Genesis of a "Null" Lymphocyte Population with Natural Killer Cell Characteristics in Bone Marrow of Normal and Tumor-Bearing Mice

by

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To my Mother, the memory of my Father, and to Guylaine

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ABSTRACT

Mouse bone marrow was shown to contain a population of null lymphocytes which lack molecular markers of both B and T lineages (μ^-, B220$^-$, Thy1$^-$). Null cells represent 12-14% of all bone marrow small lymphocytes. Studies on population dynamics have revealed that these null cells turnover rapidly with a production rate almost 1/3 as great as that of B lymphocytes. Some newly-formed null lymphocytes express natural killer (NK) cell-associated markers, including NK1.1. Some small lymphocytes in the bone marrow express low intensities of Thy1. These Thy1^{lo} cells are also rapidly renewing and some express NK1.1, suggesting that these cells, in addition to null lymphocytes, form part of the rapidly generated lineage of NK cells. During growth of a transplantable tumor, there were marked increases of limited duration in the numbers of null, NK1.1$^+$, and Thy1^{lo} cells in the bone marrow. The increase in null cells was further enhanced in tumor-bearing mice given indomethacin. These findings suggest that the production of null small lymphocytes in the bone marrow includes the neogenesis of NK cells, possibly playing an important role against naturally occuring malignancies.

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RESUME

Dans la moëlle osseuse de souris il y a une population de lymphocytes qui n'ont pas les marqueurs membranaires B220, μ et Thy1 qui caractérisent les lymphocytes des lignées B et T. Ces cellules appelées "null" (N) forment 12 à 14% de la population des petites lymphocytes de la moëlle. Des études sur la cinétiques des cellules lymphoides de la moëlle osseuse a montré que le renouvellement des cellules N est rapide et que leur taux de production est près du tiers de la production des lymphocytes B. Certains lymphocytes N expriment des marqueurs membranaires qui caractérisent les lymphocytes NK (natural killer), en particulier le marqueur NK1.1. Certains petits lymphocytes de la moëlle osseuse montrent une faible réactivité au marqueur Thy1. Ces lymphocytes Thy1-positifs se renouvellent rapidement et certains expriment le marqueur NK1.1 ce qui indique que ces dernières cellules sont de la lignée des cellules NK. Au cours de la croissance d'une tumeur transplantée sur la souris, il y a une augmentation marquée, mais d'une durée limitée, du nombre des cellules N, ou de cellules NK1.1- et Thy1-positives dand la moëlle osseuse. L'augmentation des cellules N a été plus marquée chez des souris porteuses de tumeur et injectées d'indométhacine. Ces résultats indiquent que la production de cellules N dans la moëlle osseuse de souris est associée à la genèse de cellules NK et par conséquert ces lymphocytes N peuvent jouer un rôle dans l'élimination de cellules cancéreuses nouvellement formées.

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Titre: Genèse de lymphocytes N (null) possedant des caractéristiques des lymphocytes NK (natural killer) dans la moëlle osseuse de souris normales ou porteuse de tumeur.

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INTRODUCTION

The cells and molecules of the immune system recognize infecting organisms, malignant cells and foreign substances and they respond to eliminate them from the body. As a consequence of a primary exposure to such antigens, the immune system "remembers" the encounter so that subsequent exposures to the same antigens are dealt with more efficiently.

The primary cells of the immune system which are capable of recognizing and responding to antigens are lymphocytes. Like other blood cells of mammals, lymphocytes are derived ultimately from self-perpetuating stem cells in the bone marrow (Wu et al, 1968; Abramson et al, 1977; Phillips et al, 1978; Sprangrude et al, 1988a). Lymphocytes circulate through the blood and lymphatics and are distributed in peripheral lymphoid organs throughout the body. Lymphocytes are characterized by a high nuclear to cytoplasmic ratio, a spherical pachychromatic nucleus and a small amount of undifferentiated cytoplasm at one pole of the cell (Yoffey et al, 1965; Osmond, 1975; Rosse, 1976).

Production of lymphocytes in the bone marrow

In the bone marrow of adult mice approximately one-quarter of all the nucleated cells are lymphocytes. These cells range in size from 5.5 μm to 14μm in mean nuclear diameter in smears (Miller & Osmond, 1975). Approximately 80%

of bone marrow lymphoid cells measure less than 8 μm and are termed small lymphocytes (Miller & Osmond, 1975). The remaining large lymphoid cells (>8 μm) have a leptochromatic nuclear appearance (Rosse & Yoffey, 1967; Miller & Osmond, 1973; Rosse, 1976). The number of bone marrow lymphocytes shows characteristic fluctuations with age, being most abundant in infancy, reaching a peak in early adulthood and declining with advancing age (Miller & Osmond, 1975; Osmond, 1975; Rosse, 1976).

The life history of bone marrow lymphoid cells has been examined by administration of tritiated thymidine (^{3}H-TdR) in vivo, to label DNA synthesizing cells and their progeny in radioautographic preparations (Leblond et al, 1959; Osmond & Everett, 1964; Miller & Osmond, 1975). Small lymphocytes show no immediate labeling with the DNA specific isotope and thus are not in DNA synthesis, ie. not proliferating, whereas large lymphoid cells are in DNA synthesis (Miller & Osmond, 1973; Osmond et al, 1973; Rosse, 1976). Selective in vivo labeling of bone marrow and in vitro study of cultured lymphocyte-rich fractions of bone marrow cells have shown that the proliferating large lymphoid cells located in the bone marrow give rise to non-proliferating small lymphocytes which are being renewed continuously (Osmond & Everett, 1964; Yoshida & Osmond, 1971).

During ^{3}H-TdR infusion in the mouse, bone marrow small lymphocytes show a rapid exponential increase in the labeling

index for the first three days, followed by a slower appearance of labeled small lymphocytes, revealing two populations of cells; (1) a major population (75-95%) which are rapidly renewed from proliferating precursors in the bone marrow, and shown to have a half renewal time ($T_{\frac{1}{2}}$) of 14-24 hr, and (2) a minor population (6-20%) of slowly renewed cells, mainly recirculating to and from the blood stream (Miller & Osmond, 1975; Osmond, 1975). The relative proportions of these populations vary as a function of age. As the age of the animal increases, the relative proportion of slowly renewing population of cells in the bone marrow increases also (Miller & Osmond, 1975). The total lymphocyte production in the bone marrow organ throughout the body is considerable, equivalent to approximately 10^8 cells per day in mice (Osmond, 1975). The fate of newly-formed small lymphocytes includes a rapid random migration out of the bone marrow compartment. This was demonstrated by intramyeloid radioisotope injection in situ, showing the local production of newly-formed small lymphocytes migrating into peripheral tissues including the spleen and lymph nodes (Brahim & Osmond, 1970, 1976). Electron microscopic studies show some small lymphocytes crossing the walls of the marrow sinusoids (Osmond & Batten, 1984; Jacobsen et al, 1990). Within the secondary lymphoid organs, foreign antigens are recognized and effective immune responses are elicited (Benner et al, 1981).

Lymphocyte lineages

Lymphocytes fall into 3 main classes, namely B cells, T cells and cells lacking both B and T lymphocyte characteristics, termed "null" cells.

B lymphocytes arise from the bursa of Fabricius in birds and the bone marrow in mammals (Lafleur et al, 1972; Osmond & Nossal, 1974a,b; Osmond, 1975; Phillips et al, 1978; Cooper, 1981; Landreth et al, 1981). They mediate humoral responses by giving rise to antibody forming cells. B lymphocytes are characterized by the presence of surface immunoglobulin M (sIgM or sμ), a molecule which can recognize and bind specific antigens leading to B cell activation (Cambier, 1988).

Stem cells in the bone marrow give rise to precursor cells which migrate to the thymus and differentiate into T lymphocytes (Stutman, 1978; Erzine et al, 1984; Sprangrude et al, 1988b, 1989). The T cells are responsible for cell-mediated immune responses including graft rejection, defenses against tumor and virus infected cells (Stutman, 1978; Marrack & Kappler, 1987). T cells in mice are characterized by a 25 KDa glycoprotein surface antigen, Thy1, which is a member of the immunoglobulin gene superfamily (Reif & Allan, 1964; Williams, 1985; Marrack & Kappler, 1987).

B lymphocyte production in the bone marrow

The continuous large scale production of small lymphocytes in the bone marrow represents the primary genesis

of B lymphocytes. This was first demonstrated by the in vivo administration of ^{3}H-TdR and exposing cell suspensions to radioiodinated antibodies against surface IgM, the specific B lineage marker (Osmond & Nossal, 1974a,b). Such surface labeling studies revealed that 50% of the small lymphocytes in the bone marrow show varying densities of surface immunoglobulin, representing B lymphocytes. An additional 5-7% express Thy1 antigen, representing T lymphocytes (Osmond & Nossal, 1974a; Rosse, 1976). However, almost half of the small lymphocytes in the bone marrow have neither sIgM nor Thy1 ($s\mu^{-}$,Thy1^{-}) and were thus originally referred to as "double negative" or "null" cells (Osmond & Nossal, 1974a,b; Rys & Vassalli, 1974; Rosse, 1976; Press et al, 1977).

Kinetic radioautographic studies revealed that the range of sIgM densities on bone marrow small lymphocytes represents a maturation sequence (Osmond & Nossal, 1974a,b). When first formed from cycling large lymphoid precursor cells, the small lymphocytes in the bone marrow do not express detectable $s\mu$. In fact, null cells are the first to show ^{3}H-TdR incorporation, reaching 90% within the first two days. After a post-mitotic lag period of 1 to 1.5 days, these small lymphocytes progressively express $s\mu$, leave the bone marrow at various stages of maturity and complete their maturation elsewhere, notably in the spleen (Osmond & Nossal, 1974b; Yang et al, 1978). As $s\mu$ is expressed, receptors for the Fc portion of Ig (FcR) and the third component of complement

(C3R) are also expressed on newly-formed B lymphocytes within the bone marrow (Yang et al, 1978).

The identity and fate of all the sμ⁻Thy1⁻ small lymphocytes in the bone marrow remain unknown. Many null cells are newly-formed immature B lineage cells (pre-B cells) destined to develop sμ. The radioautographic kinetic studies demonstrate that newly-formed sμ⁻ small lymphocytes undergo a post-mitotic maturation gradually expressing IgM on their surface. When sμ⁻ cells from the bone marrow were cultured in vitro for a few days (Osmond & Nossal, 1974a) or were injected into syngeneic mice, sμ soon developed (Ryser & Vassalli, 1974). However, previous data has not excluded the possibility that some null cells may form a cell lineage in the bone marrow distinct from B cells and T cells. Double marker studies by Chan and Osmond (1979) using SRBC rosetting, differential centrifugation techniques and in vivo ^{3}H-TdR labeling showed the rapid turnover in the bone marrow of a population of null lymphocytes expressing FcR, suggesting that null cells in the bone marrow may include a distinct lineage of lymphocytes.

The aim of this thesis is to attempt to resolve this controversy.

Precursors of B lymphocytes

Lafleur et al (1972) introduced the functional term, precursor B (pre-B) cells to describe large cells which

require a significant period of time after being transplanted into irradiated recipients before being able to mediate a specific humoral immune response. The immediate precursors of B lymphocytes, ie. pre-B cells, have been recognized as cells which do not express $s\mu$ but contain the μ heavy chain of IgM in their cytoplasm ($c\mu$) as shown by double immunofluorescence labeling on fixed cells (Raff et al, 1976; Landreth et al, 1981; Cooper, 1981; Opstelten & Osmond, 1983). Pre-B cells become responsive to specific foreign antigen only after they have expressed sIgM molecules as mature B cells. The size of individual $c\mu^+$ pre-B cells in the bone marrow varies widely. A variety of kinetic analyses using metaphase arrest, ^{3}H-hymidine labeling, and hydroxyurea deletion have shown that large $c\mu^+s\mu^-$ pre-B cells in the adult bone marrow are cycling rapidly. These divide to give rise to small $c\mu^+$ pre-B cells which mature into $s\mu^+$ B cells (Rusthoven & Philips, 1980; Cooper, 1981; Landreth et al, 1981; Opstelten & Osmond, 1983; Park & Osmond, 1987). Post-irradiation and post-cyclophosphamide regeneration support the concept that $c\mu^+$ pre-B cells become $s\mu^+$ B lymphocytes in the bone marrow (Burrows et al, 1978; Osmond & Owen, 1984). Pre-B cells are not found in the normal spleen or thymus, even during post-irradiation regeneration (Burrows et al, 1978; Raff et al, 1976). A cell line developed by Paige et al (1978a), termed 70Z/3, has provided one of the first pieces of evidence that $c\mu^+s\mu^-$ pre-B cells are the immediate precursors of $s\mu^+$ B cells.

Ontogenically, successive waves of $c\mu^+$ pre-B cells occur in the liver (day 13 of gestation), spleen (day 15 of gestation) and in the bone marrow (day 19 of gestation). The numbers of pre-B cells in the bone marrow rose progressively thereafter whereas the waves of pre-B cells in the liver and spleen were transient, all disappearing by approximately 2 weeks after birth (Vilardi & Cooper, 1984).

These findings have demonstrated that many $s\mu^-Thy1^-$ null small lymphocytes in the bone marrow are B lineage cells (pre-B cells) which contain $c\mu$ but have not yet expressed $s\mu$. However, it is not known whether the null cells in the bone marrow exclusively represent $c\mu^+$ pre-B cells or may also include cells of another lineage.

With the use of monoclonal antibodies (mAb), several B cell lineage-associated antigens have been found on the surface of relatively undifferentiated precursor cells. These antigens are useful for the detection, enumeration, and manipulation of cells which are destined to become B lymphocytes. These markers include Lyb2 (Kincade et al, 1981), BP-1 (Cooper et al, 1986), DNL 1.9 (Dessner et al, 1981), ThB (Eckhardt & Herzenberg, 1980), and AA4.1 (McKearn & Rosenberg, 1985).

