AN ANALYSIS OF NATURAL KILLER CELL ACTIVITY IN ACUTE MURINE GRAFT-VERSUS-HOST REACTIONS

A Thesis presented to the
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by

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Glossary of Abbreviations

ADCC - antibody dependent cell cytotoxicity
AIDS - acquired immunodeficiency syndrome
ASGM1 - asialo-gangliomannoside 1
BAL - bronchoalveolar lavage
BC - backcross
BMT - bone marrow transplantation
CAM - chorioallantoic membrane
CMV - cytomegalovirus
Con A - concanavalin A
CPM - counts per minute
Cr - complement receptor
CTI - cold target inhibition
CTL - cytotoxic T lymphocyte
DTH - delayed type hypersensitivity
FITC - fluorescein isothiocyanate
GVH - graft-versus-host
HBSS - Hank's balanced salt solution
HVG - host-versus-graft
IFN - interferon
IL-1 - interleukin - 1
IL-2 - interleukin - 2
LAK - lymphokine activated killer
LGL - large granular lymphocyte
LPS - lipopolysaccharide
MAF - macrophage-activating factor
MHC - major histocompatibility complex
NK - natural killer
NKCF - natural killer cytotoxic factor
NS - natural suppressor
PBL - peripheral blood lymphocyte
PGE - prostaglandin E
PFC - plaque-forming cell
PHA - phytohemagglutin
SCID - severe combined immunodeficiency disease
SI - stimulation index
SpI - spleen index
SRBC - sheep red blood cells
TLI - total lymphoid irradiation
TNF - tumour necrosis factor

ABSTRACT

The kinetics of NK-cell activation, cell surface phenotype, buoyancy shift of the NK-effector cells, de novo target cell lysis, and target cell specificity of natural killer (NK) cells were examined in (C57BL/6 X A/$_J$)F_1-hybrid mice with acute graft-versus-host (GVH) reactions induced by intravenous injection of 50 X 10^6 parental strain lymph node and spleen cells. Results showed that there was a marked increase in NK-cell activity directed against YAC-1 tumour cells. In the lung, this activity remained elevated over almost the entire course of the reaction whereas in the spleen, it was only transiently increased during the early stages of the reaction and then fell to normal values. Lymph node NK-cell activity rose during the lymphoproliferative phase of the reaction and was sustained throughout the reaction. During the reaction, NK cells acquired the ability to kill P815 targets, cells that are normally insensitive to NK-cell lysis. The level of P815 killing never reached that achieved against YAC-1 cells, but was significantly higher in the lung than in the lymph nodes or spleen. Antibody and complement depletion experiments showed that both anti-YAC-1 and anti-P815 activity could be depleted with antiserum to the asialo-GM1 cell surface marker, but was unaffected by anti-Lyt-1.2, anti-Lyt-2.2 and anti-L3T4 treatment. Anti-YAC-1 activity was partly sensitive to depletion with anti-Thy-1.2. Cytotoxicity to P815 target cells acquired during the reaction was completely abrogated by anti-Thy-1.2. These findings suggest that during the reaction two phenotypically distinct types of NK cells are activated; a conventional, Thy-1.2-negative cell that kills only YAC-1 targets, and a Thy-1.2-positive cell with a broadened spectrum of lytic activity. This is further supported by cold target inhibition studies in which both cold YAC-1 and P815 tumour cell targets inhibited the anti-P815 NK-cell activity but cold P815 targets only partially reduced the anti-YAC-1 NK-cell cytotoxicity. The enhanced YAC-1 and de novo P815 NK-cell lysis

during the GVH reaction were not physically separable on a discontinuous Percoll gradient. These findings have led to the proposition that the Thy-1-positive NK-cell may be generated in response to Interleukin-2 released during the lymphoproliferative phase of the reaction and may represent a type of lymphokine-activated killer cell.

1.0 INTRODUCTION

Graft-versus-host (GVH) reactions are known to be one of the major obstacles to successful bone marrow transplantation (BMT) in man. Because BMT is now frequently used to treat a wide variety of haematologic diseases including: aplastic anemia (1), thalassemia major (2), haematological malignancies (3) and also ataxia telangiectasia, Chediak-Higashi disease, Fanconi's anemia, Blackfan-Diamond anemia, osteopetrosis and Wiskott-Aldrich syndrome (4,5) the incidence of clinical GVH is increasing. In most instances, GVH reactions are either experimentally or iatrogenically induced (6-8), but they may occur naturally due to materno-fetal transfusion of lymphocytes during pregnancy or birth (9,10).

Experimental GVH reactions have also been used to study ontogeny and regulation in the immune system, and have served as models for diseases of disordered immunity such as severe combined immunodeficiency (SCID), systemic lupus erythematosus (SLE), Sjogren's syndrome, collagen vascular disease, polymyositis, primary biliary cirrhosis and scleroderma (11). The GVH reaction has also been suggested as a model to study the pathobiology of acquired immune deficiency syndrome (AIDS) (12-15).

1.1 Historical review of GVH reactions.

Despite their close association with BMT, GVH reactions were observed and described well before the first BMT was done in humans. These original experiments were performed by Murphy (16) who transplanted fragments of rat tumours and/or tissue from normal adult chicken organs through windows cut in the shells of seven day fertilized eggs. The graft tissue was placed in contact with the chorioallantoic membrane (CAM). After 17 days some of the transplanted eggs showed cellular nodules on the CAM around the graft and splenomegaly in the chick. Nodules were discovered in the embryonic dermis and epidermis. Murphy discovered that the changes always occurred following transplantation of spleen tissue, less often with liver, rarely after kidney and never with muscle or cartilage. He explained this observation in terms of cellular stimulation to the spleen and other mesodermal tissues (17) but did not attribute them to GVH reaction as such. Morten Simonsen recently reviewed Murphy's experiments in detail (18) and concluded that the immunological concept of the GVH reaction was perhaps too advanced to allow him or his contemporaries to have identified it even though it was technically possible at the time.

Murphy's cellular stimulation idea was expanded into the mistaken hypothesis that some undefined subcellular substance released from the more mature and dominating cells in the graft resulted in stimulation of organ-specific growth (19). The precise explanation for Murphy's observations and the conceptualization of the GVH reaction awaited the 1950's when researchers began to investigate biological mechanisms involved in allograft rejection and tolerance.

The convincing demonstrations of the GVH reaction ultimately came from experiments of immunological tolerance (20-24) that were initiated and influenced by the work of Medawar (25). Many of the difficulties and failures encountered in their studies of acquired tolerance of grafted

cells begged answers. Independently, Simonsen demonstrated that when adult spleen cells were injected into outbred embryonic chicks the engrafted chicks always died (22). Studies originally conducted by Billingham and Brent in immunologically immature neonatal mice to investigate mechanisms of tolerance showed that inoculation of newborn mice with isologous, homologous or heterologous adult spleen cells caused a syndrome of: skin graft acceptance, splenomegaly and mortality (26,27). The mortality was preceded by emaciation, fur loss, diarrhea, and atrophy of lymphoid tissue that they called "runting syndrome". Other experiments by Barnes showed that lethally irradiated CBA mice recovered when reconstituted with syngeneic spleen cells; but, when A strain cells were employed, recovery was followed by a destructive "secondary" disease (23). Trentin and van Bekkum examined this phenomenon using inbred mouse strains and F_1-hybrid mice (24,28). When irradiated parental mice were reconstituted with F_1-hybrid cells permanent recovery was seen, whereas the transfusion of parental strain cells into irradiated F_1-hybrid mice resulted in high mortality. The investigators concluded that in the latter combination the transferred cells reacted against the host because it was antigenically different. In 1959 the GVH reaction was recognized clinically following therapeutic human bone marrow transplantation, first performed by Mathe (29).

By 1960 results from several investigators identified lymphocytes as important cellular mediators in this "runting" or "secondary disease" (21-24,28-31). An earlier hypothesis of a viral etiology for GVH reactions was dispelled by experiments using an outbred chick model (23,32). Injection of whole blood from adult chickens into chicks resulted in GVH reactions. When the blood was separated into sera, erythrocytes and leukocytes, only the leukocyte fraction could produce the reaction. Further treatment of the leukocyte fraction sufficient to disrupt the cells but insufficient to destroy an admixed bacteria (*B. coli*), prevented the reaction (22).

Work throughout the sixties and seventies further defined and identified much of the phenomenology of the reaction (reviewed in 6,33). This included many *in vitro* and *in vivo* observations about the state of the immune functions in mice with GVH reactions (34-43).

The "GVH reaction" quickly became an important immunological concept, the validity of which was questioned only by those who had developed it (44). The first full analysis and review of the phenomenon was presented by Billingham and Brent (27) following the *Third Tissue Homotransplantation Conference* held at the New York Academy of Sciences in 1958. They interpreted the GVH reaction as an immunological disease that could arise when specific conditions were met (22).

1.2 Conditions of induction of GVH reactions.

Simonsen was the first to unequivocally define the three immunological requirements that had to be met in order to elicit a GVH reaction (22,27). These can be summarized as follows:

1) The graft must contain immunologically competent cells.
2) The host must be sufficiently different genetically from the graft so as to be perceived antigenically foreign.
3) The host must be immunologically incapable of rejecting the graft.

These requirements are all satisfied in the setting of allogeneic bone marrow transplantation in man and in three animal models: parabiotic intoxication, radiation chimera and F_1-hybrid disease (6,11,20,21,33,45).

1.3 Experimental models of GVH reactions and associated terminology.

Almost as confusing and problematic as the recognition of the GVH reaction itself was the development and standardization of its related terminology. Initially, the GVH reaction had several names as it was

described in terms of its varying experimental conditions or the various symptoms it produced. The various historical descriptions can be summarized as follows: (1) The transfer of maternal lymphocytes to the offspring resulted in "runt disease" characterized by growth retardation, wasting, alopecia and lymphoid destruction. (2) "Secondary" or "homologous" disease developed following resolution of radiation sickness known as "primary disease". This latter experimental scenario most closely resembles the events following human BMT . (3) The crossing of the circulation between parent and F_1-hybrids resulted in "parabiotic intoxication" and produced anemia, wasting, lymphoid destruction, epithelial lesions and amyloidosis. (4) F_1-hybrid disease, characterized by severe, usually fatal illness, epithelial lesions, lymphoid destruction, immune-complex nephritis and lymphomas, is the consequence of the infusion of parental strain lymphoid cells into the unirradiated F_1-hybrid host.

There are three widely used models of systemic GVH reactions. They are the radiation chimera, the F_1-hybrid and the natural materno-fetal transfusion which is essentially an F_1-hybrid model.

The radiation chimera most closely resembles BMT in that it involves the irradiation of the host followed by the intravenous injection of donor lymphoid cells. Variations of this model also incorporate other pharmacological immunosuppressive or cytoreductive pre-treatment chemotherapies of the host animal.

Of all the animal models, the F_1-hybrid is the "cleanest" immunologically because it uses only histoincompatibility to meet the three requirements. Lymphoid cells from either parental strain are injected either intravenously or intraperitoneally into the F_1-hybrid host. Radiation is unnecessary because the F_1-hybrid does not recognize the parental cells as foreign whereas the infused parental cells recognize the haplotype of the other parent on the F_1-hybrid cells. The reaction can be directed across the entire major histocompatibility complex (MHC), to only

certain regions of the MHC, or to minor histoincompatibility differences only.

Differences in the behavior of radiation chimeras and F_1-hybrid models of GVH disease allow the effects of the graft alone to be factored from the overall clinical/experimental picture which is of course a combination of both graft-related and pretreatment effects. In other words, using the F_1-hybrid model only changes due to the effect of the graft can be observed in the absence of confounding factors such as radiation, cytoreductive chemotherapy, etc.

1.4 Clinical conditions and considerations.

Unlike the controlled conditions of the experimental F_1-hybrid model human bone marrow recipients require immunoablative pre-treatment therapy to avoid graft rejection, even in the case of the immunodeficient patient. This is primarily achieved by total body irradiation with or without the addition of cytoreductive chemotherapy (46-49).

With the success of the pre-treatment of the recipient to prevent graft rejection, the GVH reaction is now the major obstacle to BMT as an effective and safe therapeutic modality. Host-versus-graft (HVG) reactions are usually only a concern when BMT is used to treat aplastic anemia, while relapse of the primary disease and failure of engraftment plagues the leukemias (reviewed in 3). Acute graft rejection has been attributed to prior sensitization of the host to donor antigens by transfusions with donor or donor-related blood products in the pre-transplant period (50).

In clinical bone marrow allotransplantation, recipient survival depends upon the survival of the complement of graft stem cells. Studies of peripheral blood, bone marrow and immune function profiles during this period reveal that specific cell-type numbers, mixed lymphocyte responsiveness, antibody-dependent and lectin-mediated cellular

cytotoxicity and natural killer (NK) cell activity recover relatively rapidly. However, for several months following transplantation, the host appears clinically immunocompromised as evidenced by increased episodes of opportunistic infections (i.e. CMV) which appears to be unrelated to GVH reaction. BMT patients are commonly maintained through this period by antibiotics and granulocyte transfusions, occasionally supplemented with transfusions of specifically sensitized donor lymphocytes (47,49,51-53). Recovery may be complicated by the occurance of GVH disease particularly if immunosuppressive or cytoreductive chemotherapies are required to control GVH reactions.

The only salutory effect that has been ascribed to the GVH reaction is an anti-leukemic effect (54). The ability to manage the GVH reaction to the benefit of the patient without excessive damage is not yet reliable enough to therapeutically exploit this effect.

1.5 Immunogenetics of the GVH reaction: Role of the MHC.

The major factor that determines both graft rejection and GVH reactions is the degree of antigenic disparity at the MHC (55-58). A direct relationship has been demonstrated between the severity of the reaction and the degree of MHC incompatibility between the host and graft (76).

The principal effector cell of the GVH reaction is believed to be the class I MHC-restricted cytotoxic T lymphocyte (CTL)(59). Evidence to support this view comes from the demonstration that depletion of mature reactive T-lymphocytes from the graft prevents the occurrence of acute GVH reactions (57).

In general, "conventional" cytotoxic T cells express the CD8+ (OKT8+, Lyt-2+) phenotype and are restricted to class I MHC determinants (60,61). Alloreactive helper T-cells, the cells which respond during the

MLR, express the CD4+ (OKT4+, L3T4+) phenotype and are class II MHC-restricted (60,61).

Lyt-1+ T-cells (predominantly T-helper cells) are also involved in lethal GVH reactions (56). In experiments using irradiated C57BL/6 recipients and Balb/C donors in vitro pre-treatment of donor marrow with anti-Lyt-1 results in an 80% survival rate at 100 days following transplantation compared to 100% mortality by day 35 without this pre-treatment.

Studies of GVH reactivity with different types of H-2 differences revealed that I-region alloantigen differences stimulate the strongest mixed lymphocyte reaction (MLR) while K- and D-region differences alone produce weak MLRs (62-65). Similar observations have been made for the human MLR (66). In mice, the degree of in vitro mixed leucocyte reactivity appears to correlate with the severity of the GVH reaction in vivo (68).

In humans, human leukocyte antigen (HLA) matching is determined through serological typing of the class I (A,B) loci and in vitro mixed lymphocyte reactivity between host and donor for class II (D,DR) loci (3). Almost invariably, genetically identical donors can not be found, but partially matched donors have been employed with reasonable success (47).

1.6 Immunogenetics of the GVH reaction: Role of minor histoincompatibility antigens.

GVH reactions induced across multiple minor histocompatibility antigen differences have been well characterized (69). In clinical settings, the reaction to minor histocompatibility determinants may be lethal but more often tends to be chronic (3).

There appears to be a difference in the clinical appearance and histological lesions of GVH reactions produced with non-MHC differences compared with those induced across the MHC (reviewed in 70). Furthermore

in mice, during GVH reactions to non-H-2 haplotypes, the development of characteristic skin lesions depends on the strain combination employed (69).

In studies of GVH reactions in neonatal mice, GVH reactivity is minimal unless the donor has been presensitized (69). This observation may be explained from studies with adult mice showing that mature T cells are required to produce lethal GVH reactions to non-MHC loci(69). The CTL's induced by minor histocompatibility antigen differences are class I restricted and are greater than 98% Thy-1 positive. Class II restricted T-cells may amplify this CTL response, but these cells do not appear to be essential for actual induction of the reaction (61,69).

The severity of GVH reactions to minor histocompatibility antigens appears to be under complex multigenic control (70). In experiments using F_1-backcrosses to map different minor histocompatibility antigens it has been shown that individual minor histocompatibility determinants have weak effectiveness in causing GVH reactions. However, when pairs of linked markers are examined some have synergistic effects, increasing the severity of the GVH reaction while others have a suppressive effect, diminishing it. Differences in suppression of the GVH reaction have also been associated with the Pgm-1 marker, a sex-associated gene in homozygous donors. This pattern of cumulative and suppressive effects suggests that the number and significance of these non-H-2 loci may be substantial (70). The results from various combinations of non-H-2 genetic differences studied so far are summarized in Figure 1.

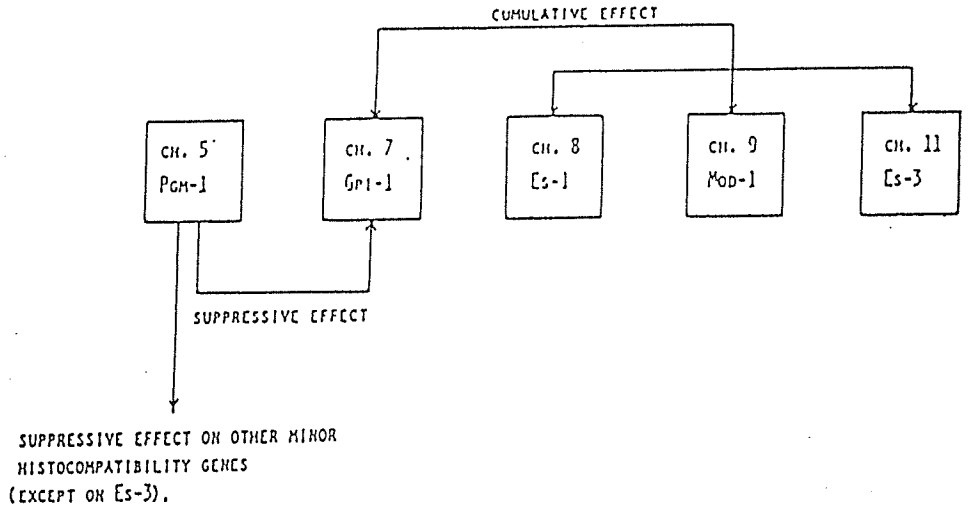


FIGURE 1 Interactions between non-H-2 genes linked to Pgm-1, Gpi-1, Es-1, Mod-1 ans Es-3 chromosomal markers. Lethally (1100r) irradiated adult (DBA/2 x B10.D2)F_1-mice were grafted (i.v.) with 10^7 bone marrow and 8 x 10^6 spleen cells from F_1 x B10.D2 backcross (BC) donors were typed individually for their allelic constitution at the chromosomal markers indicated, and in each case the mortality induced by cells from homozygous donors (incompatible with F_1 recipients) was compared to that induced by cells from heterozygous donors (compatible with F_1 recipients). The authors defined as "cumulative effects" the two observations: 1) cells from BC donors homozygous at both Mod-1 and Gpi-1 markers induce greater (percent) mortality than do cells from BC donors homozygous at Mod-1 alone; 2) cells from BC donors homozygous at both Es-1 and Es-3 (but not at either alone) induce greater mortality than that induced by cells from BC donors heterozygous at these same markers. The authors defined as "suppressive effects" the additional observations: 1) mortality associated with donor homozygosity at Gpi-1 is decreased by simultaneous homozygosity at Pgm-1; 2) homozygosity of BC donors at Pgm-1 considerably decreases the mortality, provided that they are also heterozygous at Es-3.

From Halle-Pannenko O., Pritchard L.L., Bruly-Rosset M., Berumen L. and R. Motta 1985. Parameters involved in the induction and abrogation of the lethal graft-versus-host reaction directed against non-H-2 antigens. Immunol. Rev. 88:59

1.7 Role of B-cells in GVH reactions.

Cytotoxic lymphocytes are not the only effector cells mediating GVH reactions. Anti-host antibodies are known to contribute to the manifestations of GVH disease in non-MHC induced reactions since B-cell proliferation and differentiation is seen in mice with GVH disease (70) and autoantibodies appear in the serum of these mice (71). Gleichman et al postulate that autoantibody formation may be due to abnormal T-B cell cooperation (71). In GVH reactions donor T_h cells react to host alloantigens that are physically separate from the antigens that the host B cells see. Such "cooperation" provides non-specific help or stimulation (via T-cell derived B-cell growth and differentiation factors) to all host B cells. According to this model the occurence of autoantibodies depends upon the quality and type of antigen. If an antigen allows multi-point binding and promotes cross-linking of Ig receptors (such as DNA or cell surface markers) high titres of autoantibodies will be generated as in SLE-like GVH reactions. However, if there is a lack of antigen or an unsuitable antigen (such as low avidity monovalent Thyroglobulin) there will not be adequate cross-linking of Ig receptors to trigger antibody production.

