was observed.

(ii) PGE1 and PGE2 pretreatment of GVH immunosuppressed splenocytes only partially restored T-cell proliferative responses when partial thymic medullary regeneration was observed.

(iii) PGE1 and PGE2 pretreatment of GVH immunosuppressed

splenocytes was most effective in restoring T-cell proliferative function when complete thymic medullary regeneration was observed.

(33) The severe depression of splenic NK cell activity during the intermediate phase of the GVH reaction could be accounted for by, at least three mechanisms:

(i) Depletion of nucleated cells in the spleen
(ii) Inability of splenic cells to produce lymphokines which may be required for maintaining lytic functions of NK cells
(iii) Lack of expression in the expression of NK cytotoxic potential.

(34) The severely depressed splenic NK cell activity could be boosted partially by *in vivo* administration of Poly I.C

(35) The severely depressed splenic NK cell activity could also be partly restored by *in vitro* treatment of splenocytes with PGs and Poly I:C. The combination of PGs and Poly I C treatment was more effective in restoring depressed splenic NK cell activity then either PG or Poly I:C treatment alone

(36) GVH reactions also induced IFN production which could be detected in the serum of GVH-reactive mice. Serum IFN was detected as early as day 4, peaked by day 12, and then declined to undectable levels by day 30 after GVH induction.

(37) On days 30 and 35 after GVH induction, the GVH-reactive mice produce only 25-30% of IFN as compared to the IFN produce by normal mice in response to *in vivo* administration of Poly I:C.

(38) The depression in IFN production by the GVH mice could be attributed to, at least in part, the depletion of nucleated cells in the spleen of GVH mice.

(39) The restoration of depressed splenic NK cell activity *in vitro* was independent of the degree of thymic medullary dysplasia.

(40) The GVH reaction induced splenomegaly and tissue damage are two distinct features of the GVH reaction mediated by seperate mechanisms.

By employing various parent-->F1 GVH combinations of bg/bg and +/bg mice the following results were obtained:

(1) GVH reactions augment splenic NK cell activity of both the host and donor origin.

(2) The augmented splenic NK cell activity of donor origin, but not the augmented splenic NK cell activity of host origin, correlate with the development of moderate-severe GVH-associated tissue damage.

(3) The degree of augmented splenic NK cell activity does not correlate with the degree of splenomegaly.

(4) The bg/bg PLC, like the +/bg PLC, possess the ability to induce GVH-associated splenomegaly, however, the bg/bg PLC, unlike the +/bg PLC, are deficient in their ability to induce GVH-associated tissue damage.

(5) The bg/bg PLC possess the ability to induce early severe suppression of the PFC response to SRBC in both the bg/bgF1 and +/bgF1 mice. However, the bg/bg PLC induced only partial suppression of T-cell mitogen responses.

(6) In the bg/bg and +/bg parent into F1 hybrid GVH combinations severe suppression of the PFC response can be observed in the absence of thymic dysplasia. However, severe

suppression of the T-cell mitogen responses is observed only in the presence of thymic dysplasia.

(7) The parental B6 bg/bg mice possessed the effector mechanisms responsible for allograft rejection but lacked the mechanisms that induce GVH-associated tissue damage.

(8) The parental bg/bg animals possessed a greater T-cell proliferative and IL-2 producing capacity and a lower B-cell proliferative capacity than parental +/bg mice.

** NOTE: The above summary outlines the original work contained in this thesis.

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