Kincade et al (1981) immunized rats with a mouse B lymphoma line, producing a mAb, designated as 14.8. This antibody bound to normal $s\mu^+$ B cells, $c\mu^+$ pre-B cells, μ^- early B lineage cells, and some peripheral T cells (Kincade et al,

1981; Landreth et al, 1982, 1983; Park & Osmond, 1987). Biochemical analyses have shown that mAb 14.8 precipitated a 220KD glycoprotein (B220) found on the surface of B lineage cells (Kincade et al, 1981; Coffman & Weissman, 1981) which cross-reacted with a family of polymorphic lymphocyte antigens called Ly5 (Omary et al, 1980). MAb 14.8 is thus a useful tool in distinguishing both B cells and precursor cells committed to the B lineage.

Thy1-bearing cells and the production of T lymphocytes

The bone marrow contains stem cells which have extensive self-renewal capacity and multiple differentiation options. Fluorescent activated cell sorter (FACS) analyses showed that certain bone marrow localized uncommitted stem cells bear low levels of Thy1 antigen ($Thy1^{lo}$) but lack the various surface markers that characterize cells of defined differentiated hematolymphoid cell lineages. The stem cells are devoid of the lineage specific markers of B lymphocytes (B220), granulocytes (GR-1), myelomonocytic cells (Mac-1), and mature T cells (L3T4 and Lyt2) (Muller-Sieburg et al, 1986; Sprangrude et al, 1988a) and are thus referred to as lineage-negative (Lin^-) cells. These $Thy1^{lo}$ Lin^- stem cells account for 0.1 to 0.3% of nucleated cells in the bone marrow. When transferred into irradiated recipients, they can give rise to all hemopoietic lineages, including both B lineage cells (Muller-Sieburg et al, 1986; Sprangrude et al, 1988a) and

cells which home to the thymus via the blood stream and differentiate into mature functional T lymphocytes (Ford et al, 1966; Erzine et al, 1984; Sprangrude et al, 1988a,b). It has been suggested that some congenital anomalies in cell-mediated immunity are due to defects within a subpopulation of thymus-destined bone marrow stem cells (Bosma et al, 1983).

The thymus is the central lymphoid organ for the production, differentiation, and dissemination of T lineage cells (Metcalf, 1964; Stutman, 1978; Scollay et al, 1984). In the young adult mouse, 10-20% of all thymocytes are rapidly proliferating. The daily production far exceeds the actual thymic emigration to the periphery (Scollay et al, 1984). During the maturation in the thymus, thymocytes acquire Thy1 antigen on the cell surface.

Immunofluorescence studies have shown that the thymic cortex accounts for most (90%) of the lymphocyte content in that organ and contains cells bearing high levels of the Thy1 antigen ($Thy1^{hi}$). In the medulla, however, thymocytes have the mature T cell surface phenotype, expressing medium densities of Thy1 and are believed to be the immediate source of thymic emigrants (Shortman et al, 1987). Cell sorting analysis has revealed that $Thy1^{lo}$ Lin^- stem cells in the bone marrow have a tenfold lower Thy1 antigen density per cell than on thymocytes (Muller-Sieburg et al, 1986; Sprangrude et al, 1988a,b).

Stutman (1978) coined the term "post-thymic precursor" (PTP) to describe cells which are immunologically incompetent

thymus-processed precursor cells exported from the thymus to the periphery, subsequently giving rise to competent T cells. There is evidence that these cells which continuously leave the thymus are short lived migrants and express high levels of Thy1 (Cantor et al, 1975; Stutman, 1978; Rocha et al, 1983). The expression of high densities of Thy1 antigen provides a useful marker in mice for the detection of T lineage cells in lymphoid tissues throughout the body, including the bone marrow.

In addition to the Thy1 antigen, peripheral T cells in mice express either the surface markers L3T4 or Lyt2,3, but not both (Cantor & Boyse, 1975; McKenzie & Potter, 1979). Classically, L3T4 molecules on T cells are associated with a helper function during the initiation of an immune response whereas cells bearing Lyt2 have a cytotoxic or suppressor function activated either during or after their recruitment into an active immune response (Cantor & Boyse, 1975; Scollay et al, 1984).

Many long-lived Thy1$^+$ peripheral T lymphocytes recirculate through the blood and lymph (Sprent & Basten, 1973; Osmond, 1975; Press et al, 1977), patrolling the various lymphoid tissue compartments of the body, ie. the spleen and lymph nodes. Some T cells recirculate through primary lymphoid organs such as the thymus (Michie et al, 1988; Hirokawa et al, 1989) and the bone marrow (Osmond, 1975; Press et al, 1977). Radioautographic studies in mice have suggested

a mean life span of approximately 16 weeks for recirculating T cells and 6 weeks for B cells (Sprent, 1977).

Null lymphocytes: natural killer (NK) cells, natural cytotoxic (NC) cells and natural suppressor (NS) cells

In the spleen and other peripheral lymphoid tissues, null lymphocyte populations include cells which can spontaneously lyse transformed cells of hemopoietic origin without compatibility with the major histocompatibility complex (MHC). These cells, termed natural killer (NK) cells, lack B and T cell surface markers ($s\mu^-$, $Lyt2^-$, $L3T4^-$) and gene rearrangements and are viewed as forming a primitive recognition mechanism whose primary function is to provide an initial barrier of defense against newly arising neoplasms and viral infections (Roder & Haliatis, 1980; Hercend et al, 1982; Lauzon et al, 1986; Lanier et al, 1986). NK cells may also be involved in the regulation of normal hemopoietic cells in the bone marrow and thymus (Hansson et al, 1979; Hansson et al, 1981; Riccardi et al, 1981; Miller et al, 1988) and certain B cell functions including antibody production (Michael et al, 1989). Experiments using irradiated mice reconstituted with bone marrow and ^{89}Strontium have shown that splenic NK cell function depends on cells derived from the bone marrow and requiring an intact marrow microenviroment for their differentiation (Haller & Wigzell, 1977; Haller et al 1977;

Kumar et al, 1979). However, very little is known about the endogenous production of NK cells in the bone marrow, at the cellular level.

Mature NK cells, purified by fractionation procedures based on adsorption to target cells or by sedimentation, have been described as being medium to large lymphocytes with a pale cytoplasm containing specific azurophilic granules. This cell type has been termed large granular lymphocytes (LGL). LGL mediate NK activity in both humans (Timonen et al, 1979) and rodents (Reynolds et al, 1981; Kumagai et al, 1982). Cells with LGL morphology are not exclusively NK cells however. Both non-MHC restricted cytotoxic T lymphocytes (CTL) (Lanier et al, 1986) and MHC restricted CTL (Biron et al, 1986) are also morphologically LGL.

The target cell used in most conventional NK assays is the Moloney virus-induced T cell lymphoma, YAC.1 (Kiessling et al, 1975; Roder & Kiessling, 1978; Roder et al, 1978; Herberman, 1982). NK cells destroy their target cell by two general mechanisms. In an initial binding phase the NK cells form close contacts with the target cell surface. This recognition phase, mediated by yet a fully uncharacterized receptor, can be examined morphologically at the single cell level (Roder and Kiessling, 1978; Roder et al, 1978, Pollack and Rosse, 1987). It has been suggested, however, that certain cell-surface glycoproteins, ie. T200, or specific carbohydrate moieties on NK cells may be important in target

cell recognition and binding (Gilbert et al, 1988). Target cell binding is followed by a lytic phase in which cytolytic granules are released from the NK cell directed towards its target. This cytotoxic reaction can be measured in vitro, by a chromium^{51} (^{51}Cr) release assay (Kiessling et al, 1975; Herberman, 1982; Carpen & Saksela, 1988).

Mature spleric NK cells display a variety of cell surface molecules including those identified by mAbs NK1.1 and NK2.1 (Burton et al, 1981, Koo & Peppard, 1984; Hackett et al, 1986; Koo et al, 1986), the glycosphingolipid asialo GM1 (Habu et al, 1981; Beck et al. 1982), and Fc receptors (FcRγII) (Herberman, 1982; Perussia et al, 1989). Some NK cells also demonstrate low levels of Thy1 (Herberman et al, 1978). Thy1 appears to be a marker of NK cell activation (Hurme & Sihvola, 1984; Hackett et al, 1986). All NK cells, however, lack Lyt1 and Lyt2 (Minato et al, 1981). Only NK1.1 and NK2.1 antigens are regarded as lineage specific markers (Glimcher et al, 1977; Burton et al 1981; Hackett et al, 1986; Koo et al, 1986) and thus can be used to identify cells which belong to the NK lineage.

NK cell function appears to represent a defence mechanism against certain spontaneously developing tumors in vivo (Roder & Haliotis, 1980; Herberman 1982; Lanier et al, 1986; Lauzon et al, 1986). However, the efficiency of this system is impaired in many host-tumor systems (Habu et al, 1 1; Lala et al, 1985). Lala et al (1985) have shown that during the

development of transplanted or spontaneous tumors in mice, both null lymphocytes and NK activity in the spleen rise transiently with increasing tumor growth. The decrease in NK activity is caused by an inactivation rather than a disappearance of NK cells and is mediated by the production of prostaglandins by the host's macrophages (Lala et al, 1985; Parhar & Lala, 1985; Lala et al, 1986). In the spleen, null lymphocytes include cells of the NK lineage (Kiessling et al, 1975; Herberman et al, 1979; Hercend et al, 1982) and both cell types respond selectively, in vivo, to tumor-bearing (Parhar & Lala, 1985; Lala et al, 1986). The question arises whether some of the null lymphocytes in the bone marrow may also be sensitive to tumor growth and have the potential to become NK cells.

Natural cytotoxic (NC) cells are null cells which are similar to NK cells functionally, except that their target cells are solid tumors (Stutman et al, 1978; Paige et al, 1978b; Stutman & Cuttito, 1980). It is not known whether NC cells represent a separate lineage or are a subpopulation of NK cells.

Natural suppressor (NS) cells residing in adult bone marrow and neonatal spleen are also phenotypically null cells (Duwe & Singhal, 1979; McGarry & Singhal, 1982; Oseroff et al, 1984; Maier et al, 1986). NS cells can spontaneously suppress both in vitro mixed lymphocyte reactions (Dorshkind et al, 1980; Oseroff et al, 1984) and antibody production (Duwe et

Singhal, 1979; Bains et al, 1982). They may also be an important regulatory component of self-tolerance (Muruako & Miller, 1980). Unlike NK and NC cells, however, the suppressive activity of NS cells is not associated with a lytic activity, but rather with a suppressive soluble factor (Duwe & Singhal, 1978; Mortari & Singhal, 1988). NK, NC and NS cells thus have a number of properties in common. Without any apparent priming, they can suppress certain immune responses in a genetically unrestricted manner. However, their precise lineages and developmental pathways are as yet unclear.

Null lymphocyte production in the bone marrow

The previous studies quoted above have shown that the bone marrow continuously produces many null small lymphocytes lacking sμ and Thy1. These cells include post-mitotic pre-B cells containing cμ destined to become B lymphocytes. The question arises, however, whether all of these null small lymphocytes in the bone marrow are committed to the B lineage or may include other functional lineages of the immune system also known to be derived from the bone marrow, eg. NK cells. Previous studies, based on immunofluorescence labeling of bone marrow cells, have not addressed this problem.

Objectives

The current work aimed to determine whether the large number of small lymphocytes generated in mouse bone marrow are destined solely for the B lineage or may in part belong to another lineage. The objectives were (1) to identify and quantitate null small lymphocytes, lacking B and T lineage phenotypic markers, (2) to examine their ontogenic development, kinetic properties and production rates, and (3) to examine other phenotypic properties and population responses of null small lymphocytes which might reflect their functional significance.

Section I:

This section was designed to study the expression of free cytoplasmic μ heavy chains of IgM by cells in the mouse bone marrow with lymphoid morphology. A radioautographic immunolabeling technique was developed to detect cμ in permanent hematologically-stained preparations. The incidence and size distribution of Thy1$^+$ cells were measured and the intensity of Thy1 expression was quantitated. Radioautography was used throughout most of the work because traditional immunofluorescent techniques and phase contrast microscopy do not allow precise morphological identification of cells labeled with the lineage specific antibodies. The results reveal the presence in the bone marrow of a significant population of null small lymphocytes which lack B and T

lineage specific markers (ie. $c\mu^-s\mu^-Thy1^-$). Furthermore, many $Thy1^+$ small lymphocytes in the bone marrow were mostly low intensity ($Thy1^{lo}$) compared to those in both the spleen and thymus which were mainly of high intensity ($Thy1^{hi}$).

Section II:

This section describes an alternate approach of revealing $c\mu^-s\mu^-Thy1^-$ null small lymphocytes in radioautographic preparations by labeling the B lineage-associated B220 glycoprotein detected by mAb 14.8. This section also addresses the question of whether these null small lymphocytes in the adult bone marrow have turnover kinetics which are distinct from those of 14.8^+ and $Thy1^+$ small lymphocytes. Furthermore, the ontogenic development of 14.8^-Thy1^- null small lymphocytes was examined in fetal and post-natal life. The results confirm the presence of a population of null small lymphocytes lacking B and T lineage characteristics (14.8^-Thy1^-), which appear early during fetal development. They were shown to have an exceptionally rapid turnover rate compared to 14.8^+ and $Thy1^+$ cells, representing a large continuous production of cells. The $Thy1^{lo}$ cells were kinetically distinct from $Thy1^{hi}$ cells.

Section III:

The possibility that NK cells may be contained within the population of bone marrow null lymphocytes was explored by

means of double immunolabeling techniques. The results show that some null small lymphocytes in the bone marrow express NK cell related properties including FcR, the ability to bind tumor target cells, and the NK specific marker, NK1.1. A proposed model for the differentiation of NK lineage cells in the bone marrow, including the role of Thy1 expression, is discussed.