1.8 Delayed Type Hypersensitivity in GVH reactions.

Delayed type hypersensitivity (DTH) or hypersensitivity type IV refers to cell-mediated hypersensitivity reactions which require at least twelve hours to occur. DTH is a complex phenomenon which is still not well understood. Clinically DTH is seen upon re-exposure to an antigen such as nickel, poison ivy, and tuberculin or persistent exposure to micro-organisms or immune complexes. It manifests as a thickening of the skin or the development of a granuloma. In mice and guinea pigs DTH is monitored as footpad swelling. Experimentally DTH can be transferred by sensitized T cells (T_D or T_{DTH}) which stimulate an inflammatory response

at the site of injection. Depletion of T cells with anti-Thy-1 and complement in a transfer inoculum prevents the DTH response in the recipient (116).

The contribution of DTH to the pathobiology of GVH reactions may be important as a correlation between the pattern of GVH reaction mortality in the non-H-2 donor-recipient mismatch and DTH activity in GVH reactions has been demonstrated (73,74).

Anti-host DTH activity was determined by challenging a naive secondary (strain A) recipient with lymphoid cells from the allogeneically reconstituted primary (irradiated strain B infused with strain A) hosts and measuring % specific increase foot thickness (72). Using several strain combinations in the radiation chimera GVH model Benner et al have shown that anti-host DTH activity precedes the clinical signs of the GVH reaction and peaks around day five (72). Delayed type hypersensitivity activity occurs maximally in marrow transplants which are either I-region-incompatible or alternatively H-2 matched but incompatible at the Mls locus. By inoculating various combinations of irradiated hosts with pre-treated T cells it was determined that anti-host DTH is mediated by long-lived recirculating Lyt1+2- T-cells and can be amplified by sessile splenic Lyt1+2+ T-cells that react to host H-2 K/D or non-H-2 alloantigens other than the Mls loci antigens (72). The presentation of H-2I alloantigens to GVH-activated DTH T-cells must be in combination with syngeneic H-2 K/D molecules to elicit a maximal anti-host DTH response.

1.9 Other factors modulating the severity of GVH reaction.

GVH reactions are influenced or modulated by a multiplicity of factors, some of which have been identified. An important factor in all transplant regimes appears to be the number of lymphocytes transfused. In MHC incompatibility, transfusion of a large number of lymphocytes correlates with rapid onset of 'acute' lethal GVH reactions (33). Transfusion of fewer lymphocytes results in a delayed GVH (33). This

reaction is lethal and has clinical symptoms similar to those of acute reactions. Delayed GVH reactions have been separated from chronic GVH reactions (56) because they represent an indolent version of acute reactions.

In MHC-compatible, non-MHC incompatible, donor-recipient transplants, the occurrence of acute vs. chronic GVH reactions can also be attributed to the differences in the number and types of donor lymphocytes transfused (70).

Children are most successfully transplanted, while adults, especially those over 30 years of age, have a much higher mortality rate (60%) (53).

The various treatments used to prepare human BMT recipients may also have an effect on the incidence and severity of GVH disease. For example, radiation may cause subliminal tissue damage to dividing cells, especially in the skin and gut that may alter the antigenicity of these cells and thus their vulnerability to injury by GVH effector mechanisms (77).

Clinical experience with syngeneic marrow transplants has shown that male recipients engrafted with female cells may develop GVH reactions directed against Y chromosome associated histoincompatibility antigens. Conversly, it has been shown that female cells can be given to recipients of either sex, whereas male cells cannot because they can, when injected into female recipients, be rejected by the host (82). Female BMT recipients have a greater incidence of GVH disease if they have been presensitized by pregnancy (78,79,80). Likewise in the murine GVH reaction, males engrafted with female cells differing at class I loci have a higher mortality rate than female recipients (81).

1.10 Histopathology of GVH disease.

The lesions produced by GVH reactions are characterized by tissue necrosis and mononuclear cell infiltration. These changes have been described in a variety of organs including: skin, lung, liver, thymus,

spleen, pancreas, esophagus, lymph node, salivary gland, lacrimal gland, and intestinal tract (46,51,52,84,85,86). Examples of some of these can be seen in figures 10-14.

Lymphoid tissues were originally thought to be the principal targets of GVH reaction, but epithelial cells are now also recognized as important targets. It has been suggested that Ia antigen expression on epithelial cells during GVH reactions may serve as the antigenic structure recognized by effector cells in the graft (6). Evidence in support of this notion comes from experiments showing that in irradiated mice the expression of Ia antigen on epidermal keratinocytes and gut epithelium is associated with severe tissue damage during GVH reactions (87,88). Despite this circumstantial evidence, the precise role of these antigens is not understood.

Clinically, skin involvement is found in the majority of cases (33). The cutaneous lesions are not diagnostic (51) and consist of generalized erythema or maculopapular rash with bullae and eventual desquamation which may be followed by irregular pigmentation (3,33,46,51,52). Histologically acute reactions show necrosis of the basal cell layer, satellite cell dyskeratosis, acantholysis, heaping of the keratinocytes, and mononuclear cell infiltration. Bulla formation may occur as consequence of destruction of the dermal-epidermal junction (89,90). Depletion of Langerhans cells has also been observed (91-93).

In chronic GVH reactions skin lesion are characterized by basal cell vacuolar degeneration, dermal fibrosis and edema. The dermal collagen bundles often stain positively for IgG (94). In both the acute and chronic cutaneous GVH reaction the cellular infiltrate consists almost entirely of mononuclear cells, most of which resemble lymphocytes. Chronic reactions often contain more eosinophils and plasma cells than do acute reactions (47).

Pathologic changes in the liver are reflected by increased serum bilirubin, transaminase and alkaline phosphatase (47). The pattern of

liver injury differs to some extent between acute and chronic reactions (95), but both show mononuclear cell infiltrates. Early onset acute reactions show periportal and midzone hepatocellular necrosis, characterized by foci of acidophilic cells. In late onset acute GVH reactions bile duct injury predominates. Ductal epithelial cells show dysplasia and nuclear pyknosis. A lymphocytic infiltrate is seen both around bile ducts and within the duct walls. In chronic GVH reactions, the liver shows portal fibrosis.

Injury to the gut is characterized clinically by diarrhea, nausea, vomiting, and frequently, abdominal pain and ileus (47,53). The histopathology ranges from focal dilatation and degeneration of the mucosal glands to frank mucosal ulceration. The gut lesions resemble changes occurring in ulcerative colitis and consist of flattening of the villi, cellular fragmentation, disintegration of the crypts, crypt abscess formation, necrosis of epithelium, and mucosal and submucosal mononuclear cell infiltrates. Mowat et al. (96) demonstrated that infiltration of the mucosa by intraepithelial lymphocytes during GVH reactions required an Lyt-1^+2^- T-cell whereas the development of crypt hyperplasia required both Lyt-1^+ and Lyt-2^+ T-cells.

Intestinal lesions are associated with secondary infection. Mice kept in germ-free environments apparently do not develop these severe gut lesions associated with GVH reactions in mice housed in regular facilities (97-99). The beneficial effect of gastrointestinal sterilization and a protective environment has also been observed in BMT patients (77,100,101). These studies implicate a role for infective agents in the pathogenesis of GVH disease. It is relevant to recognise that epithelial targets are also directly or indirectly in contact with the external milieu and potential pathogens (102,103). The importance of pathogenic organisms in the pathogenesis of GVH disease is further underscored by observations that reactivation of latent viruses by pre- or post-

transplant immunosuppression may permit the expression of viral antigens that in turn may stimulate the GVH reaction (104-108).

Many BMT patients with GVH disease develop pulmonary complications. Symptoms consist of coughing, dyspnoea, wheezing and abnormal pulmonary function tests (109). A restrictive or obstructive pattern may be seen (110,111). Rarely veno-occlusive disease may occur (112). The pulmonary histopathology consists of diffuse mononuclear cell infiltrates with plasma cells in perivascular locations and within the interstitium (113). Regions of haemorrhage and microinfarcts can also be found. Alveolar walls become edematous and fibrotic and show type 2 cell hyperplasia. Bronchi show epithelial cell destruction and sloughing of mucosa. Peribronchial lymphocytic infiltration and exudation of lymphocytes and macrophages into the pleural cavities may occur. In a clinical study, cells retrieved from BMT patients with GVH reactions by bronchoalveolar lavage (BAL) contained 36% þ 10% neutrophils, 16% þ 3.6% lymphocytes and 47% þ 9.3% macrophages (114). A study by Gartner et al determined that GVH reactions stimulated a systemic resident macrophage proliferative response in the lung, liver and peritoneum. This response in the lung was associated with increased numbers of macrophages in the BAL fluid (279). Studies in mice induced with B10.BR (C57BL/10SCN x B10.BR)F_1-hybrid GVH reactions revealed that greater than 80% of BAL cells were Thy-1.2 positive lymphocytes of donor origin. Cells were either L3T4 positive (38% BAL cells) or Lyt-2 positive (43% BAL cells) (115).

The interstitial pneumonitis of BMT patients has also been attributed to cytomegalovirus (CMV) infection. CMV has also been implicated in the pathogenesis of interstitial pneumonitis in mice with GVH disease (115,117). Bystander injury to tissue due to a host response to viral infection may be a factor that contributes significantly to the severity of lesions in GVH reactions (51).

Kidney damage in mice with GVH reactions has also been observed (113). Changes include lymphocytic and plasma cell infiltration of the

interstitium. Glomeruli show mesangial proliferation, narrowing or occlusion of capillary loops, lobulation of the tufts and thickening of the basement membranes. Tubular epithelial cells may show vacuolation.

Lymphoid organs show a different pattern of histopathology than the organs already described. In both acute and chronic conditions there is an initial lymphocyte loss which is later followed by repopulation and a lymphoproliferative phase (113). In acute reactions, during the lymphoproliferative phase large lymphoblastoid cells arise in the T-cell areas (33). As the reaction proceeds the total number of lymphocytes decreases markedly and lymphokaryorrhexis is seen. In chronic reactions lymphadenopathy results from an increased number of plasmacytoid cells and macrophages. Large numbers of plasma cells are seen in the medullary cords and the sinuses become engorged with macrophages.

The spleen shows similar features. During acute reactions large lymphoblastoid cells infiltrate in the red pulp. The white pulp is replaced by large lymphoid cells and vacuolated macrophages. Hemorrhagic necrosis of the red pulp occurs as the size and number of splenic follicles increases. Fragmentation of reticular fibers and loss of small lymphocytes occurs in the periarteriolar zone. In chronic reactions an initial lymphocyte loss in the white pulp is followed by the appearance of numerous lymphoblastoid cells. The red pulp shows enhanced hematopoiesis. As the reaction progresses lymphoblastoid colonies appear in the red pulp and increase in number in the white pulp. Still later in the reaction the periarteriolar lymphoid regions develop edema and fibrosis (113).

Chronic and acute GVH reactions cause thymic atrophy and dysplasia (118). Thymic epithelial cell damage results in the disappearance of Hassall's corpuscles. The thymic barrier which normally excludes the entry of mature B- and T-cells is compromised by day six of the reaction. The loss of this barrier has been demonstrated by the appearance of mature fluorescein isothiocyanate (FITC) labelled donor lymphocytes in the host

thymus (119). Dysplasia and atrophy in the thymus appear to have different underlying mechanisms. Cortical atrophy can be prevented by adrenalectomy, indicating that it is stress-related, whereas generalised dysplasia is mediated by the GVH reaction per se.

1.11 Immunopathology of GVH reactions

GVH reactions cause a wide range of immune disturbances. Abberations of T- and B-cell functions and interactions can occur (120,121). Failure of T- and B-cells to interact or excessive and/or inappropriate co-operation can occur, and was discussed in Section 1.7. Both hypogammaglobulinemia (122) and hypergammaglobulinemia (123) have been observed. IgM paraproteinemia, immune-complex deposition and plasma cell hyperplasia are features of chronic reactions (124,125). Polyclonal activation of B-cells can result in the production of lymphocytotoxic antibodies (124), antibodies to viral antigens (6), autoantibodies to self, autologous and/or donor lymphocytes (6). Reactivation of latent oncogenic viruses (126), herpes simplex virus (127,128), varicella zoster virus (127-131), cytomegalovirus (104-108) and Epstein-Barr virus (132,133) are also documented consequences of GVH reactions. The incidence of lymphoid (134-136) and non-lymphoid (137) neoplasia is associated with the activation of B-cells and/or immunosuppression that accompanies GVH reactions.

1.12 Immunosuppression during GVH reactions.

Profound suppression of T- and B-cell activity accompanying GVH reactions has been well documented (34-43). The immunosuppression is permanent under most induction conditions. Functional testing of immune responsiveness has demonstrated depressed skin-graft rejection capability, plaque-forming-cell and mitogen responsiveness (6).

Immunosuppression during the GVH reactions has been divided into two phases (6); an early active phase, a consequence of active mechanisms,

and a late permanent phase, a result of GVH-induced injury to the immune system.

Several suppressor cell mechanisms have been studied. Increased prostaglandin E (PGE) production by macrophages acts as a suppressive factor during the early immunosuppressed period (118). Removal of the PGE producing cell or in vivo treatment with indomethacin, a cyclo-oxygenase inhibitor, can reverse the immunosuppression seen in the first fifteen days. These disappear or cease acting within thirty days of induction suggesting that the late, permanent immunosuppression is mediated by a different mechanism. It is believed that maturational defects in B- and T-cell lineages caused by GVH reaction-related damage to their progenitor cells and developmental microenvironments is involved. Time lines for the development of suppression and normalized quantitations of immune functions can be seen in Figure 2.

Immunosuppression during GVH reactions induced across minor histoincompatibility differences has also been attributed to natural suppressor (NS) cells (139). These large granular lymphocytes that suppress T-cell functions do not bear B, T or macrophage cell markers and are not MHC-restricted. No cytolytic activity has been ascribed to them. Their activity is quantitated by decreased target cell proliferation These NS cells are seen during hemopoietic regeneration following total lymphoid irradiation (TLI) and can be found in normal hemopoietic tissue (140). They are believed to be involved in the induction and maintenance of tolerance (141). A more recent study has suggested that, in the neonate, these cells suppress autoreactive T-cell clones to produce self-tolerance (142).

Thy-1.2$^+$ suppressor cells have been identified in mixed lymphocyte cultures of spleen cells from mice with GVH reactions and normal F_1-hybrid mice where inhibition of immune responses of the host cells was demonstrated (143). This study also demonstrated suppressor cells with features of NS cells.

The simplest mechanism for late-stage GVH-induced immunosuppression is a passive one (118). According to this hypothesis, damage to the thymic epithelium by the reaction destroys the maturational microenvironment of the thymus and the production of soluble thymic factors. These changes cause a functional defect in the maturation of T-cells (118,144,145). The consequent loss of T-cell function affects both cell-mediated and humoral immune responses (6,146).

A contributing factor to immunosuppression during GVH reactions may be the interruption of the controversial antisuppressor T cell pathway (191). Experiments demonstrated a decline in production of the serum factor which mediates antisuppression, and that antisuppressor effector T cells became unresponsive to this factor in vitro. The authors suggested that this allows for unopposed immunosuppression in the host.

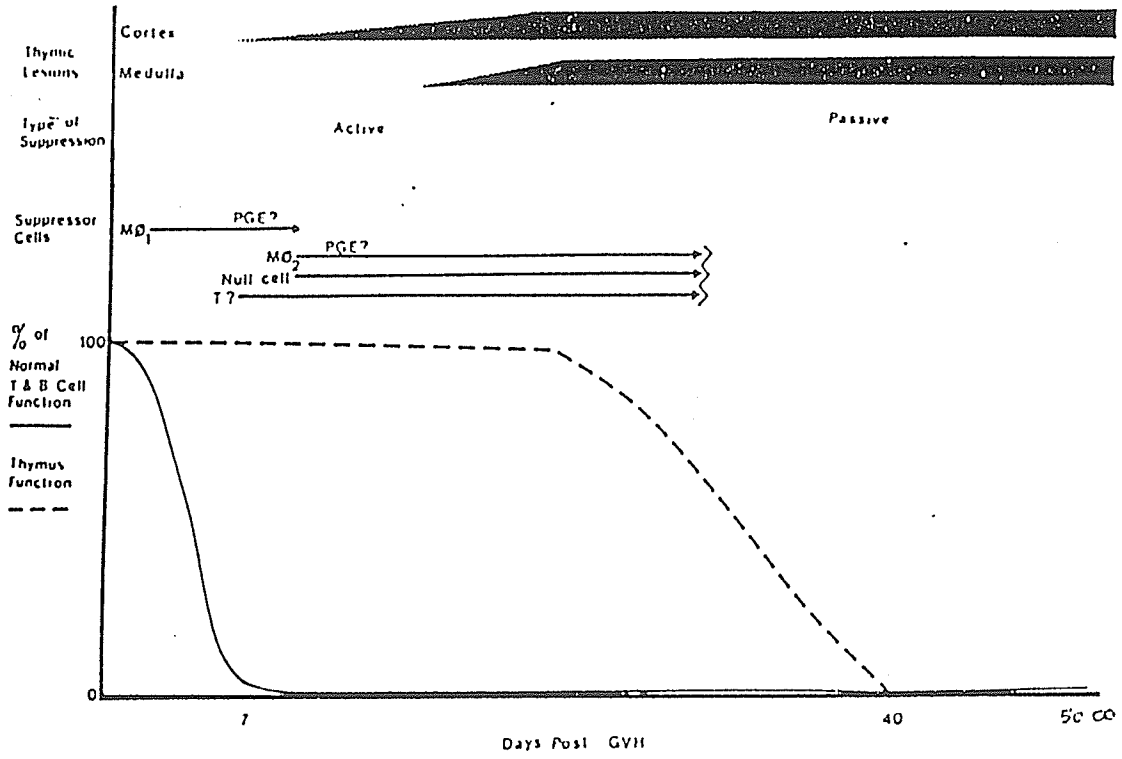


FIGURE 2 Summary of the factors that contribute to the immunosuppressed state of mice that are subject to a GVH reaction.

From Lapp W.S., Ghayur T.G., Mendes M., Seddick M. and T.A. Seemayer. 1985. The functional and histological basis for graft-versus-host induced immunosuppression. Imm. Rev. 88:107.

1.13 T-cell defects in GVH reactions.

GVH reactions directed at both class I and II antigens affect all T-cell pathways and result in failure to elicit CTL's to both self Ia + X and allo-MHC determinants. Class II differences alone produced GVH reactions in which CTL responses initiated through Ia determinants are suppressed while the class I directed T-helper path is still functional. GVH reactions produced by only class I differences appear to have functional T-helper pathways and CTL responses (156).

Allosuppression generated by class I determinants is relatively weak in comparison to the suppression resulting from both class I and II differences (61). The more severe immunosuppression seen in the latter situation is preceded by a phase of allohelp (157,158). The T-helper cell may serve to stimulate cells of the CTL and T-suppressor cell lineage thereby amplifying the suppression.

Moser et al (156) suggest that prior stimulation of specific host T-helper cells may protect them from the suppressive effects of GVH reactions. Immunization of F_1-recipients prior to transplantation using H-2 alloantigens partially prevents GVH-mediated immunosuppression.

The inability of T-cells to produce IL-2 parallels the active and passive suppressive phases of GVH reactions (6,156). There is also decreased expression of IL-2 receptors on T-cells (156). Consequently, GVH reactions cause decreased ability of splenic and thymic derived cell cultures to respond to mitogens and/or IL-2 and/or IL-1 (6). This correlates with thymic lesions (159). In addition, acute GVH reactions induce an Lyt-2,3+ cell to produce an IL-2 inhibitor, postulated to dampen or suppress T-cell activity (160).

1.14 B-cell defects in GVH disease.

B-cell function and maturation is impaired in mice with GVH reactions (147). There is a virtual absence of B- and pre B-cells in the

marrow and a concomitant 10-25 fold decrease in spleen colony forming units. A direct cytotoxic action on pre B-cells may account for the decrease in the production of progenitor cells. Damage to the putative Bursa-equivalent cell in the bone marrow stroma has also been suggested as a mechanism (148,149). This would be similar to the effect of thymic epithelial injury on T cell maturation.

1.15 Prevention and resolution of GVH reactions.

Different pre-treatments of the host and graft prior to transplantation can prevent or reduce the severity of GVH disease. Once established, GVH reactions can be suppressed pharmacologically.