Section IV:

Experiments in this section were planned to investigate in vivo whether null small lymphocyte population in the bone marrow was selectively responsive to the presence of a tumor growing elsewhere in the body. The data indicated a marked transient increase in the number of both null and $Thy1^{lo}$ small lymphocytes in the bone marrow during the growth of both ascites and solid tumors, not shown by either 14.8^{+} or $Thy1^{hi}$ cells. Many of these null small lymphocytes expressed the NK1.1 specific surface marker, suggesting that they were cells of the NK lineage.

MATERIALS AND METHODS

Mice.

Male C3H/HeJ, C57BL/6, A/SN and (C3H/HeJ x C57BL/6)F_1 mice were obtained from Jackson Laboratories, Bar Harbor, ME and used at various ages. Anti-IgM treated mice were supplied by Dr. J. Gordon, Department of Surgery, McGill University: (C3H/HeJ x C57BL/6)F_1 mice (BioBreeding Laboratories of Canada, Ottawa, Ontario) bred and maintained under specific pathogen-free conditions, were injected intraperitoneally (ip) from the first week of life, three times weekly, with an IgG-rich fraction of rabbit anti-mouse IgM (5-10 mg/0.1 ml). Control mice received a similar series of injections of an IgG fraction of normal rabbit serum (NRS). In vivo anti-IgM treatment deletes all $s\mu^+$ and functional B cells, while leaving intact T cells and B cell precursors (Manning & Jutilia, 1972; Lawton et al, 1972; Burrows et al, 1978; Osmond & Gordon, 1979).

Hydroxyurea treatment.

Mice were injected ip with hydroxyurea (HU) (1 mg/gm body weight in saline, Sigma, St. Louis, MO) at 12 hour intervals to eliminate DNA synthesizing cells by irreversibly inhibiting the enzyme ribonucleoside phosphate reductase (Young & Hodas, 1964; Rusthoven & Phillips, 1980; Miller, 1982; Freitas et al, 1986). Mice were killed by cervical dislocation at various

times between 11 hours and 73 hours after the first HU injection. The injections were given at a constant time of day to avoid variations due to the diurnal rhythm of cell proliferation. Control mice were injected with saline at the same time intervals.

Tumors.

(1) *Ehrlich ascites* (EA) carcinoma cells, orginally established from a spontaneous mammary carcinoma in mice (Ehrlich, 1906), were maintained by a weekly passage ip into C3H/HeJ mice. EA tumors grow as free cells in a serous fluid in the peritoneal cavity (Claësson & Olsson, 1977; Lala et al, 1985; Parhar & Lala, 1985). Tumor cells were harvested sterily, washed by centrifugation (200g, 10 min) , resuspended in saline and 10^6 cells/0.2 ml of saline were injected ip, the time of which was taken as day zero. Tumor-bearing as well as tumor-free control mice were used at 7-8 weeks of age. Mice were killed 3,4,5,6,7,9,11 and 13 days following tumor injection.

2) A highly metastatic subclone, H-59, of the *Lewis lung carcinoma* (LLc) cells was obtained from Dr. P. Brodt, Department of Surgery, McGill University (Brodt, 1986). LLc has been shown to cause progressive neutrophilia, anemia, thrombocytopenia, splenomegaly, and elevated marrow and splenic hemopoietic progenitors (Balducci & Hardy, 1983; Hardy & Balducci, 1986). Subcutaneous solid tumor masses, growing

in the gluteal region of LLc passaged mice, were removed sterily. Single cell suspensions were made by mincing the tumor masses with scissors and exposing them to trypsin and DNAse for 30 minutes at room temperature. Clumps and debris were removed by passing the cell mixture over a stainless steel mesh, allowing the single cells to pass through. Cells were washed by centrifugation and 10^6 cells/0.2 ml of phosphate-buffered saline (PBS), pH 7.2, were injected subcutaneously (sc) in the right gluteal region of 7 week old C57BL/6 male mice (Brodt, 1986). Mice were killed on days 5,7,9,14,16,28 and 32 following tumor injection. The time points assayed in the EA and LLc tumor systems were not the same because of the different growth kinetics of the respective tumors.

Indomethacin treatment.

LLc-bearing mice received indomethacin (Sigma) in their drinking water beginning on day 1 after tumor injection and lasting throughout the experimental period. Indomethacin inhibits the production of prostaglandins (Fulton & Levy, 1980; Fulton, 1984; Lala et al, 1986; Parhar & Lala, 1988).

Indomethacin was initially dissolved in absolute ethanol (7 mg/ml) and diluted 500 times in tap water to attain a final concentration of 14 μg/ml of drinking water. Control tumor-bearing mice were given an identical concentration (0.2%) of ethanol alone (without indomethacin) in their drinking water,

also beginning on day 1 following tumor injection. Bottles were changed twice weekly. The average daily water consumption was calculated for both groups of mice and found to be the same.

Preparation of cell suspensions

Mice were killed by cervical dislocation. Cells were pooled from groups of 3-5 mice in all experiments. The femurs were dissected out, the epiphyses were removed, and the diaphyseal bone marrow was flushed out with cold 10% (v/v) newborn calf serum (NCS) in Hepes buffered Eagle's Minimal Essential Medium (MEM; Grand Island Biological Company, Grand Island, NY) using a 23 gauge needle and 1.0 ml syringe. Cells were gently suspended by aspirating several times through the syringe needle. The spleen and thymus were removed, cut into fragments, gently teased through a stainless steel mesh screen (80 mesh/in^2) and rinsed with cold 10% NCS in MEM.

NCS (1.0 ml) was layered under cell suspensions and was left standing on ice for 5 minutes so that large cell clumps and debris settled to the bottom of the tube. The suspension above the interface was transferred into another centrifuge tube. NCS (1.0 ml) was again layered at the bottom and the cells were centrifuged (400g, 7 min at 4°C) through the NCS to remove any fine debris and fat. The cell pellets were resuspended in 10% NCS in MEM. Nucleated cells were counted using an electronic particle counter (Coulter Counter, Model

B, Hialeah, FA) after lysing the red blood cells (RBC) by the addition (6 drops) of Zap-Oglobulin (Coulter Electronics). The cell concentration was adjusted to 40 x 10^6 nucleated cells/ml in 10% NCS in MEM.

Antisera.

1. B lineage:

Hybridoma cells producing the rat mAb 14.8 (IgG_{2a}), originally developed by Dr. P.W. Kincade, Oklahoma Medical Research Foundation, Oklahoma City, OK (Kincade et al, 1981; Landreth et al, 1983) were obtained from the American Type Culture Collection (ATCC; Nashville, MD). Cultures of hybridoma cells were grown in our laboratory, supernatants containing the mAb (IgG_{2a}) were purified on an anti-rat kappa light chain sepharose column, dialysed against PBS at pH 7.2 (PBS), concentrated by Amicon Ultrafiltration, frozen for storage, and used at a dilution of 1:100. Goat anti-mouse μ chain antibody, purified on an affinity column, was a gift from Dr. M.D. Cooper, University of Alabama, Birmingham, AL. Rhodamine isothiocyanate (TRITC)-conjugated affinity purified goat anti-mouse μ (Kirkegaard & Perry, Gaithersburg, MD) was diluted 1:10 in PBS at pH 7.2.

2. T lineage:

Hybridoma cells producing the rat mAb anti-Thy1.2 (IgM) (clone AT83A; ATCC) were grown in our laboratory, affinity

column-purified, concentrated, dialysed and diluted 1:20. The alloantiserum anti-Thy1.2 (AKR anti-C3H) was provided by Dr. A. Ahmed, NIH, Bethesda, MD) and used at 1:20 dilution. The rat mAb anti-L3T4 (IgG_{2a}) (clone GK1.5; ATCC) was used as an ascites fluid and diluted 1:50. The affinity column-purified rat mAbs anti-Lyt1.2 and anti-Lyt2.2 (IgG_{2a}) were purchased from Becton Dickenson, Mountain View, CA, and used at 1:20 dilution.

3. *NK lineage:*

The mouse mAb anti-NK1.1 (IgG_{2b}) was obtained from Dr. G.C. Koo, Merck, Frosst and Dohme, Rahway, NJ, and used as a hybridoma culture supernatant fluid at 1:20 dilution (Koo & Peppard, 1984). Affinity-purified rabbit anti-asialo GM1 antibody (IgG) (Wako Chemicals, Dallas, TX) was diluted 1:50.

4. *Secondary antibodies:*

Affinity purified antibodies, goat anti-rat IgG and goat anti-rabbit IgG (heavy and light chain specific; Kirkegaard and Perry), and rabbit anti-mouse IgG (γ heavy chain specific; Cappel Scientific, Malvern, PA) were radiolabeled by a modification of the chloramine-T method (Greenwood et al, 1963) and used at a concentration of 2 μg/ml. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (heavy and light chain specific; Cooper Biomedical Inc., Malvern, PA) was diluted 1:10 in PBS.

5. ***Anti-sheep red blood cells:***

Sheep red blood cells (SRBC; Institut Armand Frappier, Laval-des-Rapides, PQ), stored in Alsever's solution, were washed in sterile PBS by centrifugation, mixed with equal volumes of Freund's incomplete adjuvant (GIBCO), injected (2 x 10^8 cells/0.2 ml) ip into C3H/HeJ mice and bled 14 days later by intracardiac puncture (Yang & Osmond, 1979). After allowing the blood to clot overnight at 4°C, the serum was separated (1000g, 20 min) and heat inactivated (57°C, 30 min) to destroy serum complement. The serum antibodies were titrated and used at a 1:20 dilution.

All antibodies were ultracentrifuged at 140,000g on a Beckman Airfuge (Beckman Instruments, Palo Alto, CA) for 20-30 min to remove any aggregates (Trautman & Cowan, 1968).

Radioiodination of antibodies

Purified antibodies and protein A from Staphylococcus aureus (Pharmacia, Dorval, PQ), both at a concentration of 1 µg/1 µl, were coupled to ^{125}I by the chloramine-T method (Greenwood et al, 1963). For example, 25 µg of goat anti-rat IgG was incubated with 1.1 mCi of carrier-free Na-I^{125} (specific activity 1.5 x 10^7 µCi/mg) and 5 µl chloramine T (2 mg/ml) for 10 min on ice. This reaction was then terminated by the addition of 5 µl of potassium metabisulfite (4.8 mg/ml) and chased with 20 µl of 0.1M potassium iodide containing

potassium metabisulfite. The sample was passed over a pre-packed G25 Sephadex column (Pharmacia) which allowed for the separation of the protein-bound iodine from the unbound iodine. One minute fractions were collected from the column. Five μl samples from each fraction were analyzed on a Beckman Gamma counter (model 4000; Beckman Instruments, Irvine, CA) to count the amount of radioactivity in each sample (counts per minute [CPM]). In order to determine the efficiency of the radiolabeling procedure, 10% trichloroacetic acid (TCA; Sigma) in water was added to the 5 μl samples to precipitate all proteins. Following the centrifugation of the samples (2000g, 30 min) the supernatants were decanted and the radioactivity of the resulting pellets was enumerated (CPM) on the Gamma counter. Counts from TCA-precipitated samples were compared with untreated samples and the percent incorporation of I^{125} was determined. As a representative sample of the method of recovering radiolabeled antibody molecules, figure 1 illustrates the radioactivity (in cpm) of the total ^{125}I-goat anti-rat IgG fractions and the TCA precipitable protein. The first peak (left) represents radioiodinated protein, whereas the smaller second peak (right) is the unbound ^{125}I. The TCA precipitable protein curve indicates the actual amount (in cpm) of ^{125}I that is bound to the antibody. For example, within fraction 7, over 90% of the radiolabel is bound to the antibody, as opposed to only 5-10% in fraction 14. Since fractions 7, 8 and 9 contain the highest proportions of

precipitable protein in cpm's, these fractions were pooled, refrigerated with 0.1% sodium azide (Sigma), and were used at a concentration of 2-4 μl/ml.

Radioautographic detection of:

1. *Surface μ chains and Thy1.2:*

To detect surface μ chains on B lymphocytes, cell suspensions from C3H/HeJ mice (4 x 10^6 cell/0.1 ml) were exposed to an equal volume of ^{125}I-goat anti-mouse μ (0°C, 30 min), washed free of any unbound antisera after each incubation by refrigerated (4-10°C) centrifugation (200g, 7 min; IEC centra 7-R, Needham Heights, MA) through 6% bovine serum albumin (BSA; Sigma) in PBS. Cells were then smeared onto glass slides precoated with 0.5% gelatin and 0.05% chrome alum, fixed in pure methanol (4 min) and processed for radioautography. Similar cell concentrations, incubation times and washing procedures were used with all surface labeling protocols. Thy1.2-bearing cells were detected by incubating cell suspensions with anti-Thy1.2 alloantiserum, washed, then exposed to ^{125}I-protein A, washed again, smeared, fixed in pure methanol, and processed for radioautography.

2. *Cytoplasmic μ chains:*

Washed cells from C3H/HeJ mice were smeared onto gelatin-coated glass slides and fixed in 5% acetic acid in ethanol for

30 min at -20°C (Raff et al, 1976; Cooper, 1981) allowing penetration of the radiolabeled antibodies. Slides were then washed four times in PBS, pH 7.2, and covered with 30% normal goat serum (NGS) (GIBCO, Grant Island, NY) for 30 min at room temperature. This initial masking step was shown to be important to minimize subsequent non-specific background binding of radioiodinated goat anti-mouse μ to the fixed marrow cells. The slides were washed once by immersing them into 1% NGS in PBS (5 min) and incubated with ^{125}I-anti μ for 30 min in a humidified chamber at room temperature. The slides were washed again with 1% NGS (10 min), then dehydrated in 30%, 50%, 70% and 100% methanol for 4 min each and processed for radioautography, as shown in Figure 2. After fixing the cells with 5% acetic acid in ethanol, the ^{125}I-anti-mouse μ antibody bound to both surface and intracellular μ chains, indistinguishable in radioautographs. The proportion of cells bearing cμ chains only was obtained by either subtracting the incidence of sμ^+ cells obtained from the surface labeling protocol in normal mice or using anti-IgM treated mice which are devoid of sμ^+ B cells. In control experiments a specific binding assay was performed to assess the specificity of the radioautographic labeling by ^{125}I-anti-μ. Control slides were incubated with a 500-fold excess of either unlabeled anti-μ or an irrelevant protein such as BSA, in addition to the radiolabeled anti-μ.

3. 14.8$^+$, Thy1$^+$ and null cells:

Cell suspensions from either C3H/HeJ or C57BL/6 mice were exposed to rat mAbs 1) 14.8, or 2) anti-Thy1.2, or 3) both 14.8 and anti-Thy1.2, or 4) 10% NCS in MEM alone in control samples (30 min, 0°C). Cells were washed by centrifugation, incubated with ^{125}I-goat anti-rat IgG (30 min, 0°C), washed again, smeared on gelatin-coated slides, fixed in pure methanol (4 min), and processed for radioautography (Fig. 3).

4. NK1.1$^+$ cells:

Bone marrow and spleen cell suspensions from C57BL/6 or (C3H/HeJ x C57BL/6)F_1 mice were incubated with the mouse mAb anti-NK1.1 (IgG_{2b}) (30 min, 0°C), washed by centrifugation, then detected by ^{125}I-rabbit anti-mouse IgG (γ chain specific), washed again, smeared and processed for radioautography. NK1.1 antigen was restricted to certain strains of mice including C57BL/6 and their F_1 hybrids (Koo & Peppard, 1984). NK1.1$^-$ mouse strains including C3H/HeJ were used as negative controls. The NK1.1 antigen was first described by Glimcher et al (1977) to be specific for NK cells. Since then, Koo and Peppard (1984) have obtained a monoclonal anti-NK1.1 antibody. Hackett et al (1986) showed that NK1.1$^+$ cells sorted by the fluorescence activated cell sorter (FACS) contained all the NK activity to the YAC lymphoma target. In vivo administration of mAb anti-NK1.1 soon after birth, a protocol mimicking anti-IgM treated mice (Osmond & Gordon, 1979) specifically depleted

mature cytotoxic NK cells but not their precursors (Koo et al, 1986).

5. *Asialo GM1:*

Bone marrow and spleen cell suspensions from C3H/HeJ and anti-IgM treated mice were incubated with a rabbit antibody directed against the glycosphingolipid macromolecule ganglio-N-tetraosylceramide (rabbit anti-asialo GM1) which was found to be expressed by NK cells (Kasai et al, 1980; Young et al, 1980; Habu et al, 1981; Beck et al, 1982). Cells were then washed, exposed to radioiodinated anti-rabbit IgG, washed again, smeared, fixed and processed for radioautography.

6. *Lyt1, Lyt2, L3T4:*

Cell suspensions were exposed to rat mAbs 1) anti-Lyt1, or 2) anti-Lyt2, or 3) both anti-Lyt1 and Lyt2, or 4) anti-L3T4, or 5) medium alone as control. Cells were washed and detected by ^{125}I-goat anti-rat IgG, washed, smeared, fixed in methanol and processed for radioautography.

Computation of the incidence of Lyt antigen-bearing cells

Antibody used	Labeled population
(a) anti-Lyt1	Lyt1 and Lyt1,2
(b) anti-Lyt2	Lyt2 and Lyt1,2
(c) anti-Lyt1 + anti-Lyt2	Lyt1, Lyt2, and Lyt1,2

(c) - (b) = Lyt1$^+$ cells
(c) - (a) = Lyt2$^+$ cells
(a) + (b) - (c) = Lyt1,2$^+$ cells

Radioautography

Fixed slides were dipped in melted Kodak NTB-2 photographic emulsion (diluted 2 emulsion: 1 water; Eastman Kodak Co., Rochester, NY) by a modification of the method of Kopriwa and Leblond (1962) and exposed for one to several days (depending on the antibody used) at 4°C. After developing and fixing the emulsion, the slides were immersed in PBS, pH 6.4 (5 min), 50% methanol in PBS, pH 6.4 (3 min), and 100% methanol (1 min). Slides were then stained through the emulsion with MacNeal's tetrachrome (Sigma) mixed with PBS, pH 6.4 (1:2), rinsed with PBS, pH 6.4, blotted dry and coverslipped using Eukitt mounting medium (Fisher Scientific, Fairlawn, NJ).

Analysis of cytology and radioautographs

Using light microscopy at 1000 X magnification (oil immersion) and MacNeal's tetrachrome stain, small lymphocytes were identified as cells less than 8.0 μm mean nuclear diameter when measured with an ocular micrometer. Their characteristic morphology is a high nucleus to cytoplasmic ratio with a small amount of undifferentiated cytoplasm at one pole of the cells (Yoffey et al, 1965; Miller & Osmond, 1973; Rosse, 1976). Large lymphoid cells have similar morphologic characteristics as the small lymphocytes, but they measure greater than 8 μm in nuclear diameter and have leptochromatic nuclei. The incidence of labeled and unlabeled lymphoid cells

versus other nucleated cells was determined by differential counts scanning longitudinally along the middle of each smear, ensuring sampling of all cell size and types. Over 2000 small lymphocytes were counted per smear.

The threshold of positively labeled small lymphocytes in preparations exposed to radiolabeled antibodies was determined by scoring silver grains over cell-free unit areas of approximately the same area as small lymphocytes. Counts were usually 0-2 grains per unit area and rarely more than 5 grains per unit area (0.05%, >10 grains). Cells were scored as labeled with more than 10 grains, well above background values, unless otherwise specified.

Null small lymphocytes were recorded as cells which remained unlabeled after combined exposure to both mAb 14.8 and mAb anti-Thy1.2, using a labeling threshold of 10 grains per cell, well above background. In each experiment, the proportion of null small lymphocytes was also obtained by subtracting the incidences of 14.8^+ and $Thy1^+$ small lymphocytes, determined separately, from the total number of small lymphocytes. The absolute numbers of small lymphocytes in each subpopulation per femur and spleen were quantitated by combining the incidence of either null, 14.8^+, or $Thy1^+$ cells, their differential counts in stained preparations and the total nucleated cells (TNC) per organ. The number of bone marrow nucleated cells from a single femur represents approximately 6% of total body bone marrow, and have therefore

multiplied the absolute number per femur by 15.8 (Benner et al, 1981):

1. Abs. no. of lymphocytes in a subpopulation =

(% of subpopulation) X (differential count) X (TNC/organ).

2. % of normal =

$$\frac{\text{Abs. no. of lymphocytes in subpopulation (exp.)}}{\text{Abs. no. of lymphocytes in subpopulation (contr.)}} \times 100$$

Double labeling procedures for detecting NK cell-associated markers.

Following exposure to mAb 14.8, anti-Thy1.2 and ^{125}I-anti-rat IgG, as described above, bone marrow cells were subjected to the following techniques for detecting NK cell-associated markers on null cells.

1. Immunoperoxidase labeling of NK1.1$^+$ cells:

This double labeling technique revealed null cells by radioautography while simultaneously detecting NK1.1 by immunoperoxidase in hematologically stained radioautographic preparations. Bone marrow cell suspensions from C57BL/6 mice were incubated (0°C, 30 min) with mAb anti-NK1.1, washed, then incubated with rabbit anti-mouse IgG (γ chain specific),

washed, and finally incubated with biotinylated goat anti-rabbit IgG (1:100 dilution; ABC Vectastain, Burlingame, CA). Most of the surface labeling steps were done on unfixed cell suspensions so as to maximize the binding of antibodies while preserving antigenicity. The cells were then cytocentrifuged at 1100 rpm for 5 min (Cytospin; Shandon Elliott, Sewickly, PA) onto gelatin-coated glass slides and air dried quickly to prevent cell shrinkage. Slides were fixed in 100% methanol (30 min, 0°C), washed in PBS pH 7.2 and exposed to 3.0% H_2O_2 (10 min) to block endogenous peroxidase in erythroid and myeloid cells. A preformed complex of avidin (having 4 biotin-binding sites) linked to 3 biotin molecules, each of which was conjugated to a peroxidase molecule, was allowed to bind to the biotinylated goat anti-rabbit IgG, a reaction having an affinity constant ($10^{15}M^{-1}$) a million times higher than that for most antibody-antigen complexes. The peroxidase enzyme promoted the reduction of H_2O_2 and oxidized the chromogen 3,3'- diaminobenzidine (DAB; Sigma), resulting in an insoluble black-brown precipitate (Graham & Karnovsky, 1966). Slides were then processed for radioautography (Fig. 4). $NK1.1^+$ cells were recognized by the presence of granular DAB reaction product on the cell surface, clearly distinguished from any overlying radioautographic silver grains.

2. *Tumor cell binding:*

Using a modification of the tumor target binding assay (Roder & Kiessling, 1978; Roder et al, 1978; Pollack & Rosse, 1987), 5.0 x 10^4 prelabeled bone marrow cells were mixed (1:5 ratio) with viable YAC.1 lymphoma cells using a 96 well, round bottom microplate (Nunc, Denmark), centrifuged gently at 100g for 5 min to form a soft pellet. The cell mixture was allowed to stand for 30 min on ice and then the cell pellets were gently aspirated 5-10 times with a Pasteur pipette, cytocentrifuged (500 rpm, 5 min) onto gelatin-coated slides, fixed in methanol, and processed for radioautography (Fig. 5). Lymphocyte-target conjugates usually had one lymphocyte closely bound to one tumor target cell which was characterized by its large size and cytoplasmic projections. This technique allowed a precise identification of cells in stained radioautographic preparations in contrast to the wet mount preparations examined in phase contrast microscopy by Roder and Kiessling (1978). Bone marrow and spleen cell suspensions from age-matched A/Sn mice were used as controls because of the low NK activity and tumor cell binding capabilities of this strain as opposed to the higher NK activity of the C3H/HeJ and C57BL/6 strains (Kiessling et al, 1975; Roder & Kiessling, 1978; Herberman, 1982).

3. *Fc receptor (FcR) rosetting:*

Radioautography and rosetting techniques were combined to

study the expression of FcR on null small lymphocytes. To prepare antibody coated SRBC's, washed sheep erythrocytes (60 x 10^6 cells/2.0 ml PBS) were incubated with an equal volume of mouse anti-SRBC (30 min., 37°C) and washed four times. Prelabeled bone marrow cells were then mixed with antibody-coated SRBC's at a 1:30 ratio, centrifuged to form a soft pellet (100g, 5 min), incubated at 37°C for 30 min, and resuspended in 10% NCS in MEM. Cell preparations were then cytocentrifuged (500 rpm, 5 min) onto gelatin-coated slides, fixed in methanol and processed for radioautography (Yang & Osmond, 1979) (fig. 6). Erythrocytes attached to individual labeled and unlabeled small lymphocytes were recorded. Lymphocytes binding 4 or more erythrocytes were scored as rosettes. The incidence of control rosettes using uncoated SRBC's was subtracted from experimental rosettes, giving specific rosette indices (Yang & Osmond, 1979).

Immunofluorescent double labeling of 14.8^+ cells and μ chains

Bone marrow cells were examined by double immunofluorescent labeling to correlate the binding of mAb 14.8 with cells expressing μ chains (both $c\mu$ and $s\mu$). Bone marrow cell suspensions (4 x 10^6 cells/0.1 ml) were incubated with an equal volume of mAb 14.8 (30 min, 0°C), washed twice through NCS by centrifugation (200g, 5 min), exposed to FITC-goat anti-rat IgG (30 min, 0°C), washed again, and resuspended in a medium containing 0.15M NaCl, 2.7 mM disodium EDTA

(Fisher Scientific Co.), and 5% BSA (Sigma) in water. Cells (10^5) were then cytocentrifuged (1100 rpm, 5 min) onto glass slides precoated with 3% BSA in PBS, fixed in 5% acetic acid in absolute ethanol (12 min, 0°C) to allow penetration of the fluorochrome-labeled antibodies (Raff et al., 1976; Opstelten & Osmond, 1983), then washed four times in PBS. Excess buffer was blotted off the fixed slides, not allowing the cytospot of cells to dry, and exposed to TRITC-anti-mouse μ (30 min, in a humidified chamber at room temperature) labeling all μ chains (Opstelten & Osmond, 1983; Park & Osmond, 1987). Slides were then washed in PBS overnight, mounted in a medium containing 90% glycerol in PBS, pH 8.0, 0.1% p-phenylenediamine (Fisher Scientific Co.) to preserve the fluorescence during microscopy (Johnson & de C. Nogueira Araujo, 1981), and examined by phase contrast microscopy (x 100, oil immersion objective) using an epifluorescence microscope (Carl Zeiss of Canada Ltd., Don Mills, Ontario) with a HBO50 mercury lamp.

Individual marrow cells (>1000 nucleated cells) were recorded for the presence of 1) FITC alone (14.8^+), 2) TRITC alone (total μ; both cμ and sμ), and 3) double labeling with FITC and TRITC (14.8^+, μ^+).

Natural killer (NK) cell cytotoxic assay.

1. Tumor cell line:

The YAC.1 lymphoma line, induced by the Moloney virus in A/SN mice (Kiessling et al, 1975), was originally obtained

from Karolinska Institute, Sweden. The lymphoma was maintained, in vitro, in 10% fetal calf serum in RPMI-1640 medium supplemented with penicillin, streptomycin, and Fungizone (GIBCO), and kept at 37°C in 5% CO_2 in air. The YAC.1 tumor cell cultures were fed three times a week by replacing an equal volume of suspended cells with fresh media. Cell viabilities were done by the Trypan blue (0.4%) dye exclusion test using a hemocytometer. Non-viable cells were more permeable than viable cells and thus became stained by the dye. Target cells with a viability of >90% were used throughout the experiments.

2. *Radiolabeled tumor target cells:*

YAC.1 lymphoma cells (5 x 10^6 cells) were incubated with 200 μCi $Na_2{}^{51}CrO_4$ (Charles E. Frosst, Kirkland, Que) (37°C, 30 min), and washed three times by centrifugation (Kiessling et al, 1975).