Cytotoxic T-lymphocytes are believed to be a main effector mechanism underlying the GVH reaction. Xenogeneic antithymocyte globulin and prednisone administered to patients with GVH disease have met with some success (165,166,167). Marrow graft pretreatment with anti-Thy monoclonal antibodies and complement to deplete donor T-cells in order to remove potentially reactive mature T-cells has been successful in preventing GVH disease both experimentally and clinically (168,169). Treatment with anti-IL-2 receptor antibody has the effect of reducing the severity of the GVH reaction, presumably by affecting allostimulated T-cells (170). Other protocols to remove T-cells such as counter-current cell separation, magnetic beads or colloids coated with antibody, and ricin conjugated anti-T-cell antibodies have also been developed (reviewed in 51). Rabbit antisera to asialo-GM1 ganglioside, a cell surface marker for activated T-cells and NK-cells has been used experimentally to prevent GVH reactions from occurring in mice (171). Treatment of donor cells with L-leucyl-L-leucine methyl ester , which binds human NK cells, activated T cells, pre-CTL's and monocytes, has also prevented murine graft-versus-host-reactions (199).

Suppressor cells have been identified in many GVH and radiation chimera studies (176-178). In vitro testing of lymphocytes derived from

stable canine chimeras showed some reactivity to host antigens (179). To test whether either clonal deletion or suppressor cells established the chimerism, donor lymphocytes were infused into the stable animals. None of the dogs developed significant GVH reaction symptoms or lesions (180). This finding supports the theory of "active suppression" but does not exclude the possibility of subsequent deletion of the host-reactive clones. In a murine study, activated T-suppressor cells blocked the T-helper cells for antibody formation and the T-helper cells which induced the suppressor cells. This "functional clonal deletion" could still be demonstrated after removal of T-suppressor cells.

The use of the specific non-H-2 determinant Mls for alloimmunization has proved to be the best tolerogen to prevent murine GVH reactions. It can abrogate GVH reactivity in H-2 matched or mismatched inductions (181). Recent work with the Mls loci have identified five alleles which vary in stimulatory capacity when tested by MLC (182,183). The human equivalent has not yet been found.

Pharmacological suppression of GVH reactions have shown variable success. Immunosuppressive therapies administered clinically to "immunosuppress" the GVH reaction include methotrexate, cyclophosphamide, cyclosporin A and corticosteroids (3,51,53,185,186). Experimentally Thalidomide has been employed in a rat model (187). It may hold promise as it is non-lethal and its teratogenicity is irrelevant to the recipient who has been sterilized as a consequence of the pre-treatment irradiation.

1.16 Natural killer cells and their role in GVH reactions.

A. History and Definition.

The observation that lymphocytes derived from unimmunized hosts could lyse tumour cells led to the description of the natural killer (NK) cell phenomenon (192). Since their discovery, NK-cells have been defined as important non-MHC-restricted effectors in the early non-specific natural immune response to foreign antigen. They have been contrasted

with conventional MHC-restricted CTL's which are important effectors in the much later antigen specific adaptive immune response. However, identifying NK-cells by morphology and phenotype has proven difficult, and the definition remains functional, based on their pattern of cytotoxicity (193-198). Studies using NK cells isolated directly from hosts determined that some tumour cell targets are sensitive to NK cell lysis and others are not (203). The tumour cells were described as "NK-sensitive" or "NK-resistant". The classic NK sensitive targets in the mouse and human are YAC-1 and K562 respectively.

Identifying NK cells has been confounded by the observations that many cells of differing origin (i.e. T cells) when generated or propagated in vitro acquired the ability to lyse the "definitive" YAC-1 target (75). Similarly PBL's can be stimulated by lymphokines such as IL-2 to become lymphokine activated killer (LAK) cells, which are capable of lysing a broader range of tumour targets than classical NK cells (75). LAK effectors have been described as a heterogenous population, the majority having markers consistent with NK cells, and a minority with T cells. In fact, non-MHC-restricted killing of tumour targets has been described for several NK, CTL and LAK cell populations (75,204,205,206). LAK cells may represent effector cells for tumour surveillance and have been used clinically as an anti-tumour treatment. The role of non-MHC-restricted CTL's has been related to anti-viral immunity and tumour surveillance (206).

Therefore, the issue of identifying NK cells has been partially clarified, since NK activity has been recognized to be a component of the broader category of non-MHC restricted cytotoxicity (203). This represents essentially a reversal of the original tenet that all non-MHC restricted cytotoxicity was NK killing.

B. Markers.

NK cells are phenotypically heterogeneous. Reports of distinct phenotypic subpopulations of NK-cells may reflect different activation or maturation states of the cell (207-210). Markers associated with murine NK-cells are the IgG Fc receptor, Thy-1.2, Cr3, NK1.1, asialoGM1, NK2.1, Qa2 and Qa5 molecules (209,212,213). Studies comparing markers known to be on NK cells with and those of T cells, monocytes and granulocytes found a high degree of similarity between T cells and NK cells (203).

The identification of NK-cells by phenotype using unique monoclonal antibodies to cell surface antigens (214-216) is not yet undisputed as the NK1.1 and 2.1 molecules are still believed to be shared with T-cells (217). If this can be resolved to show specificity for NK-cells the unique genotypic rearrangement for the markers can be pursued.

C. NK - LGL Association.

Morphological examination of lymphocytes associated with NK-cell activity has revealed that most human, mouse and rat NK-cell activity is concentrated in the large granular lymphocyte (LGL) fraction (218-224). Large granular lymphocytes are distinguished by size (15-20 μm) and the presence of azurophilic granules in the cytoplasm (220,226,227). They are non-adherent and non-phagocytic cells which rapidly develop augmented effector activity in response to IFN, IL-2 and bacterial products (202). Other cell types have been associated with the LGL fraction including killer (K) cells which are responsible for antibody dependent cell cytotoxicity (ADCC). K cells, like NK-cells, have IgG Fc receptors (225).

The anatomical distribution of LGL/NK cells has been determined (223,227). LGL show a different localization and homing pattern than do T-cells. LGL are most abundant in peripheral blood and lung, followed by spleen, peritoneum, and lymph nodes. This quantitative distribution matchs the respective levels of NK-cell activity found in cells isolated

from each organ with the exception of cells isolated from (whole) lung preparations(227).

D. Ontogeny.

The "NK-cell" has yet to be ontogenically defined. Neither a stem cell nor an anatomical site and process of maturation has been clearly identified. A review by Ortaldo and Herberman suggests that NK-cells may have three possible lineages (202). A diagram of the potential origins for NK-cells is shown in Figure 3.

A T-cell lineage of NK cells is strongly suspected but the exact nature of the relationship is unclear. NK-cells have been associated with T-cells by their Thy-1.2 marker and their ability to produce and/or respond to IL-2 (232). However, T3 (CD3) antibodies do not generally lyse LGL's or inhibit NK activity which stands as evidence against the T cell lineage association (198). Some human T leukemias (233) and virally induced CTL's (234) have characteristic LGL morphology. Perforin, the cytolytic factor present in CTL's appears to be almost identical to the cytolysin found in NK/LGL cytoplasmic granules (236). A recent study has identified clones of NK-like cells with T-cell receptor gene rearrangement and expression (237). This contrasts studies of cultured and fresh LGL/NK cells in which no ß-chain rearrangement or expression can be found (238-240).

The link to the monocyte lineage is equally controversial. A study in which human myelomonocytic cells were isolated by plastic adherence found the non-myelomonocytic cells to be predominantly LGL's (241). A second study demonstrated a similar phenomenon, identifying an adherent Leu-11+ NK cell population (200). NK-cells were made adherent in a two step procedure; (1) *in vivo* stimulation with *C. parvum* or interferon followed by (2) *in vitro* stimulation with allogeneic cells or lectins (242,243). These adherent LGL/NK cells appear to share some characteristics with macrophages such as responsiveness to macrophage-activating factor (MAF) and purified recombinant αIFN (241).

The possibility of a third and independent lineage for NK-cells has been suggested, however no clearly supporting evidence can be found (203,204).

NK cells unequivocally seem to originate from a bone marrow stem cell and require a stromal environment in which to mature (229). This was examined through three different approaches. Selective elimination of marrow precursor cells *in vivo* using strontium-89 has resulted in NK activity defects but B, T and macrophage cell functions were unaffected (230). Conversely 17-ß-estradiol treatment to suppress T and B cell marrow precursors caused the NK progenitor ($NK\text{-}1.1^+$) cells to lose their lytic capability (228). The infusion of normal NK progenitor cells (marrow cells) could not restore NK function to 17-ß-estradiol marrow ablated mice (231). Finally, studies transplanting normal B, T, or NK cell marrow progenitors into the mutant C.B-17scid mouse (deficient for functional B and T cells) indicate that NK-cell progenitors are distinct from those of B and T cells (211).

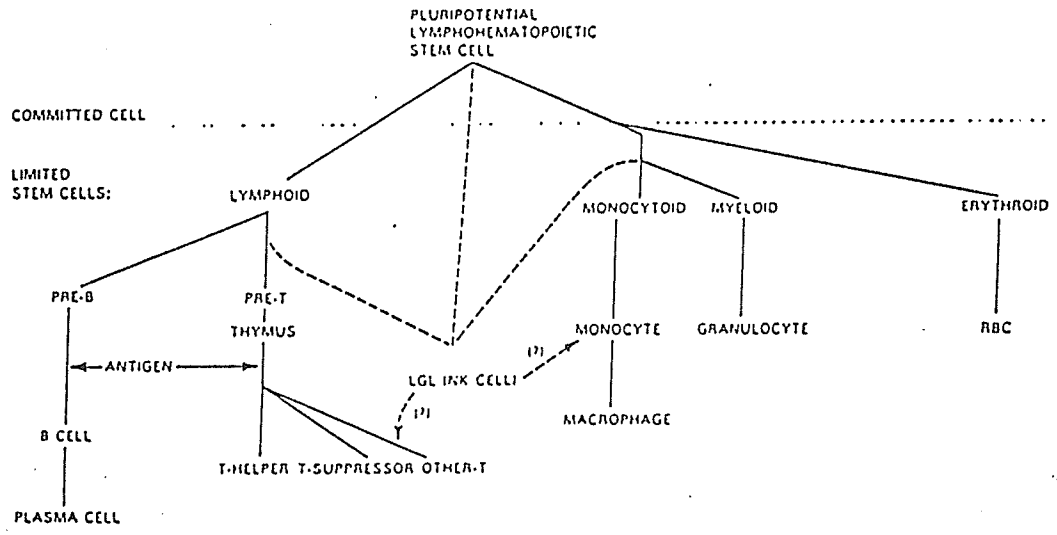


FIGURE 3 Possible alternative lineages of LGL.

From Ortaldo J.R. and R.B. Herberman. 1984. Heterogeneity of Natural Killer cells. Ann. Rev. Immunol. 2:359.

E. NK Regulation.

NK precursors and NK-cells are stimulated by interferon and IL-2. Interferon induces IL-2 receptor expression on NK-cells (60,247). The control by T-cells of NK and NK progenitor cells is not yet determined. NK cells (and non-MHC-restricted T cells) activated by IL-2 have been defined as LAK activity (75). Prostaglandin E_2 acts to suppress non-activated NK-cells and can partially suppress activated NK-cells (60). Figures 4 and 5 show schematic representations of the activation and generation of NK-cells.

F. NK Cytotoxicity.

NK-cell lysis of targets is believed to be predominantly mediated by contact and binding to target structures (244). Non-specific MHC-unrestricted killing is associated with four receptor types: a) CD2; b) $\alpha\beta$ T-cell receptor-CD3; c) $\gamma\delta$ T-cell receptor-CD3; d) CD16 (IgG-Fc receptor) (204).

The NK target structure is still elusive, but once the NK-cell has been activated a second Ca^{++} dependent phase involving microtubule rearrangement (245) and production of inositol triphosphates (246) bind the two cells and mobilize cytolysin granules. The granules exocytose into the intercellular space and deliver the "lethal hit". A second soluble factor secreted by NK-cells is NK cytotoxic factor (NKCF) which may be released upon activation to mediate cell lysis (241). Recent work suggests that NKCF is tumour necrosis factor (TNF) (247).

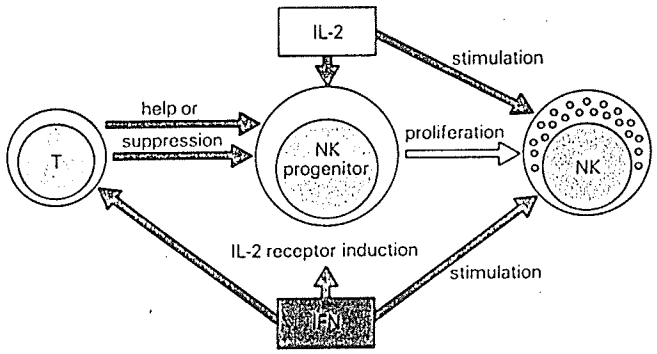


FIGURE 4 A. Schematic representation of IL-2, IFN and T cell regulatory mechanisms influencing NK cell activity. The proliferation of NK cells is under T cell control. IL-2 may also induce proliferation and stimulate the NK activity of proliferating NK cells. Interferon (IFN) induces expression of IL-2 receptors on the NK progenitor, enhancing proliferation, however, IFN also feeds back on T cells to reduce their activity.

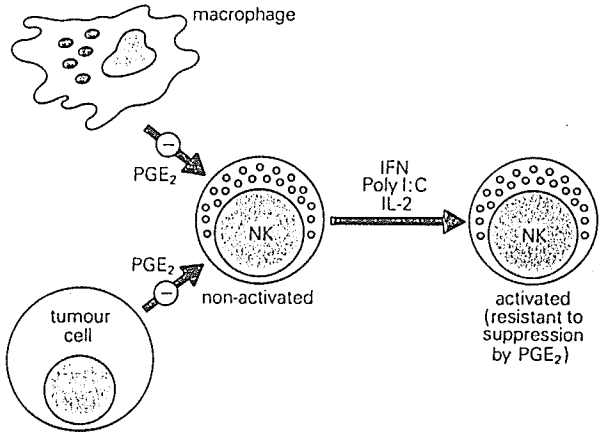


FIGURE 4 B. Involvement of IFN and PGE_2 in the regulation of NK activity. Major sources of PGE_2 are macrophages and certain tumour cells. Suppressor macrophages and tumour produce PGE_2 which suppresses NK activity. However, if NK cells are activated by IFN poly I:C (poly Ionsinic:Cytidylic acid) or IL-2, they become partially resistant to suppression by PGE_2.

From Roitt I., J. Brostoff and D. Male. 1985. Immunology. Published by Gower Medical Publishing, C.V. Mosby Co. St. Louis.

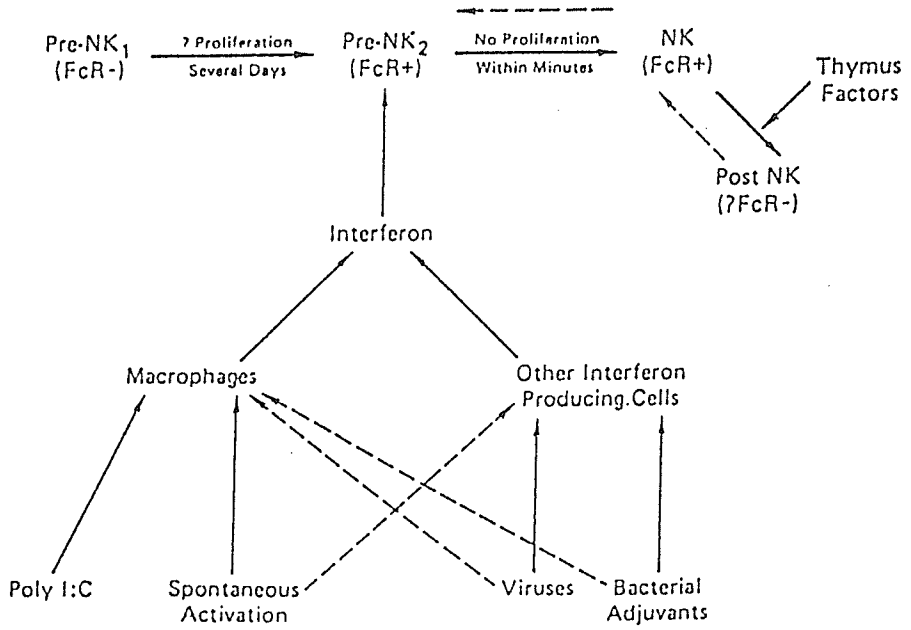


FIGURE 5. Model for generation and activation of NK cells.

From Herberman R.B., Djeu J.Y., Kay D., Ortaldo J.R., Riccardi C., Bonnard G.D., Holden H.T., Fagnami R., Santoni A. and P. Puccetti. 1979. Natural Killer cells: Characteristics and regulation of activity. Imm. Rev. 44:43.

G. NK cells in GVH disease.

NK cell involvement has been investigated in GVH reactions. The studies have not to date identified the exact function of NK cells in GVH reactions, but have described changes in the levels of NK activity. Thymic, splenic, mesenteric lymph node, pooled lymph node and intraepithelially derived NK-effector cells have shown increased NK cytotoxic activity (6,248,249). These findings have been attributed in to both increased interferon and interleukin-2 production which has been shown during the reaction (6). The stimulation of host NK cells prior to GVH induction in the F_1-hybrid model produces F_1-hybrid resistance (251,252). Studies by Ghayur et al correlated liver and pancreas histopathology to increased splenic levels of NK cell activity (155,294). They proposed a causal relationship between NK cells and GVH histopathology. A study by Charley et al (171) supports this hypothesis by demonstrating GVH disease could be prevented by the in vivo treatment of the recipient mice with anti-asialoGM1.

Clinical studies have documented activation of NK-cells in GVH reactions but have failed to elucidate the actual role of NK cells in GVH reactions. The studies indicate that NK cell activity may serve as a prognostic indicator for the reaction. Dokheler et al found that there was a positive correlation between the development of NK activity and the development of GVH reactions following transplantation (253). Elevated NK cytotoxicity for herpes simplex virus type 1 infected fibroblasts prior to transplantation was linked to increased incidence of GVH disease (254). When K562 target cells were used to assay NK activity in a similar study no correlation was demonstrated between the level of NK cytotoxicity and GVH disease (255).

2.0 STATEMENT OF OBJECTIVES AND GENERAL EXPERIMENTAL APPROACHES

The objectives of this project were as follows:

1.) To characterize the change with time in the level of NK-cell activity in lung, lymph node and spleen effector cell populations over the course of GVH reactions.

2.) To localize pulmonary NK-cell activity.

3.) To define the kinetics, level of cytotoxicity and target cell specificity of NK cells over the course of the GVH reaction.

4.) To characterize and compare the cell surface markers of NK effector cells before and during the GVH reaction.

5.) To determine whether NK cells activated during GVH reactions can be separated by their buoyant density characteristics in discontinuous gradients of Percoll.

2.1 INDUCTION AND MONITORING OF GVH REACTIONS.

2.1.1 Experimental design and rationale: The experiments described in this section involved methods to standardize the induction and monitoring of GVH reactions. The methods have been applied to all subsequent experiments reported in this thesis. GVH reactions were monitored by testing immune function and by morphological examination. Specifically, this involved quantitations of plaque-forming cell responses, mitogen-stimulated proliferative capacity, splenomegaly and examinations of histological samples at several time points over the course of the reaction.

2.1.2 General experimental methodology.

GVH reactions were induced by injection of A/J parental lymph node and spleen cells into (C57BL/6 x A/J)F_1-hybrids. In this combination differences at both class I and II loci of the MHC were present. This model resulted in an acute GVH reaction. With it resulting changes could be directly attributed to the GVH reaction, and not to irradiation or other pretreatment regimens used to prepare the host for transplantation.

A. Animals: Female A/J (H-2^a) and (C57BL/6 x A/J)F_1-hybrid (H-$2^{b/a}$) mice 6-8 weeks of age were obtained from the Jackson Laboratory, Bar Harbour, Maine. All mice were 12-14 weeks old when they were used.

B. Media and reagents: Balanced salt solutions (HBSS) were prepared from individual salts using the formulation of Hanks and Wallace (256) to which 27.5 mM TRIS-buffer was added. The solution was adjusted to pH 7.2 with HCl. Phosphate buffered saline (PBS) was made in 10X concentrated form (257). Complete RPMI-1640 medium was prepared with 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mg/ml $NaHCO_3$, 1 mM sodium pyruvate, 2mM L-glutamine, supplemented with 10% heat-inactivated fetal calf serum (all from GIBCO, Grand Island, New York) and adjusted to pH 7.3 with HCl. Concanavalin A (ConA), Phytohemagglutinin (PHA), (both from Pharmacia

Fine Chemicals, Dorval, Quebec) and lipopolysaccharide (LPS) (Sigma, St. Louis, Missouri) were used at final concentrations of 0.5, 0.5 and 1.0 μg/ml of complete RPMI-1640, respectively. Lyophilized guinea-pig complement was dissolved in restoring solution (both from GIBCO) and used at a 1:20 dilution in HBSS.

C. Induction of GVH reactions: Acute GVH reactions were induced by the injection of A/J strain lymph node and spleen cells into (C57BL/6 x A/J)F_1-hybrid mice. A single-cell suspension of A/J-strain lymph node and spleen cells was prepared by pressing the tissue through a 60 mesh stainless steel wire screen with a spatula as described by Billingham and Silvers (258). The suspension was then washed twice in HBSS and filtered through a layer of gauze to remove tissue clumps. Viable cells were counted by trypan blue exclusion and the suspension was adjusted to 1.66×10^8 cells/ml. F_1-hybrid mice were injected via the tail vein with 5×10^7 donor cells in a volume of 0.3 ml.

D. Control mice: Age and sex matched normal uninjected (C57BL/6 x A/J)F_1-hybrid mice were used as control animals in this study. Previous studies from Dr. Gartner's laboratory showed that mice injected with syngeneic cells to determine the effects of the GVH induction protocol showed none of the changes seen in mice with GVH reactions and were in all respects identical to uninjected controls (259). Therefore, only uninjected controls were used in these experiments for economic considerations.

E. Monitoring of GVH reactions: Splenomegaly, immunosuppression and diminished mitogen responsiveness served to verify the presence of GVH reactions. A histopathologic survey of tissues of experimental mice was performed to identify characteristic histopathological lesions of GVH reactions. These determinations were made on several days after the reaction was induced.

a.) **Splenomegaly:** The spleen index (SpI) was calculated as:

$$\text{Sp I} = \frac{\text{spleen weight (exp.)}}{\text{total body weight (exp.)}} \Big/ \frac{\text{spleen weight (con.)}}{\text{total body weight(con.)}}$$

Mice undergoing GVH reactions lose weight. Around day 20 body weights of experimental animals may be 3-5% lower than control. Animals experiencing particularly vigorous reactions may have terminal body weights 20% lower than control. A spleen index of at least 1.3 has been suggested as a minimum indicator for the presence of GVH reactions to allow for weight loss and the effect of the large intravenous infusion (33).

b.) Immunosuppression: This was measured in vitro using the direct splenic hemolytic plaque-forming cell (PFC) response to sheep red blood cells (SRBC) in diffusion cultures. A modification of the method described by Marbrook was used (260). A sterile single-cell suspension of 2 x 10^7 viable spleen cells/ml in complete RPMI-1640 was prepared from individual unprimed mice as described previously. Washed SRBC (National Biological Lab. Ltd., Dugald, Manitoba) at a concentration of 1 x 10^7 per ml were added to an aliquot of each cell suspension. Six cultures; three with SRBC, three with an equivalent volume of saline, were established from each experimental and control mouse. The cultures were maintained in Marbrook diffusion chambers. After four days incubation at 37°C in 5% CO_2 in air, the direct hemolytic PFC response for each culture was determined using the method described by Cunningham and Szenberg (261). For each spleen cell culture a mixture consisting of 0.5 ml diluted guinea-pig complement, 0.075 ml of SRBC in saline at a 1:5 dilution of the packed-cell volume was prepared, to which 0.10 ml of spleen cell suspension was added. This reaction mixture was pipetted into 4 Cunningham chambers which were sealed with paraffin and incubated at 37°C for 1 hour. The total number of hemolytic plaques was counted and multiplied by 10 to obtain the total number of PFC per culture. The mean number of PFC per animal was calculated by subtracting the mean number of spontaneous PFC in the cultures with saline from the mean number of PFC in cultures which contained SRBC.

c.) **Mitogen responses:** Responses to Con A, PHA, and LPS were determined as follows. Sterile single-cell suspensions in complete RPMI-1640 were made from the spleens of individual mice. One million cells in 250 1 were plated per well, in triplicate cultures, into 96-well U-bottom microtitre plates with mitogen or complete media alone and incubated at 37°C in 5% CO_2 in humidified air. After 48 hours, 1 μCi of ^{3}H-thymidine (Amersham, Oakville, Ontario) was added to each well. Fifteen hours later the cells were harvested onto glass fiber filters with a Skatron cell-harvester. The filters were then dried, placed in vials containing 3 ml of scintillation fluid, and counted in a Beckman liquid scintillation counter. The ConA, PHA and LPS responses from individual mice were expressed as stimulation indeces (SI) calculated as follows:

$$SI = \frac{\text{DPM with mitogen in culture}}{\text{DPM without mitogen in culture}}$$

d.) **Histological survey:** Samples of lung, liver, thymus, and salivary glands were removed. The tissue was fixed in 10% formalin and processed in glycol methacrylate. Histologic sections of 0.75 μm were cut from each tissue block and stained with hematoxylin and eosin.

2.1.3 RESULTS

A.) Immunosuppression and mitogen responsiveness: Table 1 shows data from a series of plaque-forming cell experiments on days 3, 7, 11, 18, 24 and 30 after induction demonstrating that mice with acute GVH reactions became completely and permanently immunosuppressed by day 18 of the reaction. Table 2 shows that there was a concomitant decrease in mitogen responsiveness to Con A, PHA, or LPS. Mice with GVH reactions developed a 3 to 4 fold increase in the spleen index by day 11. Maximal splenomegaly was accompanied by necrotic regions in the spleen as seen grossly. Splenomegaly and suppression of immune and mitogen responses are consistent features of GVH reactions in F_1-hybrid hosts (6).

B.) Histology: The histological survey revealed the presence of infiltrates of small-to-medium size mononuclear cells in lung, liver and salivary glands as the reaction progressed. Figure 10 illustrates these infiltrates in the lung. Figure 11 illustrates similar cellular ductal and periductal infiltrates in the liver. Periportal necrosis was also seen in some animals. Figure 12 demonstrates a loss of thymic architecture. Notable destruction of the cortico-medullary junction with a decrease in Hassal's corpusules accompanied by a general hypocellularity was seen. Figure 13 indicates the presence of mononuclear cell infiltration around ducts in salivary gland tissue.

C.) Mortality and clinical signs of GVH disease: Mice followed for mortality from GVH disease typically showed wasting by day 20 and developed diarrhea by day 25. These late-stage animals had a hunched posture and flaccid tails. All died of GVH disease by day 35.

The results of these experiments to monitor to GVH reaction indicated the appropriateness of the experimental methods used to induce the reactions.

TABLE 1

DATA VERIFYING THE PRESENCE OF GVH REACTION IN F_1-HYBRID MICE

Days Post Induction*	PFC Response (Mean ± SE) Experimental	P	Control	P	Spleen Index
Control	1192±119		1139±62		1.0
3	735±62	<.05	1475±105	>.05	1.6
7	325±11	<.002	1052±60	>.1	2.9
11	35±12	<.0001	1170±111	>.1	3.7
18	0	<.0001	1275±131	>.1	4.1
24	ND		ND		3.6
30	ND		ND		2.0

* 3 mice per group.

P-values were calculated using Student's T- test. Post induction groups were compared to the Control group within each column.

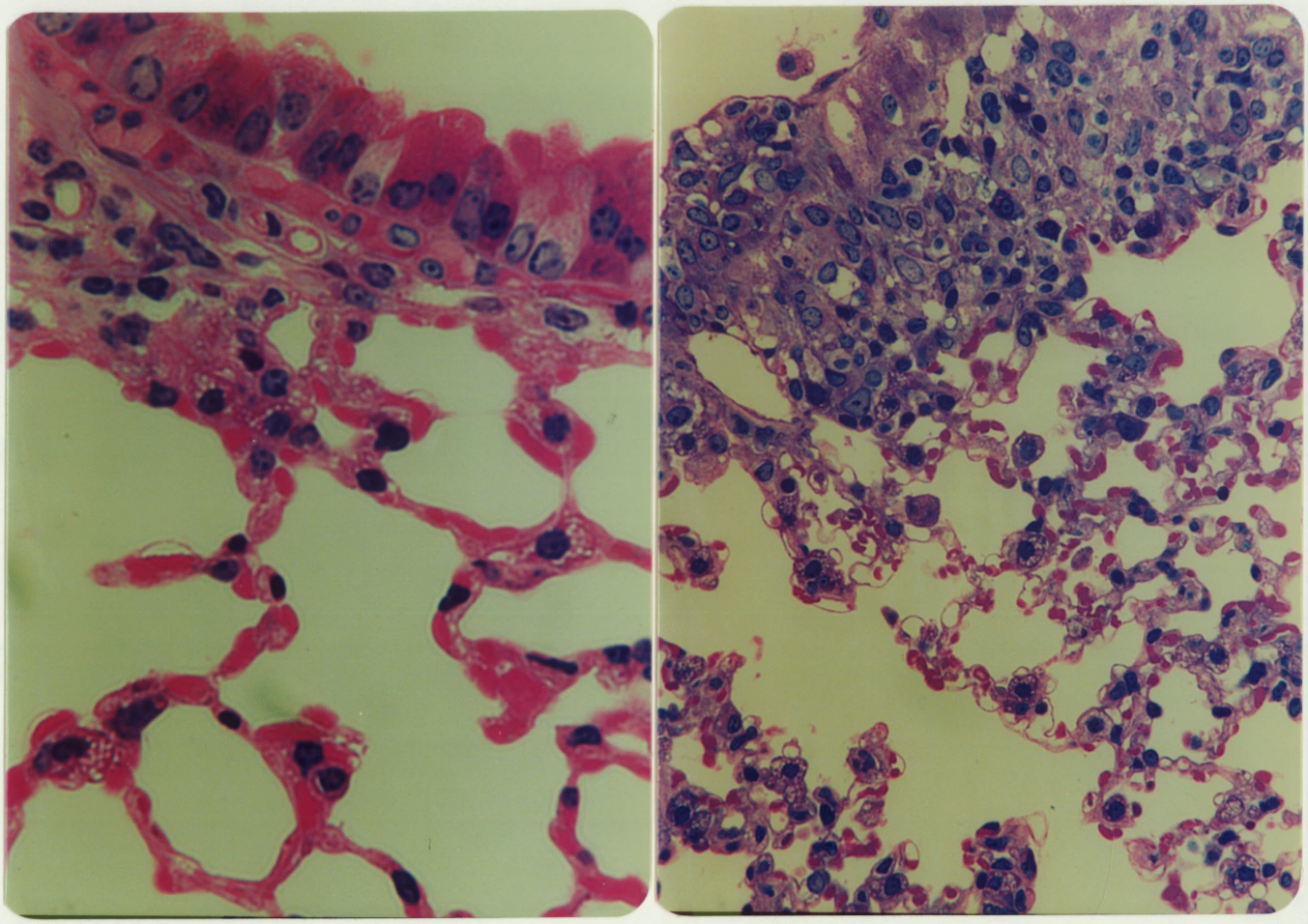
TABLE 2

DATA VERIFYING THE PRESENCE OF GVH REACTION IN F_1-HYBRID MICE

Days Post Induction*	Mitogen Response (Mean ± SE)					
	Con A	P	PHA	P	LPS	P
Control	43.8±8.1		20.7±2.1		42.9±3.0	
3	15.9±0.3	<.05	21.1±0.1	>.1	21.1±0.1	<.002
7	13.3±3.7	<.05	5.6±2.4	<.01	9.3±2.1	<.001
11	1.0±0.1	<.01	1.4±0.5	<.001	1.0±0.1	<.001
18	1.0±0.1	<.01	1.2±0.2	<.001	1.0±0.1	<.001
24	1.2±0.3	<.01	0.8±0.4	<.001	0.9±0.2	<.001
30	0.8±0.9	<.01	1.1±0.4	<.001	1.0±0.2	<.001

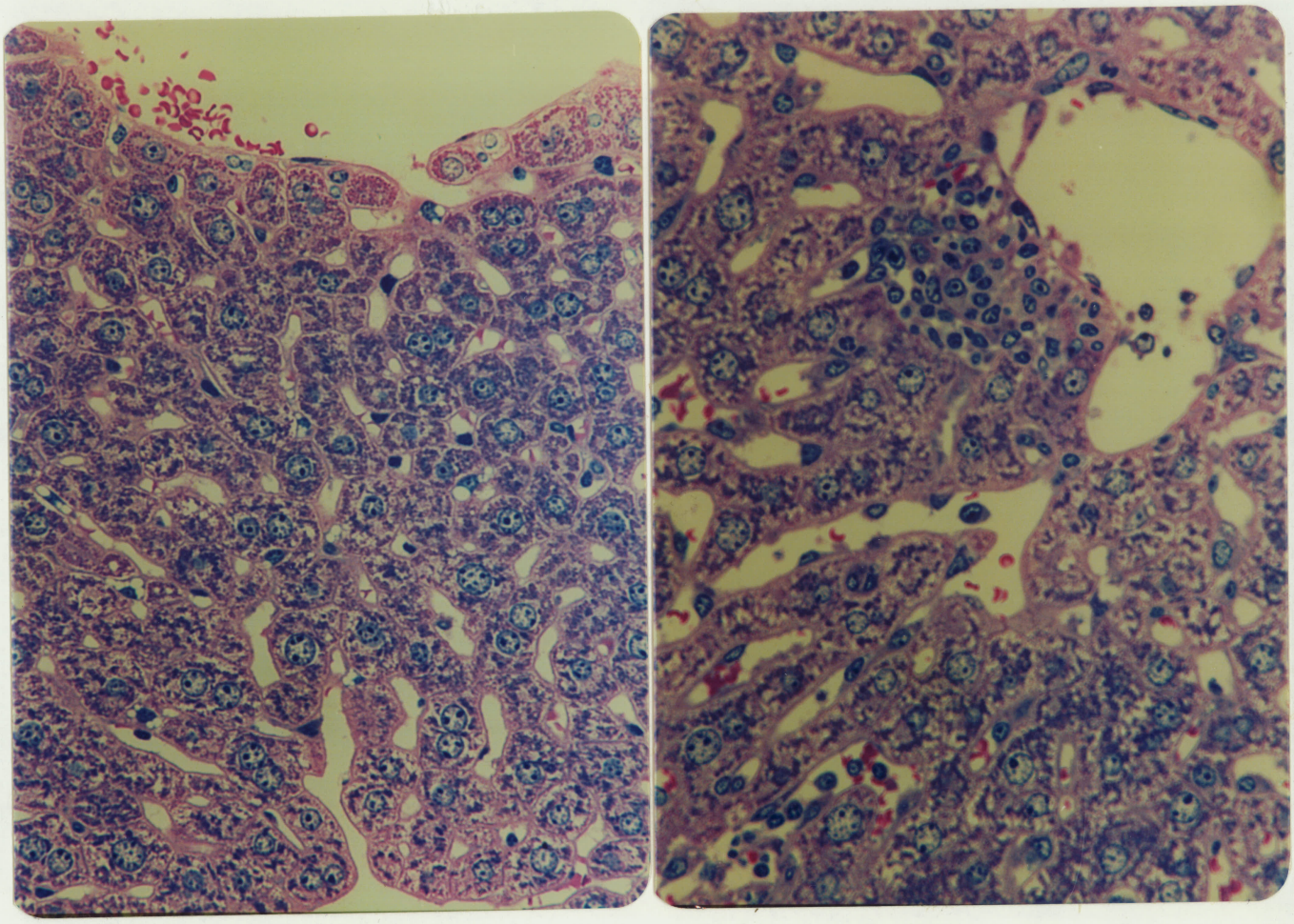
* 3 mice per group.

P-values were calculated with Student's T-test. Post induction groups were compared to the Control group within each column.



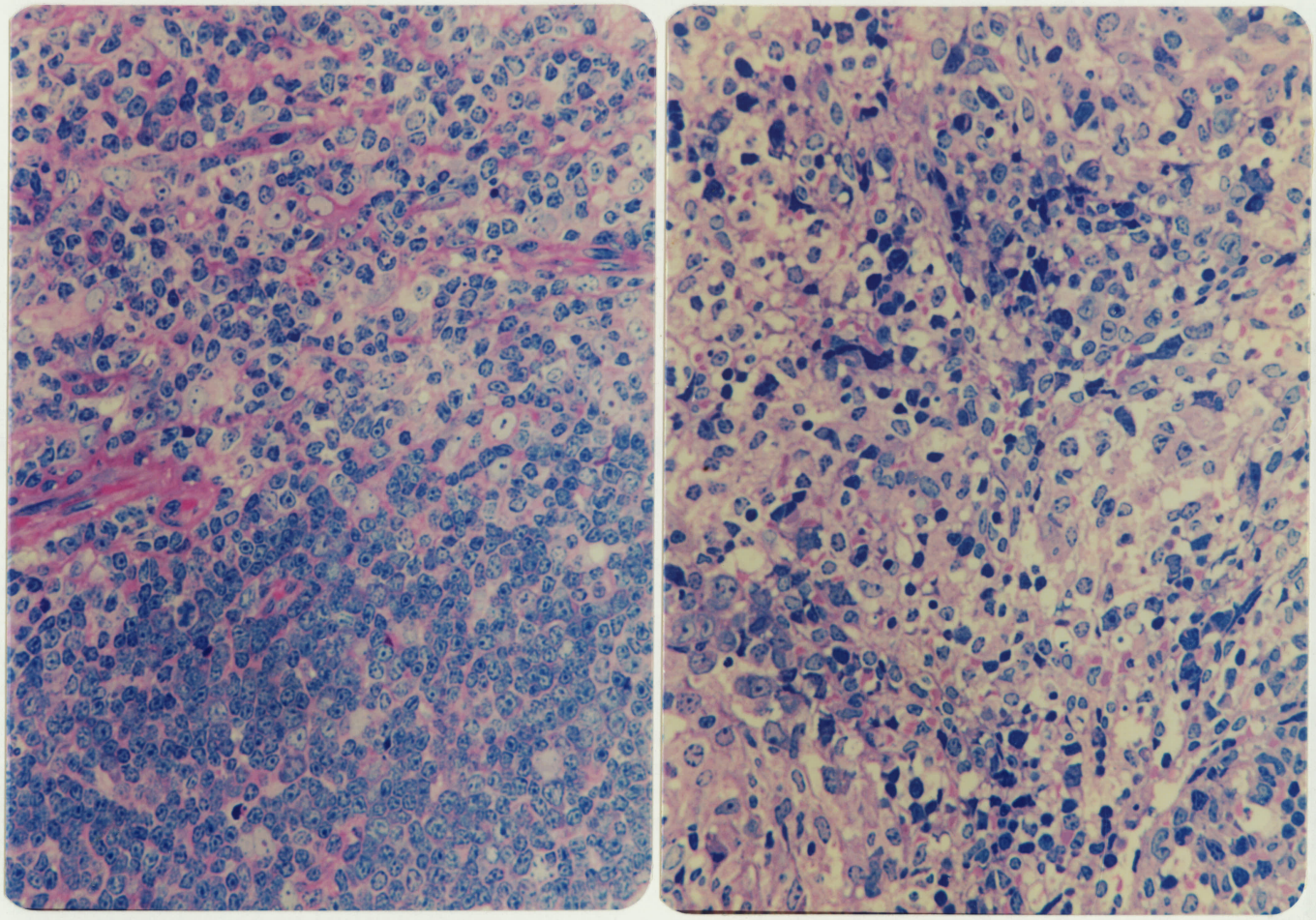
CONTROL GVH

FIGURE 10 Photographs of pulmonary GVH histopathology showing mononuclear cell infiltration and loss of architecture.