3. *Cytotoxic assay:*

NK activity was assayed in a short term ^{51}Cr release assay in round-bottomed 96 well microtiter plates (Nunc) (Kiessling et al, 1975; Herberman, 1982). A graded number of effector cells were mixed with labeled target cells ($5x10^4$) at effector to target (E:T) ratios of 20:1, 10:1 and 5:1, and the mixture was incubated for 3.25 hours at 37°C, then a further 45 min at 45°C in a humidified atmosphere at 5% CO_2 in air. Spontaneous

^{51}Cr-release (SR) from the $5x10^4$ radiolabeled YAC.1 cells was measured by omitting the effector cells. The total incorporation (TI) of ^{51}Cr (maximum release) was measured by incubating the radiolabeled YAC.1 cells with the detergent Zaponin (Coulter Electronics), causing complete cell lysis. The microtiter plates were centrifuged (400g, 10 min) and the supernatants containing the released radioactivity were harvested (10μl aliquots) and counted in a Beckman gamma counter. The degree of lysis of tumor cells was measured by the cytotoxic index and calculated by using the following formula:

$$\% \text{ specific lysis} = \frac{\text{cpm (Exp)} - \text{cpm (SR)}}{\text{cpm (TI)} - \text{cpm (SR)}} \times 100$$

Lymphocyte-rich fractions from bone marrow cell suspensions

Washed bone marrow cell suspensions were layered onto sucrose-serum continuous density gradients of 5 to 15% sucrose in 20% NCS (5% sucrose in 50% Hanks balanced salt solution in water; 15% sucrose in water). The gradients were centrifuged for 8 min (room temperature) in a 14 ml plastic tube and a slowly sedimenting lymphocyte-rich band (85-90% lymphocytes) was recovered by inserting an 18 gauge needle through the side of the tube (Yoshida & Osmond, 1971). Cells were washed, counted, and used in the NK cell cytotoxic assay.

In vitro augmentation of NK activity by interferon

Mouse interferon alpha (αIFN) was purchased from Lee BioMolecular Research Laboratories, Inc., San Diego, CA. For αIFN-induced augmentation of NK activity in vitro, 0.2 ml of bone marrow cells (either whole or fractionated) containing 20 X 10^6 cells in 1% fetal calf serum in RPMI 1640 medium (GIBCO) was added to 0.2 ml of medium containing 7.5 X 10^3 units of αIFN (Djeu et al, 1979, 1980) The cells were then incubated for 2 hours at 37°C in 5% CO_2 atmosphere and diluted in the same medium when testing the NK cell activity.

Graphics and computer analyses

The computer generated graphics were performed on a McIntosh IIx, Apple Computers, Mountain View, CA. The iterative analysis of the polynomial regression line was done on Hewlett Packard series 9000 (model 216 microcomputer), Fort Collins, CO.

Fig. 1. Radioiodination of the antibody goat anti-rat IgG. Profile of the radiactivity content (in cpm) of ^{125}I-goat anti-rat IgG fractions (o) and the TCA-precipitable counts (•) eluted from a sephadex G-25 column. The first peak (left) represents ^{125}I bound to protein, whereas the second peak (right) shows unbound ^{125}I.

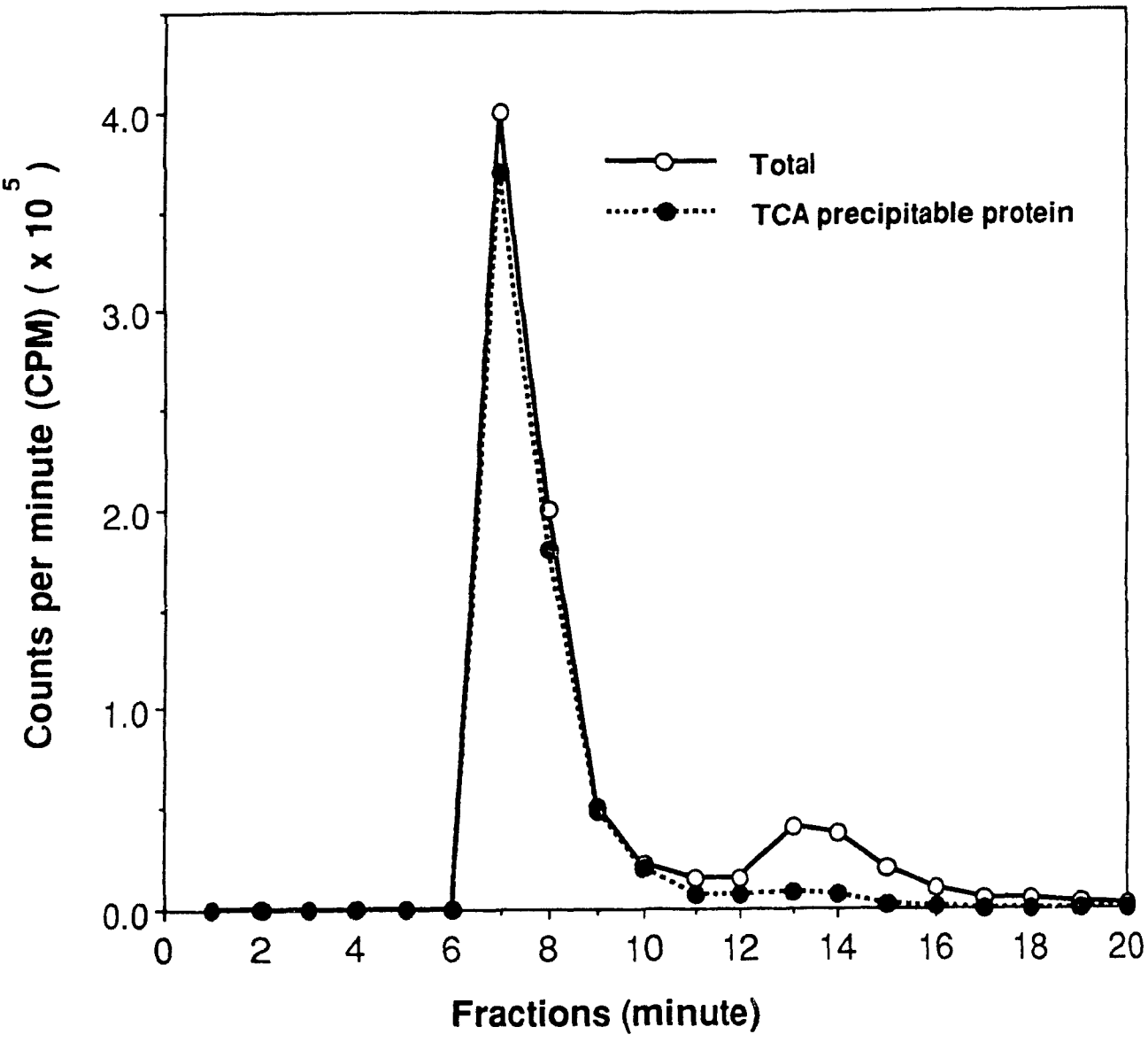

Counts per minute (CPM) (x 10 5)
4.0
3.0
2.0
1.0
0.0
0
2
4
6
8
10
12
14
16
18
20
Fractions (minute)
Total
TCA precipitable protein

Fig.2 **Radioautographic Detection of Cytoplasmic μ Chains**

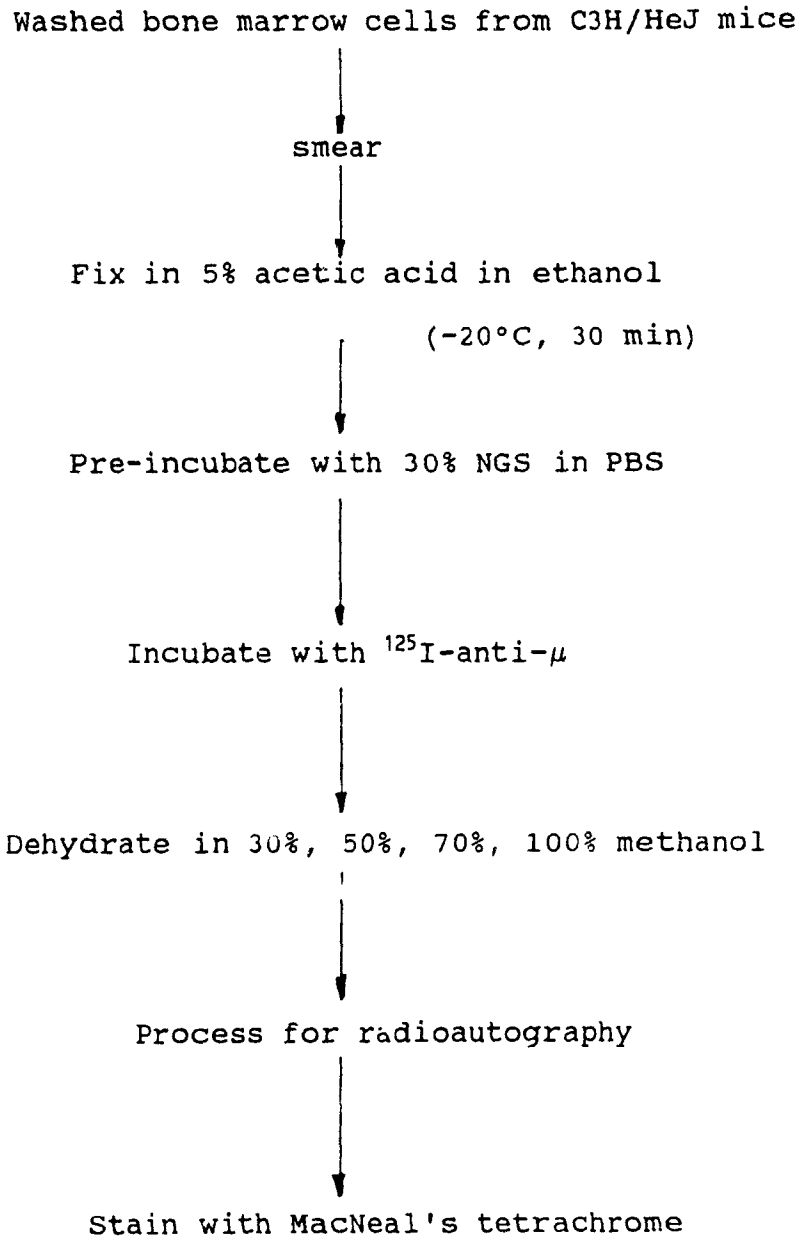
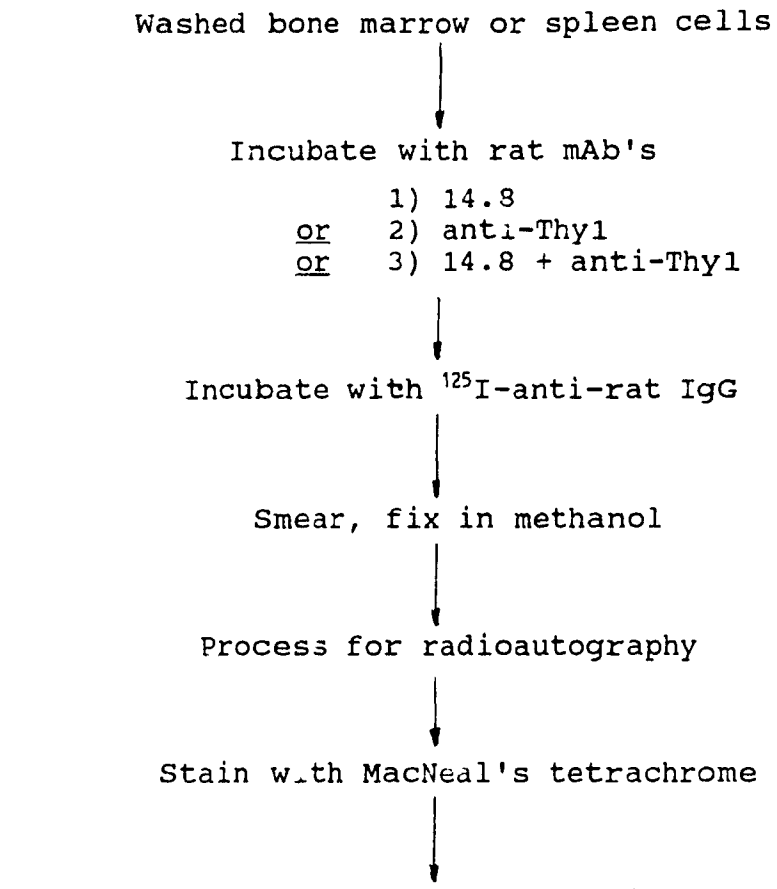


Fig. 3. **Radioautographic Detection of 14.8, Thy1$^+$ and Null Cells**



N.B. Null small lymphocytes remain unlabeled after combined exposure to mAb's 14.8 and anti-Thy1.

Fig. 4. **Double Labeling Procedure for Detecting NK1.1 on Null Lymphocytes**

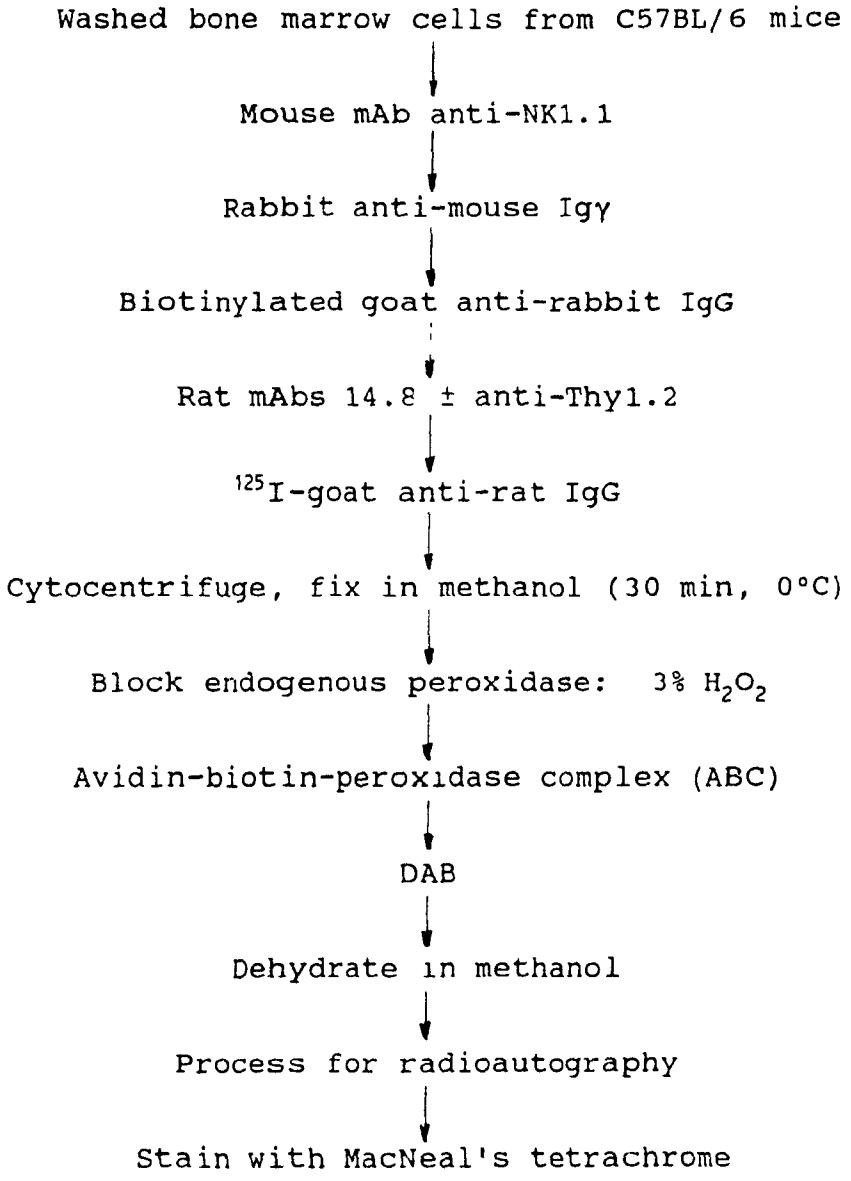


Fig. 5 **Tumor Cell Binding Assay**

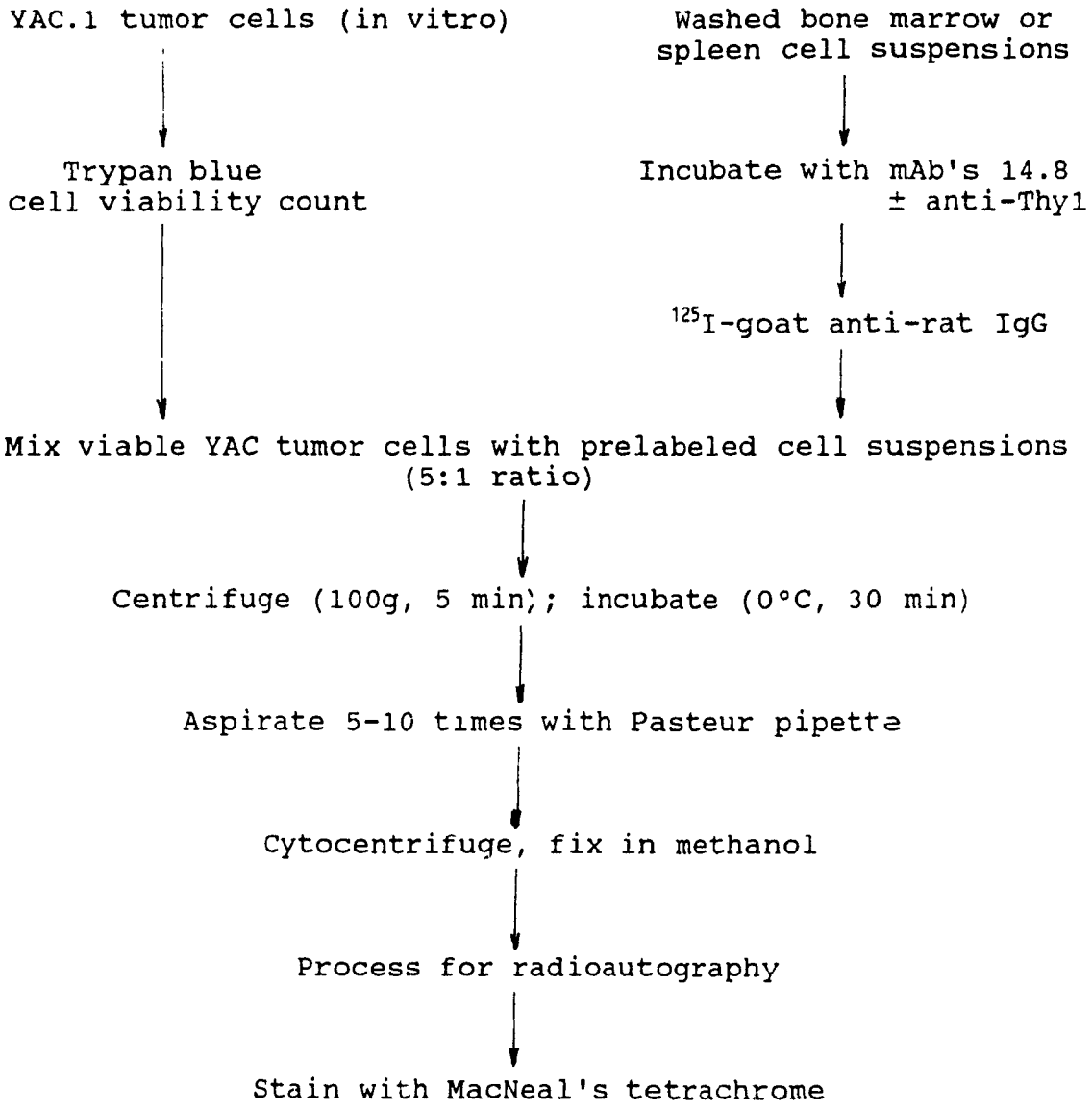
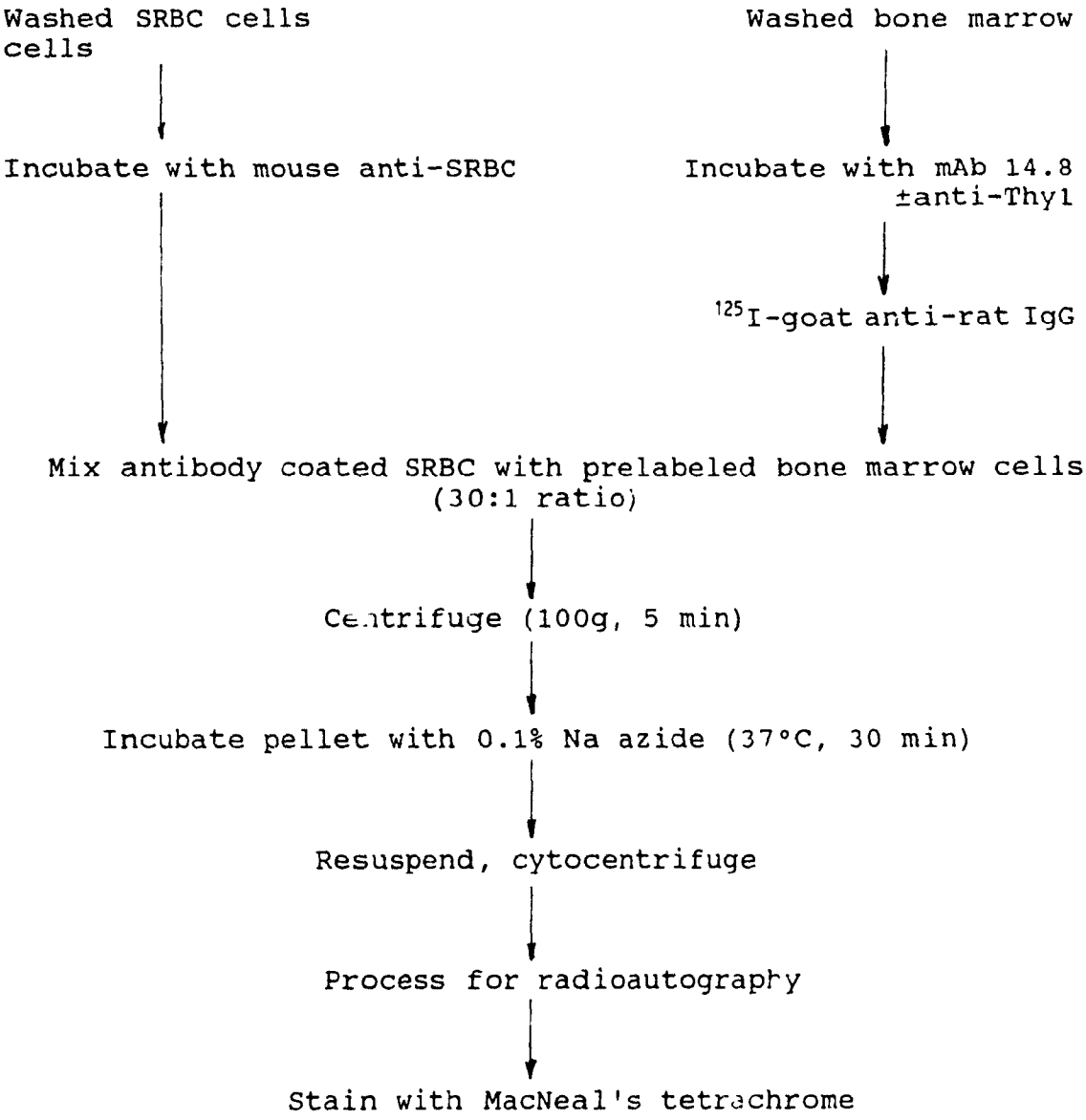


Fig.6 **Fc Receptor Erythrocyte Rosetting**



RESULTS

SECTION I

The general aim of this section was to devise a radioautographic method of detecting the presence of cytoplasmic μ chains among bone marrow lymphoid cells, in order to reveal any null lymphocytes that are devoid of B (μ chains) and T (Thy1) lineage markers.

1. Radioautographic labeling of μ chain-bearing small lymphocytes in the bone marrow

The exposure of fixed bone marrow smears to ^{125}I-anti-μ antibody resulted in an intense radioautographic labeling of marrow lymphoid cells of various sizes (Fig. 7). The population identified as small lymphocytes included a majority of cells labeled with more than 20 radioautographic grains per cell (Fig. 8). When incubated with ^{125}I-anti-μ and a 500-fold excess of nonradioactive anti-μ antibody, however, the small lymphocytes almost entirely had less than 20 grains per cell (Fig. 8). In contrast, a similar excess of an unrelated protein, BSA, produced little displacement of ^{125}I-anti-μ labeling (data not shown). Thus, under the prevailing conditions, high intensity radioautographic labeling (>20 grains per cell) represented specific binding of ^{125}I-anti-μ to μ chains, forming the criterion of positive labeling in

subsequent work. Control counts of other cell types showed only minimal (1-2%) labeling over thymus cells, when similarly fixed and incubated with ^{125}I-anti-μ, while only a few (4%) of the mature granulocytes in the marrow preparations exhibited weak, near-threshold labeling (data not shown).

2. Size distribution and incidence of total lymphoid cells, $s\mu^+$ cells, and $c\mu^+s\mu^-$ cells in normal bone marrow determined by radioautography

Figure 9a shows the size distribution of morphologically identified lymphoid cells in bone marrow smears that had been fixed and exposed to ^{125}I-anti-μ. Approximately 80% of the cells were small lymphocytes ranging in size from 5.0 to 8.0 μm nuclear diameter (mode, 7.0 μm), the remainder being large lymphoid cells, up to 15 μm nuclear diameter. Among these cells of all sizes, many but not all were labeled by ^{125}I-anti-μ, and were thus either $s\mu^+$ or $c\mu^+$ (Fig. 9a). About 80% of the small lymphocytes and 40% of the large lymphoid cells were μ chain-bearing cells by these criteria (Table 3). This technique, however, does not distinguish $c\mu$ from $s\mu$ chains.

The size distribution of $s\mu^+$ cells was determined separately by exposing viable marrow cells in suspension to ^{125}I-anti-μ before smearing and fixing (Fig. 9b). The size profile of the total lymphoid cells resembled closely that seen before (Osmond & Nossal, 1974a), while the $s\mu^+$ cells were mainly (>85%) small lymphocytes (Fig. 9b), accounting for

approximately 50% of the small lymphocyte population. The few $s\mu^+$ large lymphoid cells were lightly labeled (20 grains/cell), in contrast to the other $c\mu^+$ lymphoid cells which were heavily labeled (>100 grains/cell).

Because of the consistent cell size conditions in the two experiments, the size distribution of $c\mu^+s\mu^-$ pre-B cells (Fig. 9c) could be obtained by subtraction of $s\mu^+$ cells (Fig 9b) from the profiles of total μ chain-bearing cells (Fig. 9a). The resulting curve showed an apparently bimodal distribution of $c\mu^+s\mu^-$ cells which ranged from 5 to 14 μm nuclear diameter, accounting for approximately 30% of the cells identified as small lymphocytes and 40% of the large lymphoid cells. Collectively, the $c\mu^+s\mu^-$ cells constituted 11.8% of the total nucleated cell population of the marrow, equivalent to approximately 1.7×10^6 cells per femur.

3. Size distribution and incidence of $c\mu^+s\mu^-$ cells in the bone marrow of anti-IgM treated mice, determined by radioautography

To detect $c\mu^+s\mu^-$ cells directly by ^{125}I-anti-μ labeling, the radioautographic technique was applied to bone marrow, spleen, and thymus of mice from whom $s\mu^+$ cells had been deleted by repeated administration of anti-IgM antibodies from birth, as described previously (Manning & Jutilia, 1972; Burrows et al, 1978; Osmond & Gordon, 1979). Radioautographic cell surface labeling confirmed that no small lymphocytes in the bone marrow and spleen of the anti-IgM treated mice showed

detectable $s\mu^+$ whereas about half did so in control mice (Table 1). Of the residual small lymphocytes in anti-IgM treated mice, the large majority (>2%) were "null" cells ($s\mu^-$ $Thy1^-$) in the bone marrow and $Thy1^+$ cells in the spleen.