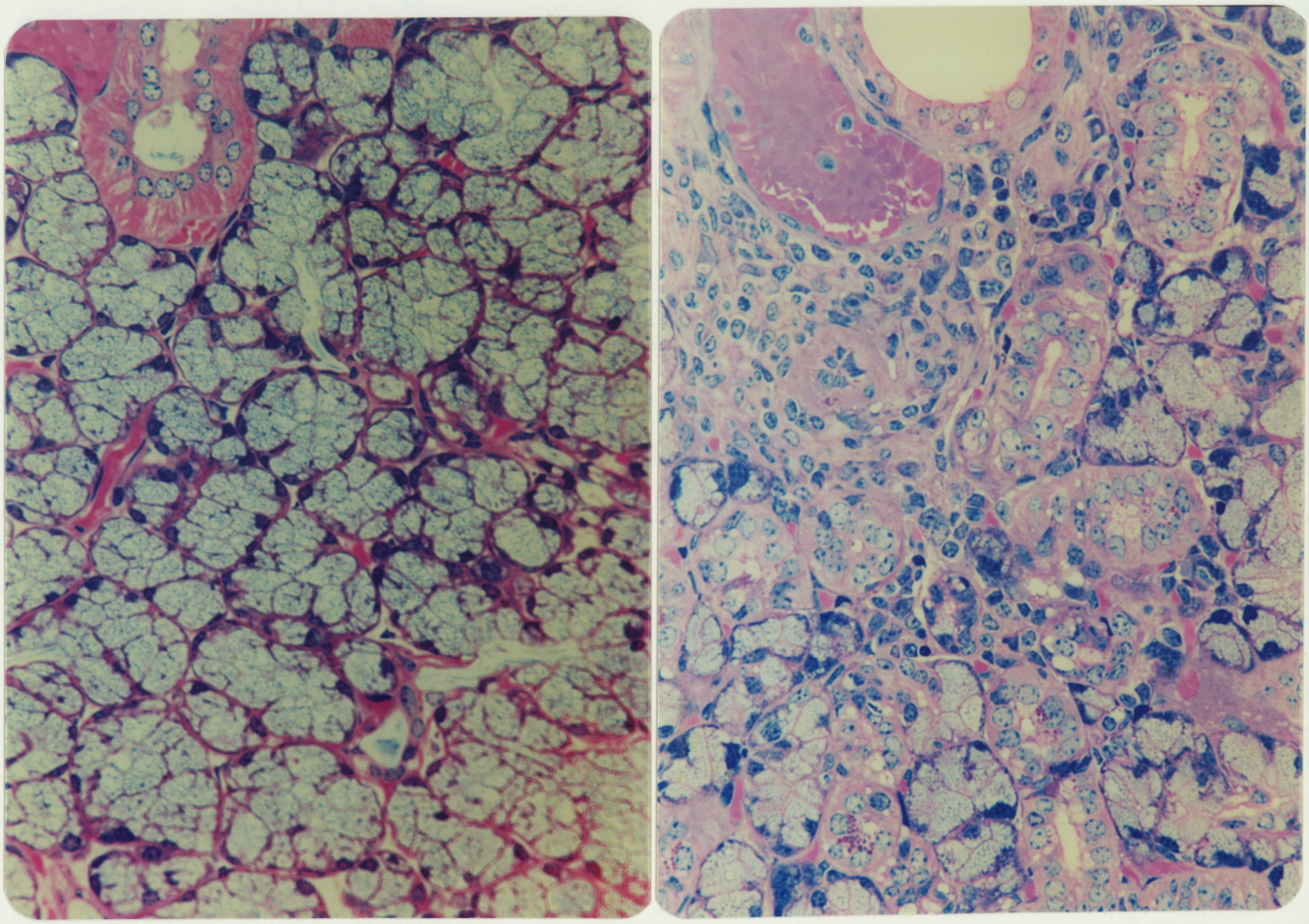
CONTROL GVH

FIGURE 11 Photographs of hepatic GVH histopathology showing ductal and periductal mononuclear cell infiltration.



CONTROL GVH

FIGURE 12 Photographs of thymic GVH histopathology showing a loss of architecture and hypocellularity.



CONTROL GVH

FIGURE 13 Photographs of salivary gland GVH histopathology showing the presence of mononuclear cell infiltration.

3.0 SPECIFIC EXPERIMENTS PERFORMED

This study was done in five parts. The original observation of changes in NK-cell activity in lung, lymph nodes and spleen as a consequence of the GVH reaction are described in Ssection 3.1. Studies to localize pulmonary NK-cell activity are reported in Section 3.2. The phenotypic characterization of the cells responsible for the NK-activity seen during the GVH reaction are reported in Section 3.3. Experiments that attempted to separate NK-effector cells over a discontinuous Percoll density gradient to determine the fraction of cells containing anti-YAC-1 and anti-P815 lytic NK-cells are described in Section 3.4. Finally, cold target inhibition experiments were done to determine whether broadened tumour cell lytic activity was mediated by one or more cells. These experiments are described in Section 3.5.

3.1 EXAMINATION OF CHANGES IN NK-CELL ACTIVITY IN THE LUNG, LYMPH NODES AND SPLEEN OVER THE COURSE OF THE ACUTE GVH REACTION IN F_1-HYBRID MICE.

3.1.1 Experimental design and rationale: The lungs are particularly vulnerable to injury during GVH reactions. Aside from infections, destructive lesions, cellular infiltrates and fibrosis are associated with GVH disease in BMT patients (119-122,255,162-164,172-174).

Because NK cells have been suggested as possible mediators of injury during GVH reactions, changes in NK-cell activity with time were measured in the lung. Groups of mice were sacrificed on days 3, 7, 11, 18, 24 and 30 after the reaction was induced. The NK activity was assayed in cells isolated from lungs, lymph node and spleen.

3.1.2 Specific methodology.

A. Tumour target cell lines: The YAC-1 Moloney virus-induced lymphoma cell line ($H\text{-}2^a$), P815 methyl chloranthrene-induced mastocytoma cell line ($H\text{-}2^d$) and human K562 chronic myelogenous leukemia cells were gifts from Dr. Arnold Greenberg. YAC-1 and P815 cells were also obtained from ATCC (Rockville, Maryland). The cells were maintained in continuous batch cultures in complete RPMI-1640 at 37°C in 5% CO_2 in humidified air.

B. Isolation of NK-effector cells: In each experiment mice were randomly selected from control and GVH groups of animals. Mice were sacrificed by cervical dislocation. The spleen, lymph nodes and lungs were removed. A small amount of hilar lung tissue and any visible hilar lymph nodes were dissected from each lung. Spleens and lymph nodes from within each group were pooled and rendered into single-cell suspensions using 60 mesh stainless steel screens and spatulas by the method already described (258). Lungs from each group were pooled and then dissociated into single cell suspensions using a modification of the method described by Stein-Streilein *et al* (262). Lung tissue was minced finely with a razor blade,

suspended in collagenase solution at a ratio of 1 lung per 5 ml of solution, and incubated for 90 min. at 37°C in 5% CO_2 in air with continuous stirring. Collagenase (Sigma) solutions were prepared by dissolving 24 units/ml in RPMI-1640 containing 5mM $CaCl_2$ and adjusted to pH 7.6. The digested lung tissue was pressed through a 60 mesh stainless steel wire screen with a spatula to obtain a single-cell suspension, which was washed in complete media. Lung, lymph node and spleen cell suspensions were then poured into nylon wool (Fenwal Lab, division of Travenol, Deerfield, Illinois) columns and incubated for 1 hr. at 37°C in 5% CO_2 in humidified air. Nylon wool columns extract most B-cells and macrophages leaving predominantly T- and NK-cells in the eluate (263). Non-adherent cells were eluted with 2-3 column volumes of complete medium. The eluted cell suspensions were then washed, concentrated and layered onto Lympholyte M (Cedarlane) density gradients. The gradients were centrifuged at 400 x g for 25 min. The cells at the interface were collected and washed twice in HBSS. These techniques yielded approximately 1.0-2.5 x 10^6, 2-10 x 10^6 and 80-200 x 10^6 effector cells per animal from the lung, lymph nodes and spleen respectively.

C. NK-cell assay: This assay is an adaptation of the standard ^{51}Cr release assay described by Grabstein et al (266). Appropriate numbers of YAC-1 and P815 tumour cell targets were labelled with $Na_2{}^{51}CrO_4$ (Amersham, Oakville, Ontario) at a dose of 50 μCI per 1 x 10^6 cells. Labelling was carried out by incubating the tumour cells for 60-75 min. in a shaking water bath at 37°C followed by three washes in HBSS. The labelled cells were resuspended in complete RPMI-1640 at a final concentration of 1 x 10^5 cells/ml for use in the assay. One hundred μl of labelled target cell suspension (1 x 10^4 cells) was plated into each well of 96-well plastic V-bottom microtitre plates with varying numbers of pre-treated lung, lymph node or spleen effector cells at four effector to target (E:T) cell ratios (100:1, 50:1, 25:1 and 12:1). Cells in each ratio were plated in triplicate. The plates were centrifuged at 15 x g for 5 min and incubated

for 4 hr. at 37°C in 5% CO_2 in humidified air. Following incubation the plates were centrifuged for 10 min at 200 x g and 100 µl of supernatant was harvested from each well. Supernatants were counted for 2 min in an LKB gamma counter. The percent lysis was calculated as:

$$\% \text{ lysis} = \frac{\text{CPM (exp.)} - \text{CPM (spont.)}}{\text{CPM (max.)} - \text{CPM (spont.)}} \times 100$$

The mean percent lysis and standard error of each triplicate was determined. Experiments were repeated a minimum of three times. The spontaneous release in all experiments never exceeded 10% of the maximum ^{51}Cr releasable.

3.1.3 RESULTS

Changes in NK-cell activity during GVH reactions in lung, lymph node and splenic effector cells: Changes in NK-cell activity were observed with time in the lung, lymph nodes and spleen over the course of the GVH reaction. These changes are shown in Figures 6 A and B. Low levels of NK-cell cytotoxicity to YAC-1 tumour cells were observed in both the lung and spleen of control mice. Control lymph nodes had very low to non-existent levels of NK-cell activity. Killing of "NK-resistant" P815 tumour cells was not seen in control mice.

In the early part of the GVH reaction, splenic cytotoxicity to YAC-1 tumour cell targets increased transiently to greater than control values (Figure 6 A). This activity peaked between days 3 to 5 and then decreased to control or slightly below control levels by day 11. The de novo ability of spleen cells to lyse P815 targets was first observed on day 5 of the GVH reaction. This anti-P815 cytotoxicity reached a maximum on day 11, and then diminished. The lysis of P815 tumour cells, like that of the YAC-1 targets, remained at low levels for the remainder of the reaction (Figure 6 B).

Anti-YAC-1 NK-cell activity in the lung showed a much greater increase. Lytic activity against YAC-1 tumour cell targets reached a

maximum of 45% lysis (E:T ratio at 50:1) on day 7 and then declined slowly to return to control values by day 30 (Figure 6 A). In the lung, cytotoxicity to P815 tumour cells reached a plateau of 13% lysis on day 7 and then declined rapidly between days 20 and 30 (Figure 6 B).

Lymph node NK-cell activity to YAC-1 tumour cell targets rose above control levels more slowly than either splenic or pulmonary NK-cell activity. It peaked by day 11 at levels of 20% lysis, and gradually declined to zero by the end of the reaction. NK-cell activity to P815 tumour cells developed in parallel to the increase of anti-YAC-1 NK-cell activity.

In contrast to the transient increase in the activity of splenic NK-effector cells early in the course of the GVH reaction, the increased level of lymph node NK-cell activity was sustained for the major portion of the GVH reaction, similarly the pulmonary NK-cell response was sustained almost over the entire course of the reaction. The de novo anti-P815 NK-cell activity appeared in all organs on day 5 and remained elevated above control values over most of the reaction.

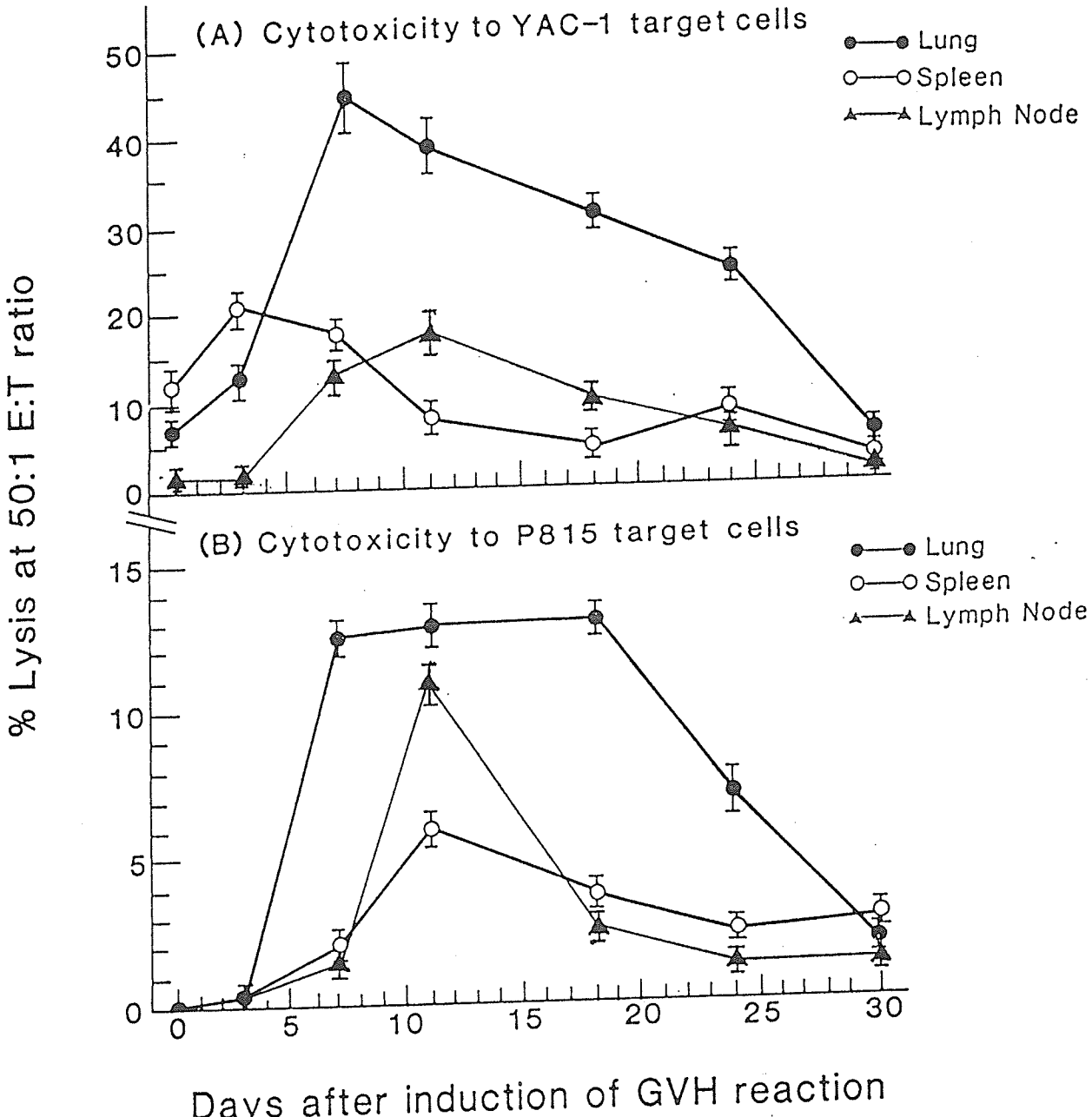


FIGURE 6 Kinetics of NK activity during acute A -> (C57/6 x A/J)F_1-hybrid GVHR. A. Cytotoxicity of NK effector cells isolated from the lung, lymph nodes and spleen to YAC-1 target cells. B. Cytotoxicity of NK effector cells from the lung, lymph nodes and spllen to P815 target cells.

3.2 INVESTIGATIONS TO DETERMINE THE ANATOMICAL LOCALIZATION OF PULMONARY NK-CELL ACTIVITY.

3.2.1 Experimental design and rationale: Results from experiments described in the previous section showed a marked increase in NK-cell activity in the lung, a non-lymphoid organ. Experiments were conducted to determine in which lung compartment this activity was localized. In a series of experiments, groups of control mice and mice with GVH reactions were treated with bronchoalveolar lavage (BAL), pulmonary vascular perfusion, or a combination of BAL and pulmonary vascular perfusion to determine whether these pre-treatments could deplete NK-cell activity from the lung. Depletion of NK-cell activity by pulmonary vasculature perfusion would suggest that circulating lymphocytes were responsible for lung NK-cell activity. Removal of NK activity by BAL would suggest that the activity was associated with the intra-alveolar monocyte population. If these combined pretreatments failed to deplete NK activity from the lung then it could be reasonably concluded that the activity was associated with cells in the pulmonary interstitium which has a rich population of resident LGL cells (223,227).

3.2.2 Specific material and methods.

Bronchoalveolar lavage (BAL) and pulmonary vascular perfusion: BAL was performed by washing the lungs 4 times with 1 ml saline through a tracheotomy. The lavage fluid was collected. The pulmonary vasculature was perfused with 2 ml saline injected into the inferior vena cava or pulmonary artery until the lungs were completely blanched. Effector cells were removed from treated lungs as described in section 4.1.2 B. NK-cell activity was measured in each of the treatment groups and in the fraction of cells lavaged from the lung.

3.2.3 RESULTS

Effect of BAL and pulmonary vascular perfusion on pulmonary NK-cell activity: BAL, lung perfusion and a combination of BAL and lung perfusion were performed on groups of normal mice, and mice with GVH reactions to determine whether these treatments could deplete NK-cell activity from the lung. Results obtained on each day of the reaction studied were identical and demonstrated that none of the treatments reduced NK-cell activity in the effector cells isolated from the lung. Table 3 shows data from normal mice and days 7 and 11 of the GVH reaction. Neither the individual treatments nor their combination had any significant effect upon the level of pulmonary NK-cell activity. The level of NK-cell activity in the lavaged cell fraction is shown. It was negligible when compared with the activity of effector cells isolated from the lung parenchyma (cells left after BAL and perfusion treatment) by collagenase digestion.

TABLE 3

EFFECT OF BRONCHOALVEOLAR LAVAGE AND PULMONARY VASCULAR PERFUSION ON PULMONARY NK-CELL ACTIVITY

Treatment	YAC-1 Targets*					
	Control	P	Day 7	P	Day 11	P
NONE	6.9±0.6		30.3±4.4		25.3±3.0	
BAL and Perfusion	7.9±0.5	>.1	44.0±2.8	>.05	32.6±1.7	>.1
BAL alone	7.2±1.0	>.1	35.0±0.6	>.1	30.2±1.2	>.05
Perfusion alone	6.2±0.7	>.05	33.7±1.4	>.05	26.7±0.9	>.1
Lavaged cells	1.0±0.4	<.001	3.3±0.7	<.01	2.8±0.2	<.002
	P815 Targets*					
	Control	P	Day 7	P	Day 11	P
NONE	0		1.9±0.5		8.5±1.0	
BAL and Perfusion	0		2.7±0.2	>.1	9.3±0.1	>.1
BAL and Perfusion	0		1.6±0.1	>.1	5.8±1.0	>.1
Perfusion alone						
Lavaged cells	0		0.4±0.1	<.05	0.1±0.3	<.002

* % lysis at 25:1 E:T ratio.

P-values were calculated with Student's T-test. Treatment groups were compared to the no treatment group within each target/day group.

3.3 CHARACTERIZATION OF THE PHENOTYPES OF THE NK OR NK-LIKE EFFECTOR CELLS ACTIVATED DURING THE GVH REACTION.

3.3.1 Experimental design and rationale: The cell surface markers of NK effector cells removed from lung, lymph nodes and spleen were determined by negative selection made using cytotoxic monoclonal antibodies or antisera and complement.

The following antibodies or antisera were used; anti-Thy-1.2, anti-Lyt-1.1, anti-L3T4, anti-Lyt-2.2 and anti-asialo-GM1. The Thy-1.2 monoclonal antibody, a pan T-cell marker, was chosen to deplete T-cells and some NK-cells. Monoclonal antibodies to the surface proteins characteristic for T-helper (Lyt-1.1 and L3T4) and cytotoxic T-cells (Lyt-2.2) were used to determine whether a T-cell response was responsible for any of the NK-activity observed during the GVH reaction. Anti-asialo-GM1 antiserum was used to confirm the presence of the asialo-GM1 cell surface antigen believed to be a universal murine NK-cell marker (203). Asialo-GM1 is also present on activated T-cells, but it was selected because it is the only antibody that can be used to deplete murine "NK-cells".

3.3.2 Specific material and methods.

Effector cell pre-treatments.

a.) Monoclonal antibodies or antisera and complement:

Monoclonal antibodies to Thy-1.2 (NEN-001, IgM), Lyt-1.2 (NEN-017, IgG_{2b}), Lyt-2.2 (NEN, IgM) cell surface antigen markers were obtained from New England Nuclear (Lachine, Que) and were used at dilutions of 1:100, 1:40, and 1:100, respectively. A second monoclonal antibody anti-Lyt2.2 (CL8992,IgM, AD4(15)) obtained from Cedarlane Laboratories (Hornby, Ont) was also used at 1:100 dilution. Anti-asialoGM1 (anti-ASGM1) rabbit antiserum obtained from Wako Fine Chemicals (Dallas, TX) was employed at a dilution of 1:100. Monoclonal antibody anti-L3T4 (IgG_{2b}) from clone YTS

191.1 (Cedarlane) was employed at a dilution of 1:500. These dilutions were chosen from the efficiency curves supplied by the manufacturers. Asialo-GM1 antisera, anti-L3T4 (YTS 191) and Thy-1.2 were all tested for cytotoxicity on Con A stimulated thymocytes using the method of Shiigi et al (281). Anti-Lyt-1.2, Lyt-2.2, L3T4 and Thy-1.2 were tested for cytotoxicity using flow cytometry to verify depletion of appropriately labelled thymus cell preparations. Thymocytes prepared for analysis of specific florescence were resuspended at 1 x 10^6 cells/ml for 1 hr. at 4°C with fluorescein isothyocyanate (FITC) conjugated anti-Thy-1.2 (Becton-Dickinson, cat. #1333, clone 30-H12), Lyt-1 (B-D, cat. #1343, clone 53.7.3), Lyt-2 (B-D, cat. #1353, clone 53-6.7), or phycoerythrin (PE) conjugated anti-L3T4 (B-D, cat.#1447, clone GK1.5) (all at 1:10 dilution) and washed prior to sampling. Low tox M rabbit complement (Cedarlane) was absorbed on agar Noble (Difco, Detroit, Michigan) (264) and diluted to 1:10 in Cytotoxicity Medium (Cedarlane).

Negative selection of subpopulations of effector cells by cell surface markers was carried out with specific monoclonal antibodies or antisera and complement as described by Henry (265). Aliquots of isolated effector cells at a concentration of 1 x 10^7 were incubated with anti-Thy-1.2, anti-Lyt-1.1, anti-Lyt-2.2, anti-L3T4(YTS 191.1), anti-ASGM1, or HBSS for 1 hr. at 4°C, washed, resuspended in an equivalent volume of rabbit complement and incubated for 1 hr. at 37°C in 5% CO_2 in humidified air. The cells were then washed and resuspended in complete RPMI-1640 for use in the NK-cell assay.

3.3.3 RESULTS.

A. Verification of the lytic capability of cytotoxic antibodies and antisera.

Verification of cytotoxic capability was carried out for some of the monoclonal antibodies. When tested with ^{51}Chromium-labelled Con A stimulated thymocytes as targets asialo-GM1 and Thy-1.2 produced greater

than 95% lysis while anti-L3T4 (YTS 191) achieved between 68-72% cell death as measured by chromium release. FACS analysis of the lytic capability of anti-Thy-1.2, L3T4, Lyt-1.2 and Lyt-2.2 antibodies showed effective removal of the respectively treated and labelled populations (Table 4).

Aliquots of anti-L3T4(GK1.5) and PBS treated cells as well as anti-Thy-1.2 treated cells were also treated with complement. PBS treated cells had a >95% viability, anti-L3T4 (GK1.5) treated cells a >85% viability while anti-Thy-1.2 treated cells showed <5% viability as measured by Trypan blue exclusion. Treatment of cells from similar T-cell cultures with anti-L3T4 (YTS 191.1) and complement produced 68 to 75% lysis or cell death as measured by chromium release.

B. **Phenotyping of NK-effector cells by negative selection:** Results of experiments in which anti-Lyt-1.1, anti-Lyt-2.2, and anti-ASGM1 and complement were used to deplete NK-cell activity from pulmonary, lymph node and splenic effector populations are shown in Tables 5 and 6. Treatment of the cells with either anti-Lyt-1.1 or anti-Lyt-2.2 did not significantly reduce either normal NK-cell activity or the increased activity produced by the GVH reaction. In contrast, anti-ASGM1 completely abolished NK-cell activity to both YAC-1 and P815 tumour cell targets. These results were the same on each of the days of the reaction we examined, but data from only days 7 and 11 are shown.

Results of experiments in which the cytotoxic monoclonal antibody anti-L3T4 from the clone YTS 191.1 was used to deplete lymph node and splenic NK-cell activity are shown in Tables 7 and 8. Pre-treatment with the cytotoxic monoclonal anti-L3T4 antibody from the clone YTS 191.1 and complement did not result in a significant reduction of lymph node or splenic NK-cell cytotoxicity to either YAC-1 or P815 tumour cell targets in both the control or GVH effector cell populations.

The effect of anti-Thy-1.2 and complement is shown in Figures 7 A and B. The data in Figure 7 A reveal that this treatment partially abolished both normal and increased levels of lysis of YAC-1 tumour cell targets. Although splenic, lymph node and pulmonary NK-cell activity were reduced, this treatment had a much greater effect on NK-effector cells from the lymph nodes and lung. This was most apparent on days 11 and 18 in the lymph node. Figure 7 B shows that anti-Thy-1.2 completely abolished the cytotoxicity to P815 tumour cells that was activated de novo in the spleen, lymph nodes and lung during the GVH reaction.

These results indicate that the NK-cells which mediate some of the YAC-1 and all of the P815 killing are Thy-1.2 positive and that these cells are not in the T-helper or cytotoxic T-cell lineages.

TABLE 4

CONFIRMATION OF THE LYTIC CAPACITY OF MONOCLONAL ANTIBODIES USED IN THIS STUDY

Treatment		Labeled	% Cells
Antibody	Complement	Antibody	Positive
-	+	Anti-Thy-1.2	98.0
-	+	Anti-L3T4	87.1
-	+	Anti-Lyt-1	84.5
_	+	Anti-Lyt-2	80.3
Anti-Thy-1.2	+	Anti-Thy-1.2	0
Anti-L3T4	+	Anti-L3T4	4.5
Anti-Lyt-1.2	+	Anti-Lyt-1	4.5
Anti-Lyt-2.2	+	Anti-Lyt-2	5.2

a. Only data from complement controls are shown. Data from media controls were identical.

TABLE 5

CELL SURFACE PHENOTYPE ANALYSIS OF NK EFFECTOR CELLS
IN LUNG, LYMPH NODES AND SPLEEN

Organ	Treatment Antibody	Complement	YAC-1 Targets* Control	P	Day 7	P	Day 11	P
LUNG	-	-	7.3±0.7	>.1	44.9±1.3	>.1	41.2±2.7	>.1
	-	+	6.6±0.5		41.7±1.8		39.6±2.6	
	Anti-Lyt-1.2	+	6.5±0.6	>.1	42.6±0.4	>.1	46.2±1.1	>.1
	Anti-Lyt-2.2	+	6.0±0.4	>.1	39.1±0.8	>.1	43.0±0.7	>.1
	Anti-ASGM1	+	0	<.001	0	<.001	0	<.001
LYMPH NODE	-	-	2.5±0.1	>.1	14.4±0.4	>.1	17.0±0.5	>.05
	-	+	2.6±0.2		15.4±0.3		19.2±0.7	
	Anti-Lyt-1.2	+	2.0±0.4	>.1	15.0±0.6	>.1	13.0±0.3	>.05
	Anti-Lyt-2.2	+	1.7±0.3	>.05	13.1±1.1	>.1	22.6±0.8	>.05
	Anti-ASGM1	+	0	<.001	1.3±0.3	<.001	3.1±0.1	<.001
SPLEEN	-	-	13.0±1.1	>.05	19.4±1.5	>.05	10.0±0.5	>.05
	-	+	10.4±0.5		15.4±0.2		8.3±0.4	
	Anti-Lyt-1.2	+	11.6±0.1	>.05	16.3±1.3	>.1	8.6±0.1	>.05
	Anti-Lyt-2.2	+	10.9±0.2	>.1	17.3±0.8	>.05	8.0±0.1	>.05
	Anti-ASGM1	+	0	<.001	0.4±0.2	<.001	1.9±0.1	<.001

* % lysis at 50:1 E:T ratio.

P-values were calculated with Student's T-test. Treatment groups were compared to the complement control within each organ/day/target group.

TABLE 6

CELL SURFACE PHENOTYPE ANALYSIS OF NK EFFECTOR CELLS
IN LUNG, LYMPH NODES AND SPLEEN

Organ	Treatment Antibody	Complement	P815 Targets* Control	P	Day 7	P	Day 11	P
LUNG	-	-	0		12.7±0.3	>.05	12.7±1.1	>.1
	-	+	0		10.5±0.2		10.5±0.2	
	Anti-Lyt-1.2	+	0		11.6±0.4	>.1	9.6±0.6	>.1
	Anti-Lyt-2.2	+	0		11.3±0.8	>.1	10.7±0.6	>.1
	Anti-ASGM1	+	0		0	<.001	0	<.001
LYMPH NODE	-	-	0		1.9±0.1	>.1	10.7±0.5	>.1
	-	+	0		1.8±0.3		12.2±0.8	
	Anti-Lyt-1.2	+	0		2.0±0.1	>.1	7.0±2.1	>.05
	Anti-Lyt-2.2	+	0		1.6±0.2	>.1	14.6±1.2	>.1
	Anti-ASGM1	+	0		0	<.01	0.6±0.2	<.001
SPLEEN	-	-	0		2.8±0.6	>.1	7.4±0.1	>.05
	-	+	0		2.0±0.2		8.0±0.2	
	Anti-Lyt-1.2	+	0		1.7±0.4	>.1	7.2±0.9	>.1
	Anti-Lyt-2.2	+	0		2.3±0.3	>.1	8.8±0.4	>.1
	Anti-ASGM1	+	0		0.3±0.1	<.002	0	<.001

* % lysis at 50:1 E:T ratio.

P-values were calculated with Student's T-test. Treatment groups were compared to the complement control within each organ/day/target group.

TABLE 7

CELL SURFACE PHENOTYPE ANALYSIS OF NK EFFECTOR CELLS IN LYMPH NODES AND SPLEEN

Organ	Treatment Antibody	Complement	YAC-1 Targets* Control	P	Day 5	P
LYMPH NODE	-	-	2.7±0.2	>.1	15.9±0.2	>.05
	-	+	2.3±0.2		13.0±1.3	
	Anti-L3T4	+	2.1±0.4	>.1	12.6±0.6	>.1
SPLEEN	-	-	11.2±0.6	>.1	23.1±0.7	>.05
	-	+	9.3±1.1		19.1±1.3	
	Anti-L3T4	+	8.2±0.8	>.1	16.2±0.6	>.1

* % lysis at 50:1 E:T ratio.

P-values were calculated with Student's T-test. Treatment groups were compared to the complement control within each organ/day/target group.

TABLE 8

CELL SURFACE PHENOTYPE ANALYSIS OF NK EFFECTOR CELLS IN LYMPH NODES AND SPLEEN

Organ	Treatment Antibody	Complement	P815 Targets* Control	P	Day 5	P
LYMPH NODE	-	-	0		2.0±1.1	>.1
	-	+	0		2.2±1.0	
	Anti-L3T4	+	0		1.3±0.6	>.1
SPLEEN	-	-	0		2.4±0.3	>.1
	-	+	0		2.5±1.2	
	Anti-L3T4	+	0		1.9±0.3	>.1

* % lysis at 50:1 E:T ratio.

P-values were calculated with Student's T-test. Treatment groups were compared to the complement control within each organ/day/target group.

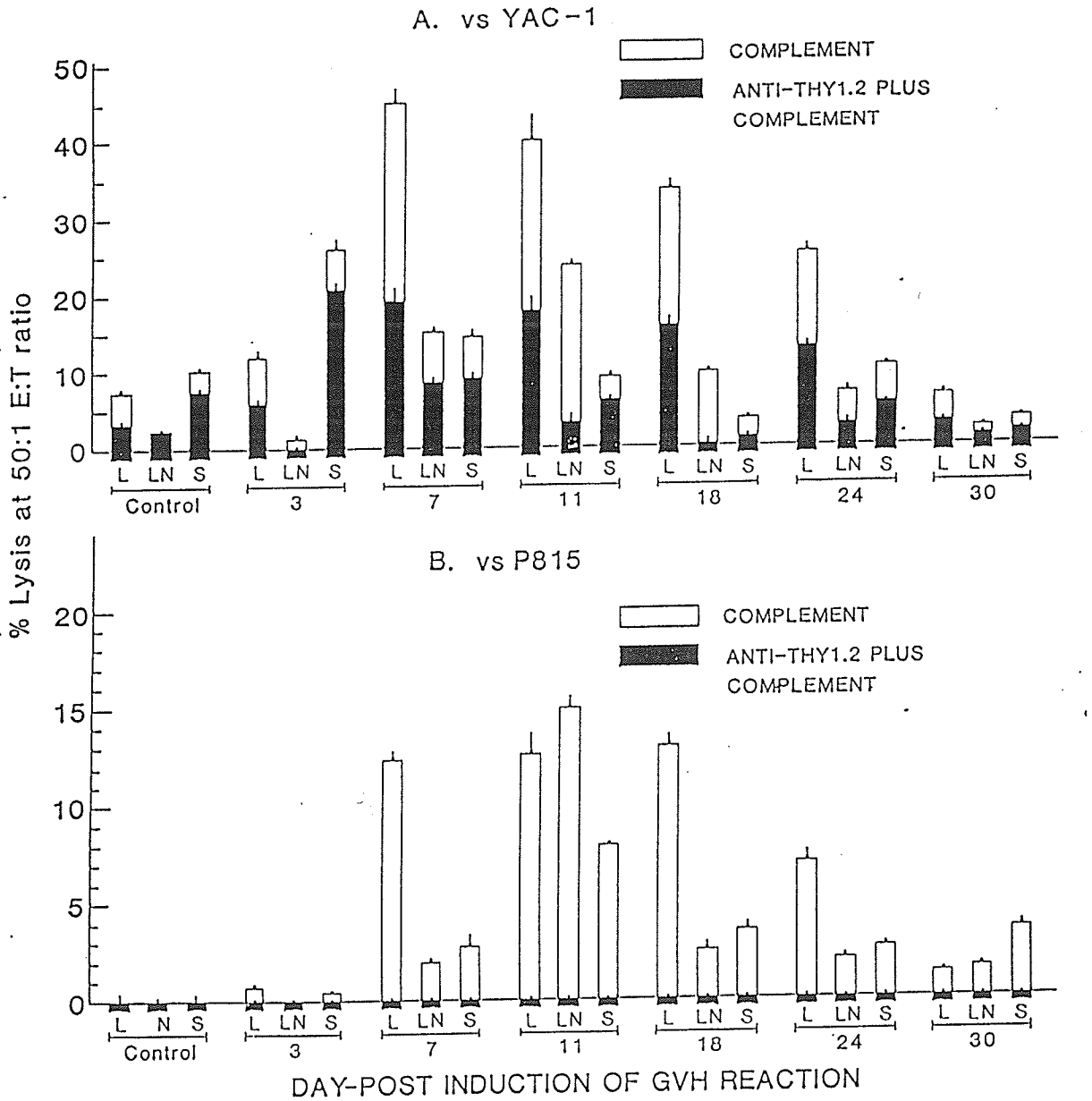


FIGURE 7 Sensitivity of NK activity to anti-Thy-1.2 and complement. Effector cells isolated from lung (L), lymph node (LN) and spleen (S) were incubated with monoclonal antibody (1:100) and agar-absorbed rabbit complement (1:10). A. Cytotoxicity of YAC-1 tumour cells. B. Cytotoxicity of P815 tumour cells.

3.4 PERCOLL BUOYANT DENSITY FRACTIONATION OF NK-EFFECTOR CELL POPULATIONS.

3.4.1 Experimental design and rationale: Effector cells from lymph nodes and spleen were separated over discontinuous Percoll density gradients. This was done because of previous reports indicating that NK-cell activity segregates to specific Percoll density fractions under some experimental conditions (272). The purpose of this experiment was to determine whether cells that mediate killing of P815 and YAC-1 tumour target cells could be physically separated by these methods.

3.4.2 Specific material and methods.

Effector cell pre-treatment.

Buoyant density separation: Lymph node and spleen effector cells were separated on discontinuous gradients (Percoll, Pharmacia Fine Chemicals). Percoll and complete media were adjusted to 285 mosm/kg H_2O with distilled water and 10X concentrated PBS. Gradients were established with seven concentrations of Percoll and media mixtures ranging from 38 to 63 percent Percoll (269-272). The Percoll gradients were constructed by layering 2 mls of each concentration with a 9" pasteur pipette at 20°C in conical 15 ml test tubes (Corning, distributed by Fisher Scientific). Alternate layers were coloured with phenol red. A maximum of 2×10^8 cells were loaded onto each gradient. The tubes were centrifuged at 300 x g for 45 minutes. The cells at the interfaces were collected and washed twice in HBSS and resuspended in complete RPMI-1640 for use in the NK assay. In parallel, aliquots of the original cell suspensions were also separated using Lympholyte M, as previously described, for comparison.

3.4.3 RESULTS.

Buoyant density gradient separations of effector cells: Discontinuous Percoll density gradients were used to segregate splenic and lymph node effector cells by buoyant density. The results are graphed in Figures 8.1 and 8.2.

Anti-YAC-1 NK-cell cytotoxicity from both control mice and mice on day 5 of GVH reactions segregate similarly, with activity clustering in the fourth and fifth fractions (Figures 8.1 A and C). Day 11 GVH splenic effector cells show a shift in the anti-YAC-1 activity towards fraction 4 (Figure 8.1 E). No control anti-P815 NK-cell activity was recorded in spleen cells (Figure 8.1 B). Splenic NK-cell cytotoxicity induced to P815 tumour cell targets in the GVH reaction segregates to the same fractions as the anti-YAC-1 activity (Figures 8.1 B,D and F).

In the lymph nodes, as in the spleen, a shift in anti-YAC-1 activity to the lighter density fractions between control and day 11 GVH effector cell populations when compared to controls (Figures 8.2 A and B). The lymph node anti-P815 NK-cell activity developing during the GVH reaction also appeared in the same fractions as the anti-YAC-1 activity (Figure 8.2 D). There was no P815 cytotoxicity in control lymph node preparations (Figure 8.2 B).

These results clearly show that there is no buoyant density difference between the NK-cells mediating YAC-1 or P815 target lysis. The results suggest that during the GVH reaction there is a shift of NK activity to the lighter density fractions. This may represent a younger NK blast cell that develops during the lymphoproliferative phase of the reaction.

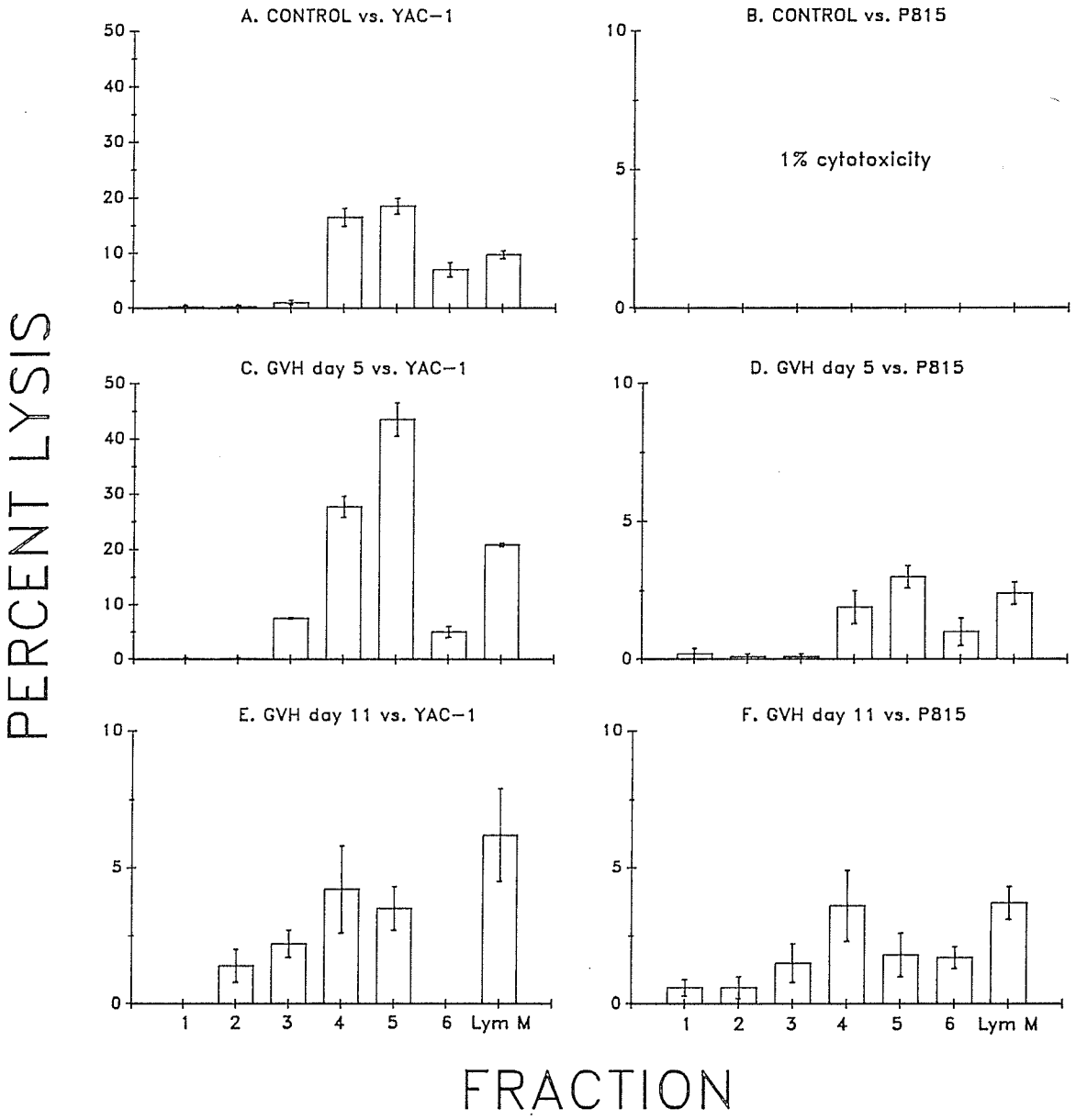


FIGURE 8.1 Percoll density separations of splenic NK-effector cells. Percent lysis at 50:1 E:T ratio. Lym M is Lympholye M. Graphs show pooled data from all experiments performed.