The elimination of $s\mu^+$ cells from the bone marrow of anti-IgM treated mice was reflected by a reduction (65% of normal) in the proportion of cells which fell under the category of small lymphocytes in a size distribution profile of total lymphoid cells (Fig. 10). Exposing fixed smears to ^{125}I-anti-μ again labeled a wide size range (5-14 μm diameter) of lymphoid cells: in the absence of $s\mu^+$ cells these represented $c\mu^+s\mu^-$ cells (Fig. 10). As in normal mice, the size distribution of $c\mu^+s\mu^-$ cells tended to be bimodal, but they accounted for almost 60% of the residual small lymphocytes and 40% of large lymphoid cells (Fig. 10). Parallel studies of spleen and thymus cells of anti-IgM treated mice showed no labeled cells by this technique as compared to NRS control mice (Fig. 11) while only 1-2% mature marrow granulocytes showed grain counts slightly above the labeling threshold (data not shown).

4. Size distribution and incidence of bone marrow lymphoid cells lacking cytoplasmic and surface μ chains

A striking feature of the present technique was that, even among small lymphocytes, a substantial number of bone marrow lymphoid cells showed no detectable μ chains either at

the cell surface or in the cytoplasm, and were thus not identifiable as B lineage cells by these criteria. The incidence and size distribution of such μ^- cells were derived by subtracting the profile of all μ chain bearing cells ($c\mu^+$ and $s\mu^+$) detected by ^{125}I-anti-μ labeling in fixed smears from that of the total lymphoid cell population. The results are shown in Fig 12. The μ^- cells in both normal mice (Fig. 12a) and anti-IgM treated mice (Fig. 12b), included both small and large lymphoid cells, ranging in size from 5 to 14 μm nuclear diameter. Compared to the total lymphoid cells, however, the μ^- lymphoid cells tended to have a broader size distribution profile, shifted to the right, predominantly medium-sized rather than small cells (Figs 12a and b). The μ^- cells accounted for a higher proportion of the lymphoid cells remaining in the bone marrow of anti-IgM treated mice than in normal mice (Table 1).

When the μ^- cell populations of bone marrow from normal and anti-IgM treated mice were normalized, their size distribution profiles coincided closely (Fig 12c). While these profiles included the small number of Thy1.2$^+$ cells (Table 1), they predominantly represented cells lacking both μ chains and Thy1.2 antigen.

5. Size distribution, incidence, and labeling intensity of Thy1$^+$ lymphoid cells

The size distribution of Thy1$^+$ lymphoid cells, ranging

from 5 μm to 13 μm in diameter, was obtained by examining smeared preparations of bone marrow cell suspensions incubated with anti-Thy1.2, and then ^{125}I-protein A (Fig. 13). Approximately 8% of the small lymphocytes and 20% of the large lymphoid cells in the bone marrow expressed the Thy1 antigen (Fig. 13, Tables 2 & 3).

The radioautographic grain count distribution of labeled Thy1$^+$ small lymphocytes (>10 grains/cell) was determined in the bone marrow, and compared to grain densities in both thymus and spleen (Fig. 14). Most (80%) of the small lymphocytes in the bone marrow bearing Thy1 had a light to moderate labeling intensity (11-40 grains/cell), only 20% being heavily labeled (>40 grains). In contrast, nearly all (90%) thymic small lymphocytes expressed high densities of Thy1 (>40 grains/cell), and Thy1$^+$ spleen cells were intermediate in labeling intensity. Large lymphoid cells in the bone marrow had almost exclusively light to moderate labeling intensity (15-20 grains/cell).

6. Incidence of Lyt and L3T4 lymphoid cells in the bone marrow, spleen and thymus

The relative incidence and absolute number of lymphoid cells expressing markers defining certain T lineage subpopulations (L3T4; Lyt$^+$) were determined in bone marrow, spleen, and thymus (Table 2). The total number of small lymphocytes of each subset was calculated on the basis of the

total cellularity of the organs tested and differential counts of cells binding ^{125}I-labeled antibodies. The radioautographic technique detected the presence of small but significant populations of small lymphocytes in the bone marrow expressing markers belonging to T cell subpopulations (Table 2). The proportion of small lymphocytes in the bone marrow bearing either L3T4 (2.5%) or Lyt2 (1.6%) did not fully account for all the Thy1$^+$ small lymphocytes (8.3%) in the bone marrow (Table 2). The proportion of bone marrow small lymphocytes bearing Lyt1+2 was statistically negligible (0.37±0.3). Some (13%) large lymphoid cells in the bone marrow were L3T4$^+$ (Table 2).

7. Size distribution and incidence of null lymphoid cells (cμ^-, sμ^-, Thy1$^-$) in the bone marrow

The size distribution of null lymphoid cells in the bone marrow (Fig. 15) was obtained by subtracting the profile of Thy1$^+$ lymphoid cells (Fig. 15) from that of cμ^- lymphoid cells (Fig. 12a). Null lymphoid cells (cμ^-, sμ^-, Thy1$^-$) had a broad size distribution resembling that observed in anti-IgM treated mice (Fig. 12).

Table 3 summarizes the incidence and number of bone marrow small lymphocytes shown by the present technique to be immature B lineage cells (cμ^+sμ^-), B cells (sμ^+), and T cells (Thy1$^+$) as well as the cells revealed by these criteria to lack these B or T lineages markers. This population of μ^-Thy1$^-$

null cells formed a substantial subset of the bone marrow lymphoid cells, comprising more than ten percent of the small lymphocytes in normal bone marrow, one third of those in the bone marrow of anti-IgM treated mice, and almost half of the large lymphoid cells in normal bone marrow (Table 3).

Fig. 7. Radioautographic detection of μ chains in bone marrow stained cell preparations. One labeled large lymphoid cell and one unlabeled small lymphocyte after exposing fixed smears to ^{125}I-anti-mouse μ antibody (x 1500).

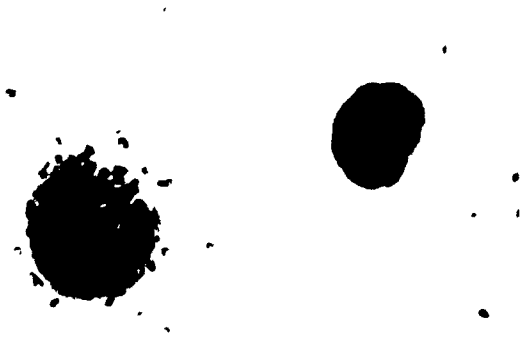


Fig. 8. Intensity of radioautographic labeling of small lymphocytes in fixed bone marrow smears exposed to ^{125}I-anti-μ antibody either alone (shaded area) or with a 500-fold excess of nonradioactive anti-μ (unshaded profile).

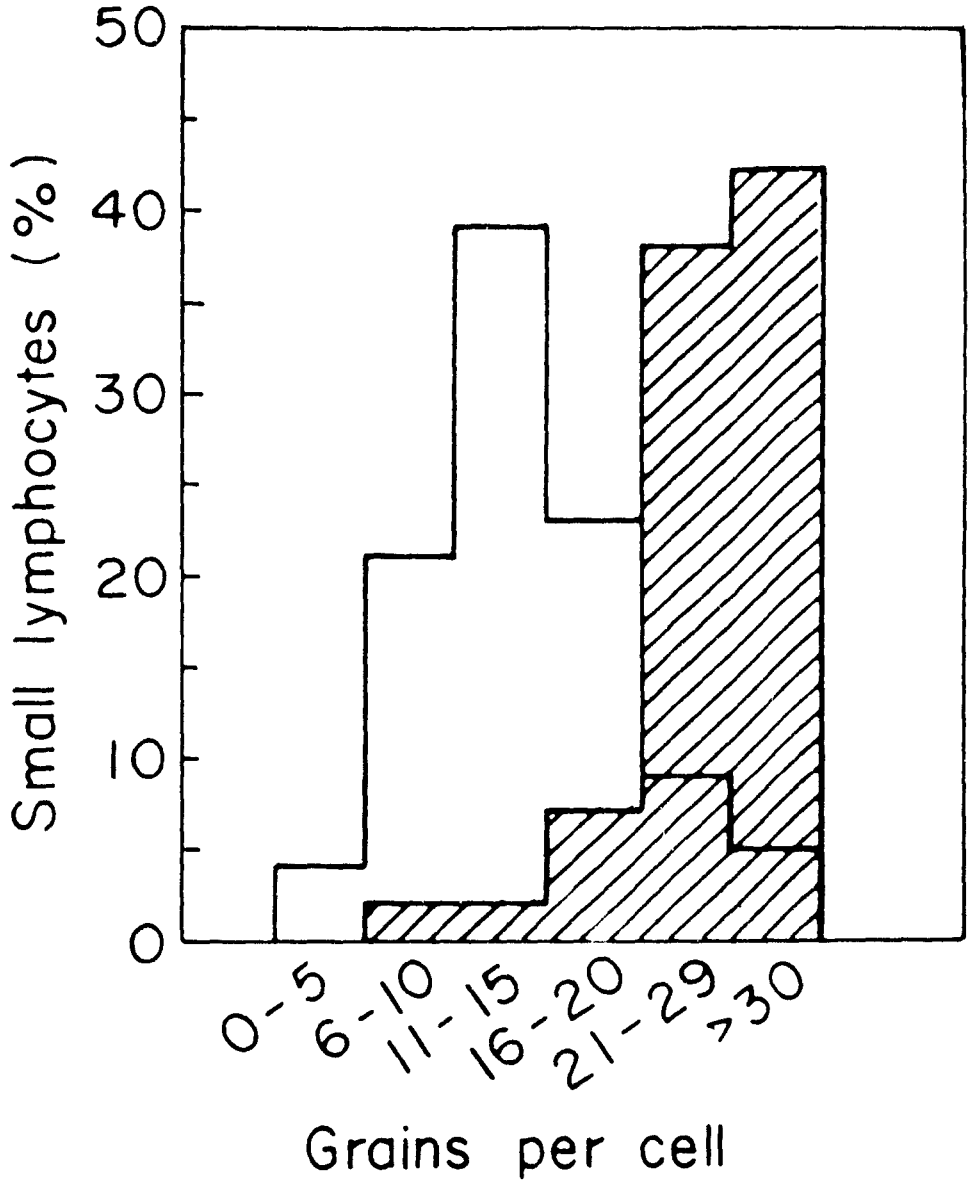

50
40
30
20
10
0
Small lymphocytes (%)
0-5
6-10
11-15
16-20
21-29
>30
Grains per cell

Fig. 9. Size distribution of μ chain-bearing cells among the lymphoid cells in normal bone marrow, as determined by radioautography. (a) Total identifiable lymphoid cells and those labeled by exposure to ^{125}I-anti-μ in fixed smears to detect both sμ and cμ. (b) Total lymphoid cells and those labeled by ^{125}I-anti-μ in suspension to detect sμ alone. (c) cμ^+ cells derived by subtracting the profile of sμ^+ cells, b, from total μ chain-bearing cells, a.

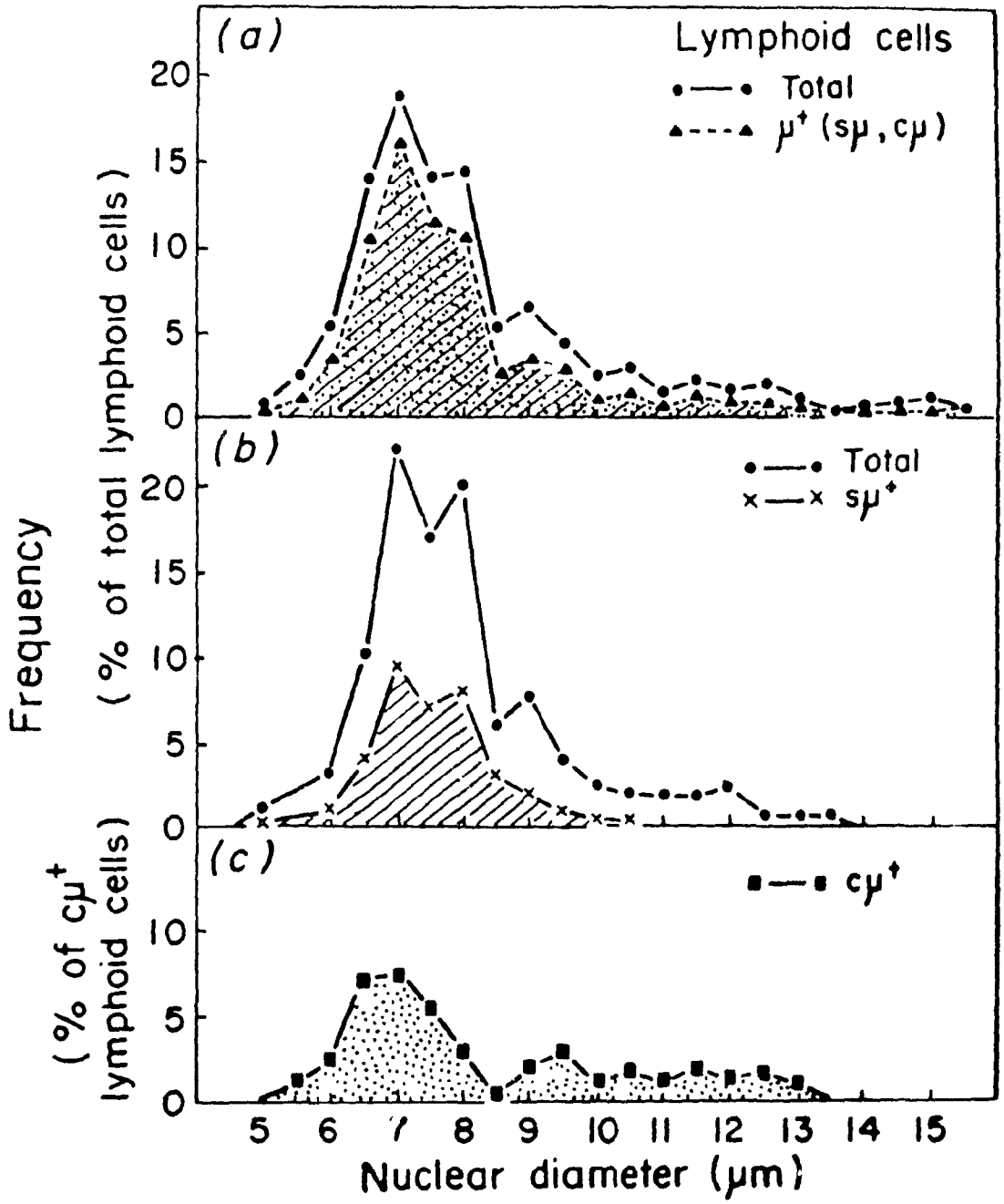

(a)
Lymphoid cells
Total
μ⁺ (sμ, cμ)
(b)
Total
sμ⁺
(c)
cμ⁺
Frequency
(% of total lymphoid cells)
(% of cμ⁺ lymphoid cells)
0
5
10
15
20
5 6 7 8 9 10 11 12 13 14 15
Nuclear diameter (μm)

Fig. 10. Size distribution of $c\mu^+$ cells (■, stippled profile) among the total lymphoid cells (●) in the bone marrow of anti-IgM treated mice, determined radioautographically by ^{125}I-anti-μ labeling in fixed smears.