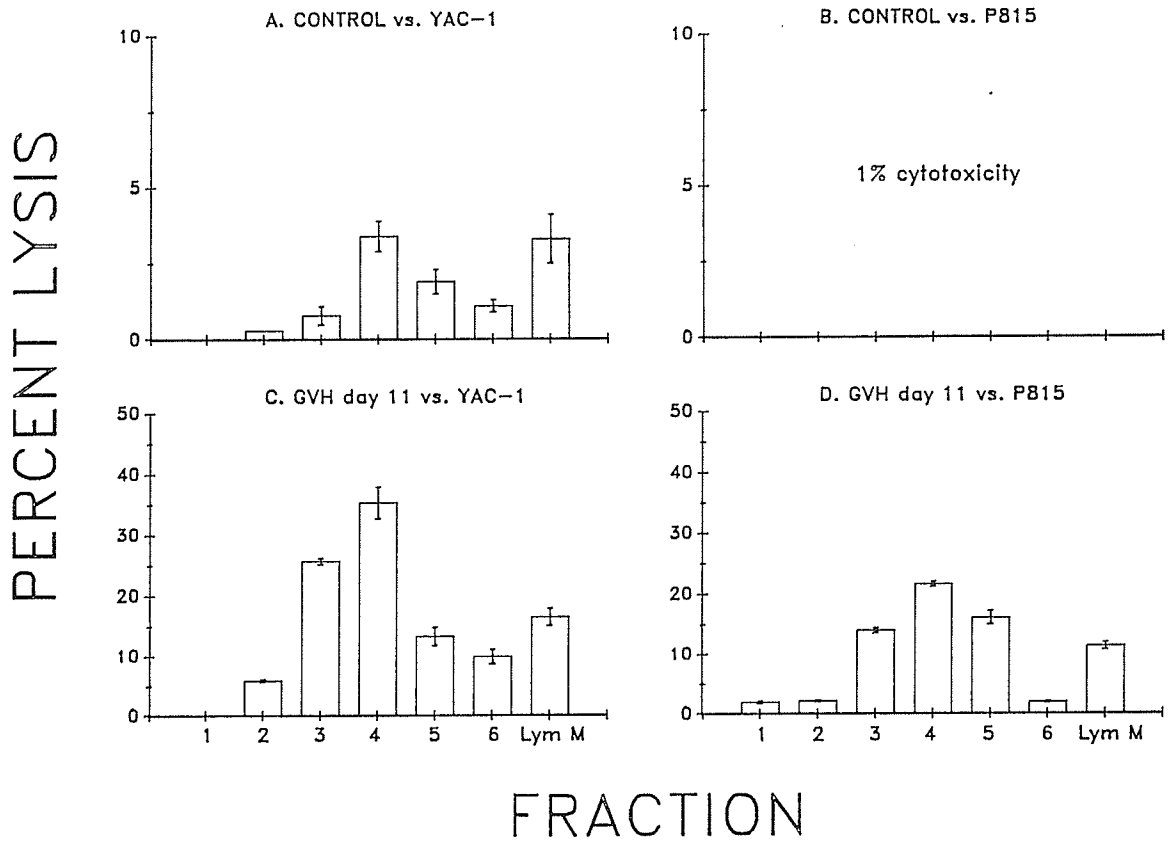


FIGURE 8.2 Percoll density separations of lymph node NK-effector cells. Percent lysis at 50:1 E:T ratio. Lym M is Lympholye M. Graphs show pooled data from all experiments performed.

3.5 EXAMINATION OF TARGET CELL SPECIFICITY OF NK-EFFECTOR CELLS BY COLD TARGET INHIBITION STUDIES.

3.5.1 Experimental design and rationale: Based on previous results it was postulated that GVH reactions induce a Thy-1.2-positive NK-cell which has the capacity to kill a broader range of targets (YAC-1 and P815) than do conventional Thy-1.2-negative NK-cells. To test this hypothesis cold target inhibition (CTI) experiments were performed in which unlabelled tumour target cells were added to NK-assay cultures to create a competitive inhibition situation (273,274). The nature of the target recognition structure for YAC-1 and P815 tumour cells has not been identified; however, even if they were distinct and separated physically the steric hinderance of cell-to-cell binding would probably preclude autonomous target binding, recognition and subsequent lysis.

3.5.2 Specific material and methods.

Cold target inhibition assay: Unlabelled (cold) YAC-1, P815 or K562 tumour cell target cells were added at several concentrations to triplicate cultures containing labelled (hot) target and effector cells (273,274). The inhibition of cytotoxic activity was recorded at cold:hot ratios of 0:1, 1:1, 3:1, 5:1, 7:1 and 10:1 in various combinations of target cell types at 50:1 and 25:1 effector:target cell ratios. The 0:1 cold:hot ratio percent lysis value was normalized to represent 100 percent lysis.

3.5.3 RESULTS.

The effect of unlabelled (cold) target cells competing for NK-cell cytotoxicity in spleen, lymph node and lung effector cell populations is graphed in figures 9.1, 9.2 and 9.3.

Control splenic NK-cell activity to labelled (hot) YAC-1 tumour cell targets was inhibited by increasing numbers of unlabelled (cold) YAC-1 tumour cell targets but was unaffected by the addition of cold P815 tumour cell targets (Figure 9.1 A). Experiments employing cold K562 tumour cells as competing targets in control groups were never able to inhibit NK-cell activity to YAC-1 or P815 tumour cell targets (Figure 9.1). In some experiments using cold K562 cells as competing targets in GVH groups an inhibitory effect could be seen (2 of 7 trials), this variability is accounted for within the SE bars of Figure 9.1. However, subsequent CTI studies by Gartner et al have consistently found K562 an inhibitory competative target. Control splenic effector cell populations were unable to lyse human K562 tumour cells, whereas GVH-derived cells were on occasion cytotoxic to K562 cells (data not shown). Further experiments by Gartner et al using GVH-derived cells have consistently demonstrated K562 cytotoxicity. None of the control NK-effector cell preparations showed anti-P815 cytotoxicity (Figures 9.1 C, 9.2 C, 9.3 C).

The increased anti-YAC-1 NK-cell cytotoxicity of the splenic GVH day 5 effector cell was inhibited by cold YAC-1 tumour cell targets in the same way as were control splenic effector cells (Figure 9.1 B). The reduced day 11 YAC-1 cytotoxicity of the reaction was likewise affected (Figure 9.1 B). The day 5 GVH YAC-1 cytotoxicity was not inhibited by cold P815 targets to the same extent as the day 11 GVH YAC-1 cytotoxicity. The de novo NK-cell cytotoxicity of splenic GVH effector cells to P815 tumour cell targets was similarly inhibited by both cold YAC-1 and P815 tumour cell targets (Figure 9.1 D).

Figure 9.2 shows the same series of experiments performed with lymph node effector cells. The NK-cell activity to YAC-1 tumour cell targets activated by the GVH reaction was inhibited almost identically by both cold YAC-1 and cold P815 tumour cell targets (Figure 9.2 B). NK-cell activity to P815 tumour cell targets stimulated by the GVH reaction was inhibited slightly more by cold P815 than cold YAC-1 tumour cell targets

inhibited slightly more by cold P815 than cold YAC-1 tumour cell targets (Figure 9.2 D). Normal lymph node effector cells demonstrated similar trends of inhibition by cold YAC-1 but not cold P815 tumour cell targets as normal splenic effector cells but the anti-YAC-1 cytotoxicity was too low to be significant (levels of 0-3% lysis at 50:1 E:T ratio).

Inhibition of pulmonary effector cell NK-cell activity is shown in figure 9.3. The increased induced NK-cell cytotoxicity to YAC-1 tumour cell targets seen during the GVH reactions was inhibited by competing cold YAC-1 tumour cells but only partially inhibited by cold P815 target cells (Figure 9.3 B). Anti-P815 NK-cell cytotoxicity seen in GVH pulmonary effector cells was inhibited by both cold P815 and cold YAC-1 tumour cell targets (Figure 9.3 D). Control pulmonary NK-cell activity followed the same pattern of the other control effector cell populations, inhibited by cold YAC-1 but not cold P815 tumour cell targets, but target cell lysis levels were too low at the lower E:T ratio used for the cold target inhibition studies to be significant (levels of 0-3% lysis at 5:1 E:T ratio).

These results show that the Thy-1.2-positive NK-cell which mediates the killing of P815 targets is also capable of lysing YAC-1 tumour cells. This cell population, as measured by lytic activity, begins to appear around day 5 after induction and expands during the lymphoproliferative phase of the reaction (day 11).

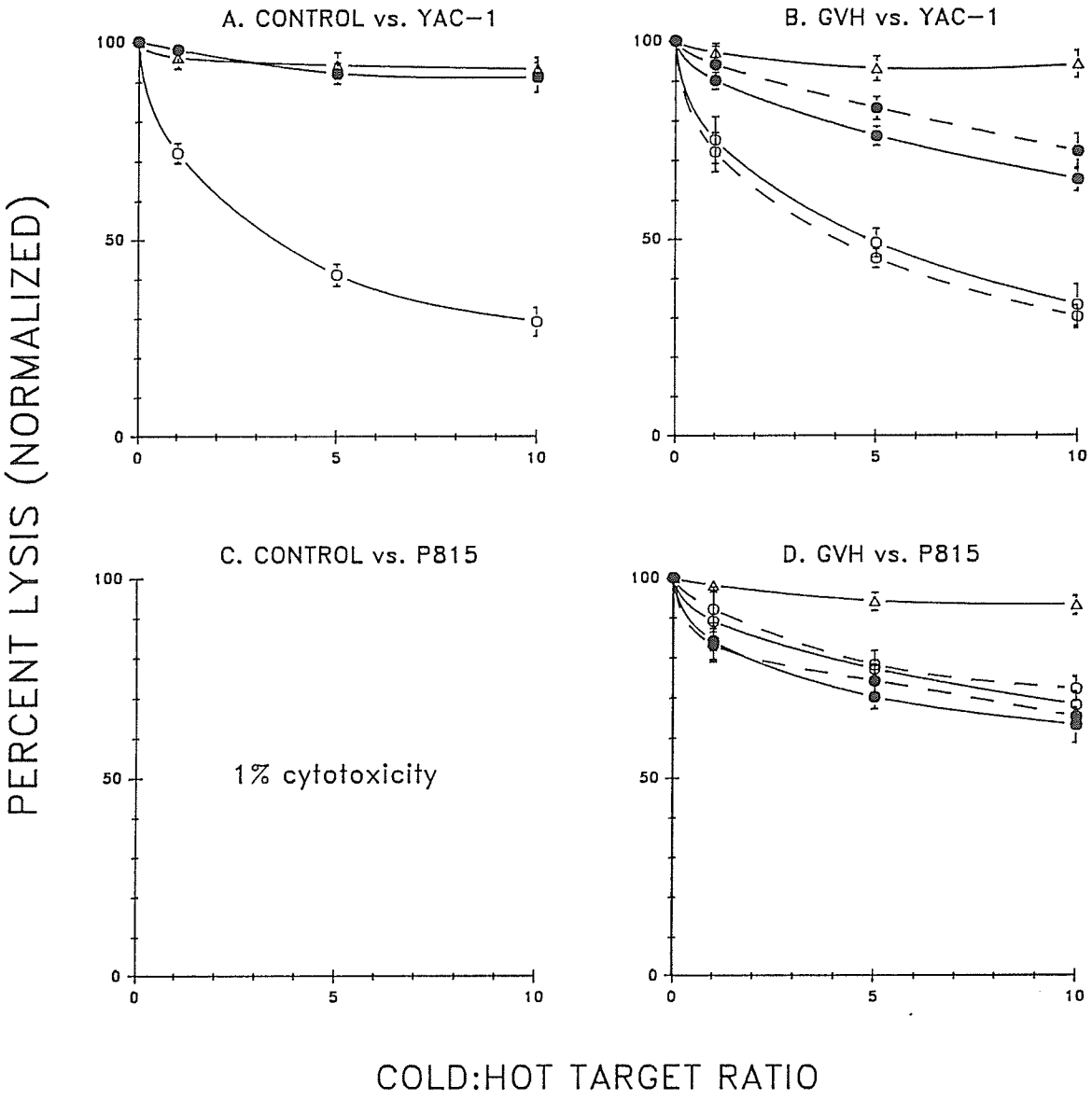


FIGURE 9.1 Cold target inhibition studies of splenic NK-effector cell populations. Effect of unlabelled (cold) YAC-1, P815 and K562 tumour target cells on NK-cytotoxicity to ^{51}Cr-labelled (hot) YAC-1 and P815 target cells by control and GVH days 5 and 11 NK-cells. Percent lysis at 50:1 E:T ratio. Control 100% cytotoxicity vs. YAC-1=9.1%, vs. P815=0%; GVH day 5 (dashed line) 100% cytotoxicity vs. YAC-1=22.5%, vs. P815=3.5%; GVH day 11 (solid line) 100% cytotoxicity vs. YAC-1=10.4%, vs. P815=7.4%. [O--O, cold YAC-1 cells; O--O, cold P815 cells; -- , cold K562 cells] The graphs show the pooled and averaged data from all experiments performed.

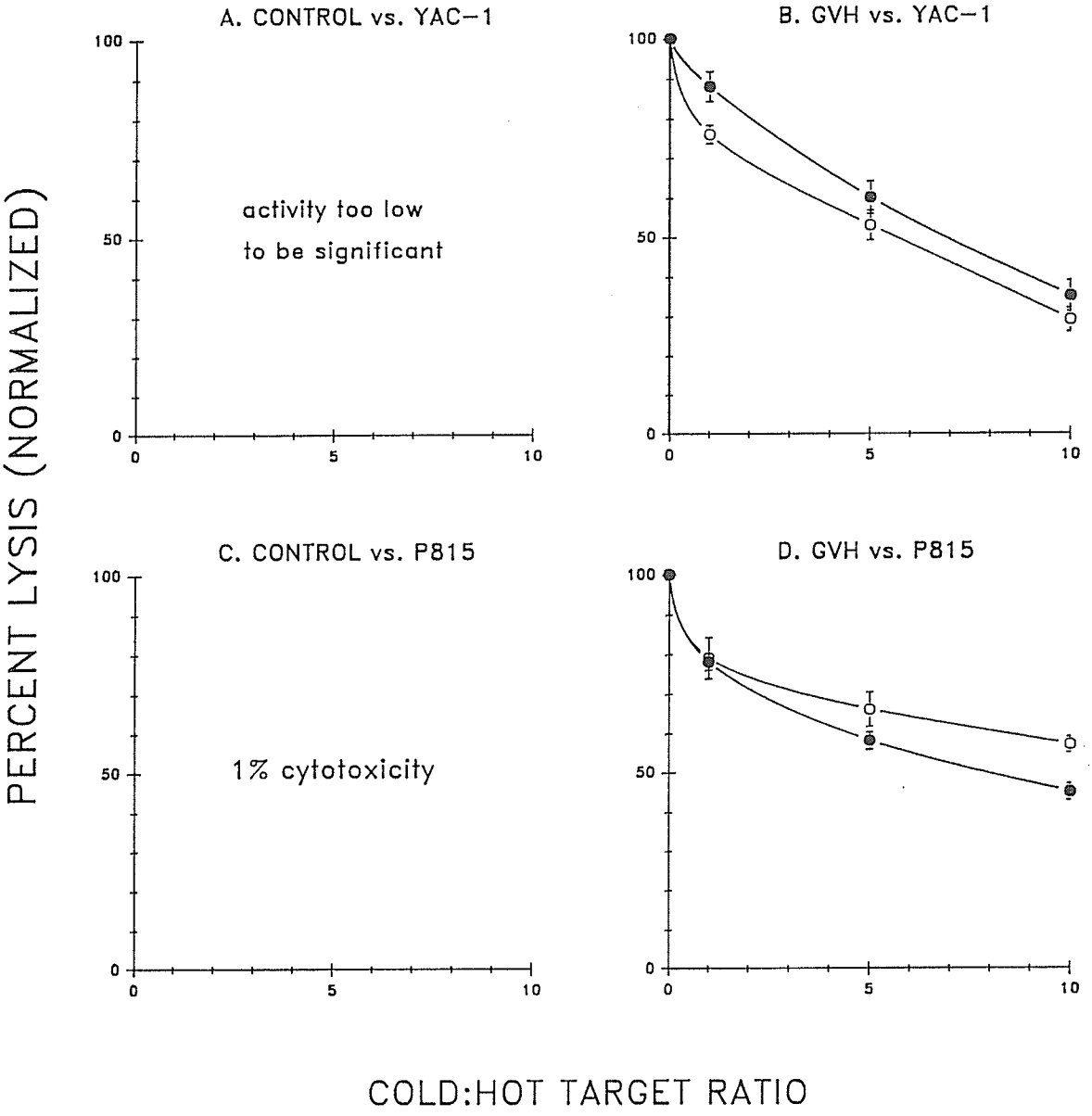


FIGURE 9.2 Cold target inhibition studies of lymph node NK-effector cell populations. Effect of unlabelled (cold) YAC-1 and P815 tumour target cells on NK-cytotoxicity to ^{51}Cr-labelled (hot) YAC-1 and P815 target cells by control and GVH NK-cells. Percent lysis at 50:1 E:T ratio. Control 100% cytotoxicity vs. YAC-1=3.0%, vs. P815=0%; GVH day 11 100% cytotoxicity vs. YAC-1=20.9%, vs. P815=11.4%. [O--O, cold YAC-1 cells; O--O, cold P815 cells] The graphs show the pooled and averaged data from all experiments performed.

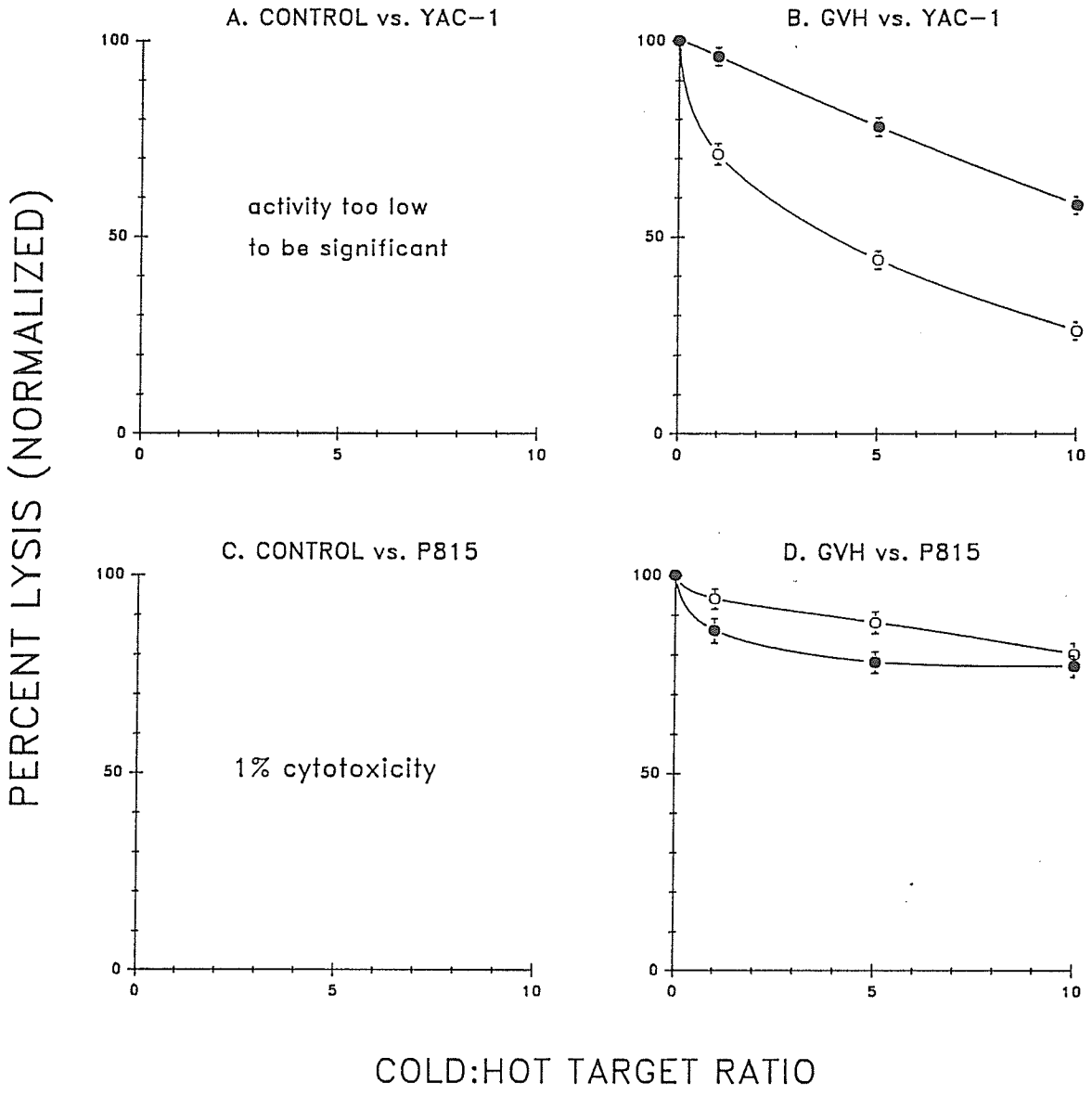


FIGURE 9.3 Cold target inhibition studies in pulmonary NK-effector cell populations. Effect of unlabelled (cold) YAC-1 and P815 tumour target cells on NK-cytotoxicity to ^{51}Cr-labelled (hot) YAC-1 and P815 target cells by control and GVH day 11 NK-cells. Percent lysis at 5:1 E:T ratio. Control 100% cytotoxicity vs. YAC-1=3.0%, vs. P815=0%; GVH day 11 100% cytotoxicity vs. YAC-1=10.6%, vs. P815=4.9%. [O--O, cold YAC-1 cells; O--O, cold P815 cells] The graphs show the pooled and averaged data from all experiments performed.

4.0 DISCUSSION

4.1 Choice of GVH model and controls.

In this study, the F_1-hybrid model was selected because it reasonably reproduces the consequences of GVH reactions in BMT recipients (58,150) and transfusion-associated-GVH (8,152). This induction protocol allows the study of GVH reactions uncomplicated by the effects of radiation or cyto-reductive therapy. The data presented in section 4.1 verify the vigour of the GVH reaction studied and the success of the induction methods at producing an acute lethal GVH reaction.

4.2 Changes in NK-cell activity in lung, lymph nodes and spleen over the course of the GVH reaction.

Previous investigations have documented a rise in splenic and thymic NK activity during GVH reactions (6). The experiments reported in section 3.1 showed that the GVH reaction stimulated a 4 to 5 fold increase in the level of NK-cell activity in the lung. Results revealed that the increase in activity was sustained for most of the reaction. Lymph node NK-cell activity rose from near zero in control mice to levels of 20% lysis of YAC-1 tumour cell targets. The appearance of NK-cell activity in the lymph nodes lagged slightly behind that of the lung but then persisted for a major portion of the reaction. In contrast, NK activity in the spleen was elevated only transiently during the early part of the reaction and then diminished to below normal values.

The increase in pulmonary NK activity may be explained by the fact that the lung is known to contain a large population of LGL cells with low NK activity (223,227). The GVH reaction may stimulate these cells to become cytotoxic. Although this is an attractive explanation, the possibility of migration of NK cells from the spleen may account for, or at least contribute to, pulmonary NK activity.

During the lymphoproliferative phase of the reaction NK-cells isolated from the lung, lymph nodes and spleen developed the ability to lyse P815 tumour cells. P815 tumour cells are normally insensitive to lysis by conventional NK-cells (221). The immunogenetics of the GVH reaction employed in these experiments (A/J -> (C57BL/6 x A/J) F_1-hybrids) results in an anti-H-$2^{b/a}$ T-cell response. This phenomenon cannot be attributed to a cytotoxic T cell responding to the MHC haplotype of the P815 since these cells are H-2^d.

The degree to which P815 tumour cells could be lysed varied with the organ from which the cells were isolated. The levels of anti-P815 activity in the lung was significantly higher than that in the lymph nodes and spleen. With the exception of the transient peak of NK-cell cytotoxicity in the lymph nodes during the reaction, the level of anti-P815 activity never approached the level of lysis achieved against YAC-1 targets.

The changes in NK-cell activities with time over the course of the reaction for the cytotoxicity mediated by lung and lymph node NK-effector cells against the two target cells were similar in profile but differed from the splenic activities, particularly in the anti-YAC-1 response.

4.3 Localization of pulmonary NK-cell activity.

The lung is a non-lymphoid organ but showed greatly increased levels of NK-cell activity during GVH reactions. Pulmonary levels of NK-activity surpassed those seen in spleen and lymph nodes. Natural suppressor cells have been demonstrated during GVH reactions in the spleen which may partly account for this phenomena (139,143). Experiments were performed to localize NK cell pulmonary activity to a specific lung compartment.

The experiments reported in section 4.2 utilized BAL and perfusion to deplete specific lung compartments of pulmonary NK-cell activity. Since neither diminished the total level of pulmonary NK-cell activity it was concluded that the cells from these compartments did not mediate a

major portion of the pulmonary NK-cell activity. An application of both also failed to remove NK activity from the lung, and therefore suggested that the NK-effector cells within the pulmonary interstitium mediated the enhanced cytotoxicity. This finding is in keeping with observations by others that the lung parenchyma, which normally has low levels of NK-activity, has a rich population of resident LGL cells (227). Nonetheless, it is possible that the effector cells may be tightly adhered to either the alveolar lining cells or to vascular endothelium and are not removable by lavage or perfusion (205,153).

The recent literature has presented a conflicting spectrum of data on the localization and activation of NK cells in the lung (205,152-154,175). The conflicting conclusions may be attributable to species differences. Experimental work done in normal mice and humans has shown that interstitial NK cells, not alveolar ones represent the active NK population (152,175) which concurs with the reported findings in this thesis. Conversely, Pritchard et al found that in the guinea pig interstitial and alveolar NK-cells were equally active (153). However, they also reported differences for local vs. systemic stimulation of NK-activity with interferon inducers (153). Pulmonary interstitial NK-cells were found to be particularly sensitive to interferon stimulation, responding to doses of interferon inducers which did not affect splenic NK-activity.

The effect of locally augmented pulmonary NK-cell activity during GVH reactions can not be precisely determined from this study. It is possible that NK-cells may, in some way, be involved in the development of pulmonary lesions.

A direct cytolytic role for GVH-stimulated NK-cells was proposed by Ghayur et al (155). In that study, histopathological changes seen in the liver and pancreas during the GVH reaction were believed to be related to the concurrent finding of increased NK-cell activity in the spleen.

In this study, characteristic mononuclear cell infiltrates were observed in the interstitium and alveolar septae. Pulmonary fibrosis was observed. In a more comprehensive study of pulmonary histopathology during acute GVH reactions, Gartner et al (259) addressed the question of the link between increased in NK-cell activity and histopathological changes. They concluded that there was no direct correlation between increased NK-cell activity in the lung and the occurrence of pulmonary lesions since NK-activity rose and peaked prior to the appearance of the cellular infiltrate. Cellular infiltrates only appeared as anti-P815 target NK-activity developed. The infiltration of the lung was most extensive at a time when the cytolytic activities of the NK-effector cell populations were declining.

4.4 Phenotypic characterization of NK-effector cells activated by the GVH reaction.

Phenotyping of NK-effector cells by negative selection with antibody and complement showed that anti-asialo-GM1 antisera depleted all of the NK-cell activity against both tumour cell targets whereas monoclonal antibodies to the L3T4, Lyt-1.2 and Lyt-2.2 cell surface markers had no significant effect on the levels of NK-mediated cytotoxicity. These results show that the cells responsible for NK-activity in GVH reactions have a cell surface marker characteristic of murine NK-cells and activated T-cells, but do not express the specific T-cell markers of CTL or cytotoxic T helper cells. This confirms that the anti-tumour cell lysis seen in the GVH reaction is not directly attributable to a T-cell response.

The phenotyping study also demonstrated that a portion of the anti-YAC-1 activity was partially sensitive to depletion with anti-Thy-1.2 and complement in both control mice and in those with GVH reactions. The proportion of pulmonary and splenic of YAC-1 target NK-activity removed by this treatment remained roughly constant over the course of the

reaction. This would suggest that the GVH reaction provided a general proliferative stimulus affecting both Thy-1.2-positive and negative NK-cells similarly. In contrast anti-YAC-1 activity in lymph nodes was almost completely depletable by the anti-Thy-1.2. This finding suggests that virtually all NK cell arising in lymph nodes during the GVH reaction are Thy-1.2 positive. The NK-activity of the lymph nodes stimulated by the GVH reaction may represent a selected, sequestered and expanding, NK-cell population as pre-induction levels of YAC-1 tumour cell lysis are normally below 5% and relatively insensitive to treatment with anti-Thy1.2 antibodies. In all three organs anti-Thy-1.2 plus complement abrogated all anti-P815 target NK-activity acquired de novo during the reaction.

These results suggest that NK cells lysing both tumour cell targets may be in the T-cell lineage. It is known that NK-cells may express Thy-1.2 but do not express other functional T-cell markers such as the alpha, beta or gamma chains of the T-cell receptor (184).

The findings suggest that GVH reactions induce and stimulate two populations of NK-effector cells: a resident Thy-1.2-negative NK-cell that acts like a conventionally defined NK-cell having only YAC-1 cytotoxicity, and a Thy-1.2-positive population that can lyse both YAC-1 and P815 tumour cells. This GVH-induced effector-cell bears striking resemblance to lymphokine-activated killer (LAK) cells (188). LAK cells are generated in the presence of IL-2 (189) and are believed to represent "transformed" NK and T-cells (190).

The activation of LAK cells during the GVH reaction is supported by the findings of a transient increase of IL-2 production in spleen cell cultures derived from mice in the lymphoproliferative phase of a GVH reaction (201). NK cells stimulated by IL-2 possess the Thy-1.2 surface marker when "transformed" into LAK cells. NK/LAK cells are able to lyse targets normally insensitive to conventional NK cell cytotoxicity (75). The kinetic profile of splenic anti-YAC-1 NK-cell activity appears to parallel changes in IL-2 production described over the course of the

reaction. The early fall of splenic anti-YAC-1 NK-cell cytotoxicity prior to the decline of all other NK-cell activity in the GVH reaction may be the result of the action of locally induced natural suppressor (NS) cells on conventional NK-effector cell (158) but not the LAK/NK-cell.

The appearance of P815 tumour cell lysis and the plateau of lung and lymph node YAC-1 tumour cell lysis show a brief lag period following the increase in IL-2 production. This would be expected if the effector cells were generated in response to IL-2. This activity may also be mediated in part by interferon, a cytokine that is able to potentiate NK-cell activity. In a study by Shah et al, the use of polyinosinic:polycytidylic acid in mice to stimulate endogenous interferon release resulted in the activation of a Thy-1.2-positive NK-cell within 24-48 hours. These cells were able to lyse both YAC-1 and P815 tumour cell targets (275). The combined effects of Interferon and IL-2 certainly could explain the pattern of NK-cell activity seen in GVH reaction.

4.5 Buoyant density analysis of NK-cell activity during the GVH reaction.

These experiments were performed to determine whether NK cells activated during GVH reactions could be separated on the basis of buoyant density in Percoll. Both control and GVH effector cells fractionated consistently to the lighter densities. In the spleen and lymph node, there was no difference between the fractionation of the anti-YAC-1 and anti-P815 NK-cell cytotoxicity; both seemed to rise simultaneously.

On day 5 the separation of splenic effector cells from both experimental and control animals were similar but by day 11 the larger portion of the NK-cell activity was found in the lighter fractions. This differed from the results in the lymph node effector cell separations where virtually no pre-induction or early phase NK-cell activity was seen. The circulating nature of the lymph node population would predict this result as a mature, more dense, resident LGL/NK-cell would not be expected

to compose a significant portion of the cellular constituents of the normal or early phase lymph node (198).

Because the spleen is normally a repository for active NK-cells and the lymph nodes are not (271), under the systemic stimulation of the GVH reaction, resident or sequestered NK-cells may be activated first (201) and then proliferate (83) during the allogeneic phase of the reaction. If this model is correct then the enhanced NK-cell cytotoxicity of the GVH reaction would then correspond to both an activated population and a newly generated population of LGL/NK-cells (163,83).

Fractions 3, 4 and 5 of the Percoll gradient are associated with LGL/NK-cells (271). In normal mice mature resident LGL/NK-cells would tend to fractionate towards the denser gradients in the LGL/NK-cell buoyancy range. Likewise, the mature resident or infused LGL/NK-cells activated in the early stages of the reaction would tend to be in the denser fractions. As the GVH reaction progresses into the lymphoproliferative phase a larger, more buoyant blast cell population would arise and appear in the lighter buoyancy fractions. The experimental results are consistent with this idea.

4.6 Cold target inhibition studies.

Results from phenotyping experiments in the proposition that GVH reactions result in the activation of both a Thy-1.2 negative population of NK cells that are able to kill only YAC-1 target cells, and a Thy-1.2 positive population with a broadened lytic spectrum. Results from the CTI experiments supported this view. The ability of either cold P815 or YAC-1 target cells to inhibit anti-P815 NK-cell cytotoxicity suggests that the cell mediating P815 cytotoxicity has a broadened spectrum of lytic activity.

The results further support and emphasize the importance of pre-GVH induction levels of NK-cell activity in the specific organs. Both the lung and spleen have a normal resident NK-cell population whereas the

lymph node generally does not. The relationship of pre- and post-induction NK activity can be deduced for the lung and spleen from the result of partial inhibition of the YAC-1 tumour cell cytotoxicity by the cold P815 tumour cell targets where two NK-cells are postulated to reside during the GVH reaction. This differs from the lymph node where an almost equal inhibitory effect seen upon the NK-cell YAC-1 cytotoxicity by both the cold P815 and YAC-1 targets where predominantly the GVH-induced Thy-1.2-positive NK-cell is found. The patterns of increase of NK activity in GVH reactions reflects the specific initial NK activity levels.

4.7 Possible role of NK-cells in GVH reactions.

Despite the fact that NK cell activation is a well described phenomenon of GVH reactions the precise role these cells play in the evolution of GVH disease remains poorly understood. It has been suggested that the role of these cells in GVH reactions is related to their cytolytic capacity.

Results from this study provide additional confirmation that NK activity increases in the spleen and lymph nodes (6,250). Other studies have demonstrated augmented NK-cell activity in the thymus (11) and in intraepithelial lymphocytes of GVH reactions in mice (249). Some have suggested that NK-cell activation in GVH reactions produced in unirradiated F_1-hybrids may be related to a preceding anti-host DTH response with NK-cells being but one component of the effector mechanisms recruited by the DTH response (250).

A study by Ghayur et al shows that the development of histologic lesions characteristic of GVH reactions in the liver and pancreas of unirradiated F_1-hybrid mice correlated with strain combinations and parental lymphoid infusion doses that caused an early peak in NK activity in the spleen (155). They suggested that a direct or indirect causal relationship between NK cells and tissue lesions may exist. Although they did not measure NK-activity in the damaged organs this premise is

supported by a study by Charley et al (171) in which GVH disease could be prevented or resolved by in vivo treatment of the recipient mice with anti-asialoGM1. Similar evidence of the necessity of NK-cells for tissue injury during GVH reactions is presented in a subsequent study by Ghayur et al (294) using NK-cell deficient Beige mice. Characteristic GVH reaction histopathology was not seen in recipient mice infused with lymphocytes from NK-cell deficient donors. Although the Beige mouse studies are cited in support of the idea that NK cells participate in the effector response they have some inherent problems. Asialo-GM1 is not specific for NK-cells, as discussed earlier, and could abrogate a possible T-cell response in the mice. The Beige mutation has several other defects, including one involving degranulation of cells. This may interfere with any cytotoxic T-cell lytic activity.

Clinical studies have primarily correlated pre- and post-transplant NK-cell activity as a prognostic indicator to the occurrence or severity of GVH disease. The conclusions of these studies regarding the role of NK-cells in GVHD vary with the timing and target cell used to assay NK-cell activity. Dokheler et al (253) found that there was a positive correlation between the development of peripheral blood NK-cell activity soon after BMT and the occurrence of GVH disease. Lopez et al (254) showed that patients with high pre-transplant levels of NK-cytotoxicity to herpes simplex virus type 1 infected fibroblasts had an increased risk of developing a GVH reaction post-transplant. A similar study by Livnat et al (255) using K562 target cells to assay NK activity could not demonstrate any correlation between NK-cell cytotoxicity pre- and post-transplant with the occurrence of GVH disease.

The findings reported in this thesis which are part of a larger investigation (259), could not demonstrate a correlation between augmented NK-cell activity in either the spleen or the pulmonary interstitium and the initial appearance or intensity of the pathological changes in the lung. Indeed the results suggested an inverse relationship between these

two parameters, and appear to refute the suggestion that injury to host tissue constituents seen during GVH reactions is attributable to a direct, cytotoxic effect of NK cells. It would seem reasonable to suggest that the in vitro tumour cell lytic activity is but one component of NK cell function which allows these cells to be identified and cytotoxicity may not be the only capacity in which NK cells act. Thus, if NK cells have a role in the pathogenesis of GVH disease, then this role may be indirect and involve activation and interaction with other potential effector cells. It is known, for example that, NK-cells release cytokines that can attract and activate macrophages (184,235) causing increased superoxide production and augmented bacterial killing. Several studies in mice with GVH reactions have shown that peritoneal and pulmonary macrophages are activated during GVH reactions. Further reports of macrophage activity during GVH reactions document increased macrophage-mediated destruction of target cells (67,280), enhanced phagocytosis and bactericidal activity (161,277), and a heightened oxidative burst potential (278). Correlative support for an NK-cell-macrophage link in mediating lung tissue injury was reported in a recent study, from this laboratory (279), where a marked increase in the number of macrophages was observed in the alveoli of mice with GVH reactions. This was associated with a proliferative response of macrophages in the interstitium of the lung (279).

As natural killer cells are becoming defined as an immunosurveillant or immunoregulatory cell in the immune system their role in the GVH reaction may be considered in a new light. It is possible that NK-cells play an indirect regulatory role in the evolution of GVH disease by stimulating macrophage activation. NK-cells activated by the stimulation provided by the GVH reaction act as aberrant immunotransducer cells (inadvertently) signalling host or self destruction. This could be achieved through IL-2, produced by allo-activated T-cells very early in the reaction stimulating an increase in NK and NK/LAK-cell activity. The GVH reaction related activation of NK cells may be supplemented by

bacterial products if infection has set in as NK cells have been shown to be stimulated by bacterial surface lipopolysaccharide (268). These activated NK cells may then release cytokines that, in turn, kindle and amplify macrophage-mediated injury to host tissue constituents (see Figure 14). This hypothesis for a role for NK cells in GVH reactions is supported by: the findings of increased IL-2 production in GVH reactions; the interpretation of Mowat et al (251) linking the DTH response to increased NK activity in GVH reactions; and the correlative studies showing an increase of NK activity preceding the appearance of GVH histopathology. The interactive link between NK cells and macrophages/monocytes is becoming more apparent. Studies of in vitro hemopoiesis show that NK cells interact with macrophages in a regulatory manner (267). The existence of this proposed mechanism in no way diminishes or replaces the central position of CTL in initiating tissue injury in the host, and indeed would probably act synergistically with CTL by transducing and amplifying the IL-2 signal between allo-reactive T cells and macrophages.

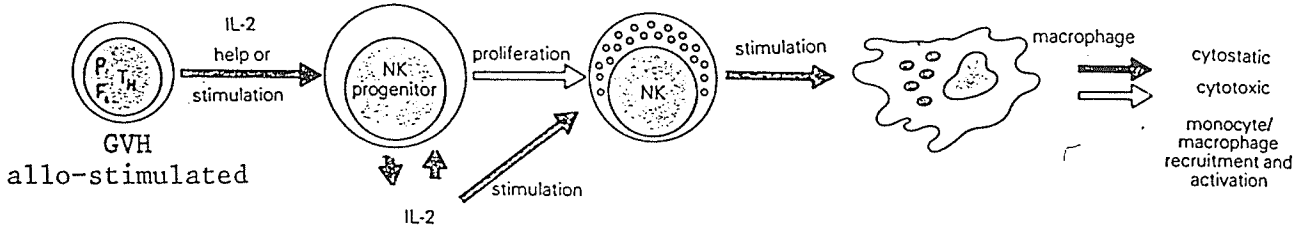


FIGURE 14 Possible role of NK cells in GVH reactions

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