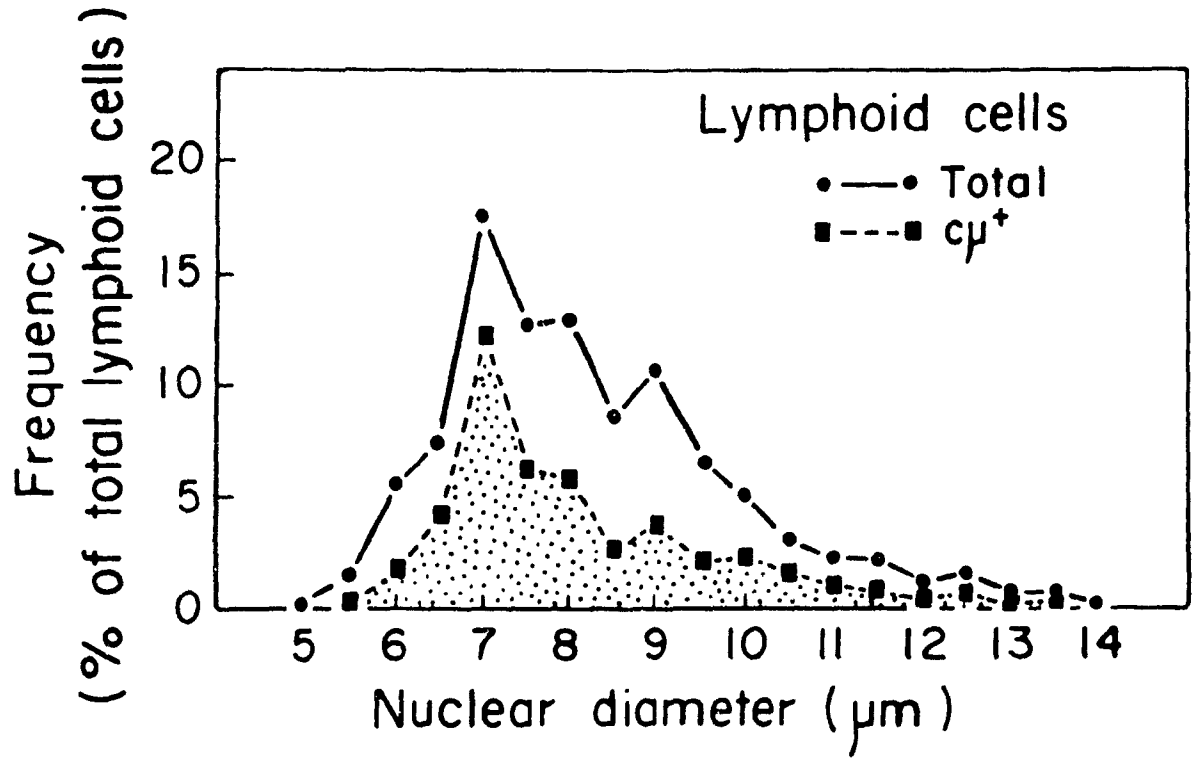

Lymphoid cells
Total
cμ+
Frequency
(% of total lymphoid cells)
0
5
10
15
20
5
6
7
8
9
10
11
12
13
14
Nuclear diameter (μm)

Fig. 11. Radioautographic labeling of μ-bearing small lymphocytes (both cμ and sμ) in the thymus, spleen and bone marrow of anti-IgM treated and normal rabbit serum (NRS) treated control mice. Labeling threshold was >20 grains per cell (shaded area).

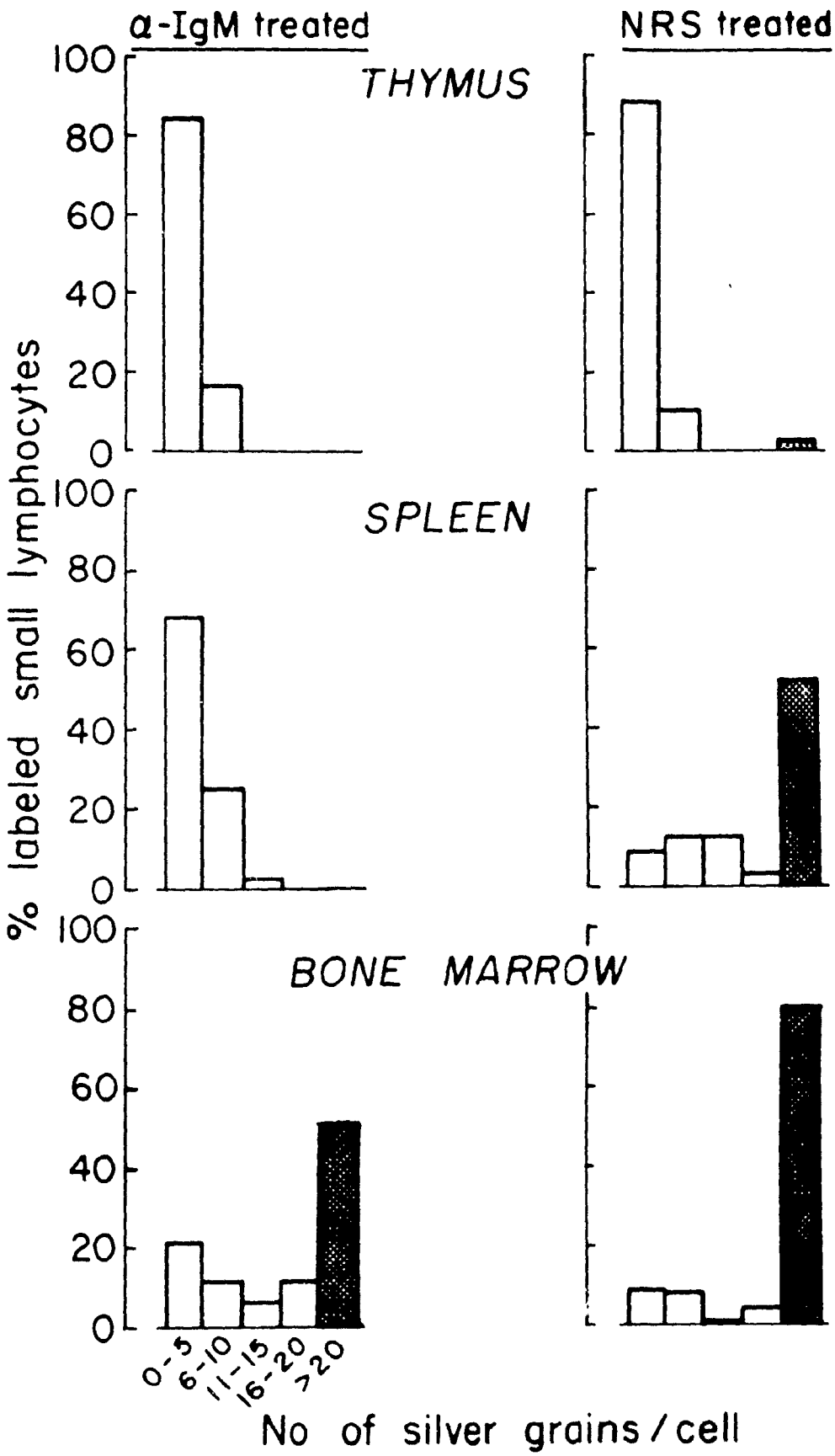

α-IgM treated
NRS treated
THYMUS
SPLEEN
BONE MARROW
% labeled small lymphocytes
100
80
60
40
20
0
0-5
6-10
11-15
16-20
>20
No of silver grains / cell

Fig. 12. Size distribution of cells lacking either surface or cytoplasmic μ chains among the total lymphoid cells in the bone marrow of (a) normal mice and (b) anti-IgM treated mice, (c) normalized size distributions of populations of μ^- lymphoid cells in bone marrow of normal and anti-IgM treated mice.

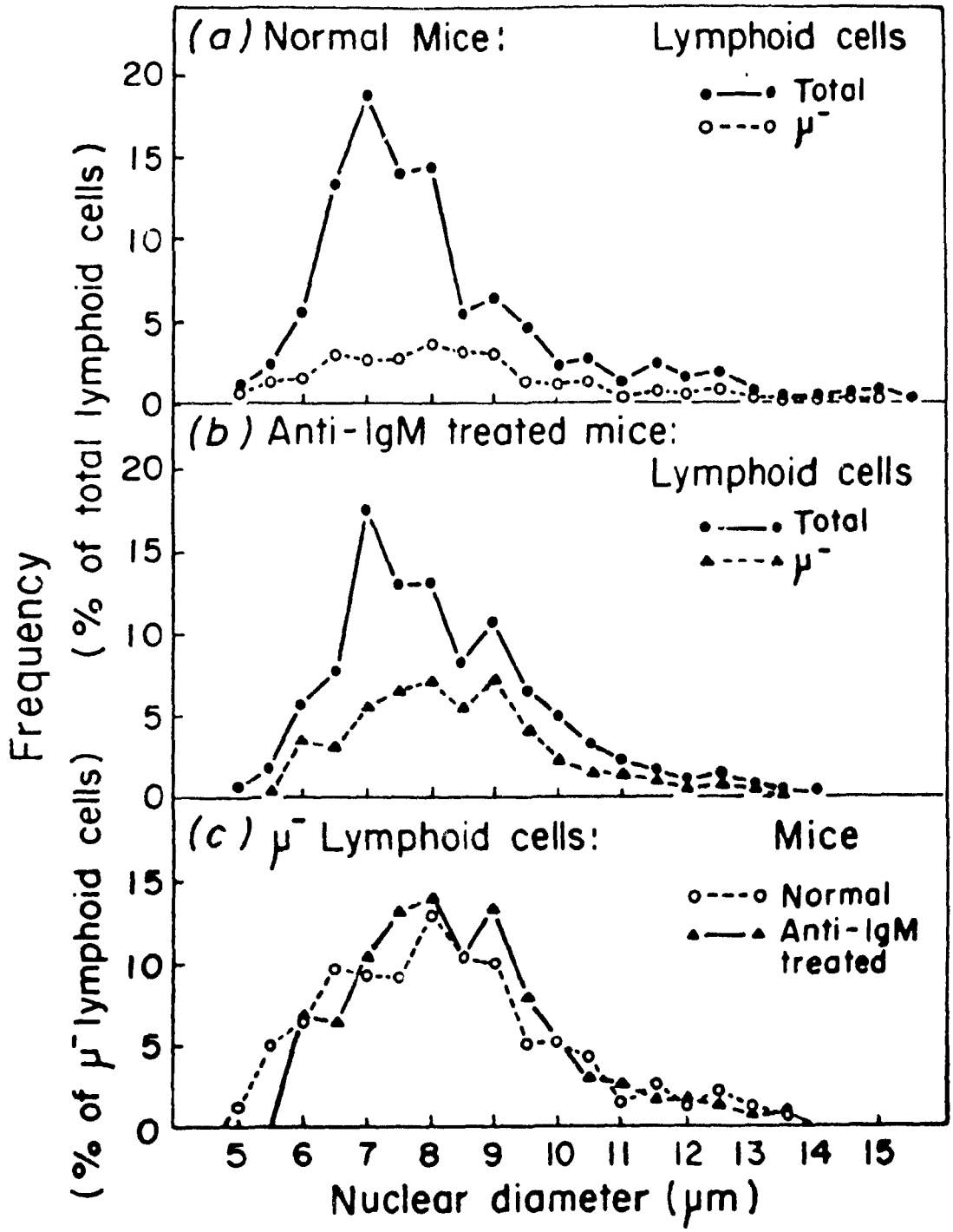

(a) Normal Mice: Lymphoid cells
Total
μ⁻
(b) Anti-IgM treated mice: Lymphoid cells
Total
μ⁻
(c) μ⁻ Lymphoid cells: Mice
Normal
Anti-IgM treated
Frequency (% of total lymphoid cells)
(% of μ⁻ lymphoid cells)
0
5
10
15
20
5 6 7 8 9 10 11 12 13 14 15
Nuclear diameter (μm)

Fig. 13. Size distribution of Thy1$^+$ lymphoid cells (o) among the total lymphoid cells (•) in the bone marrow of normal mice, determined radioautographically by exposing cell suspensions to anti-Thy1.2 and ^{125}I-protein A.

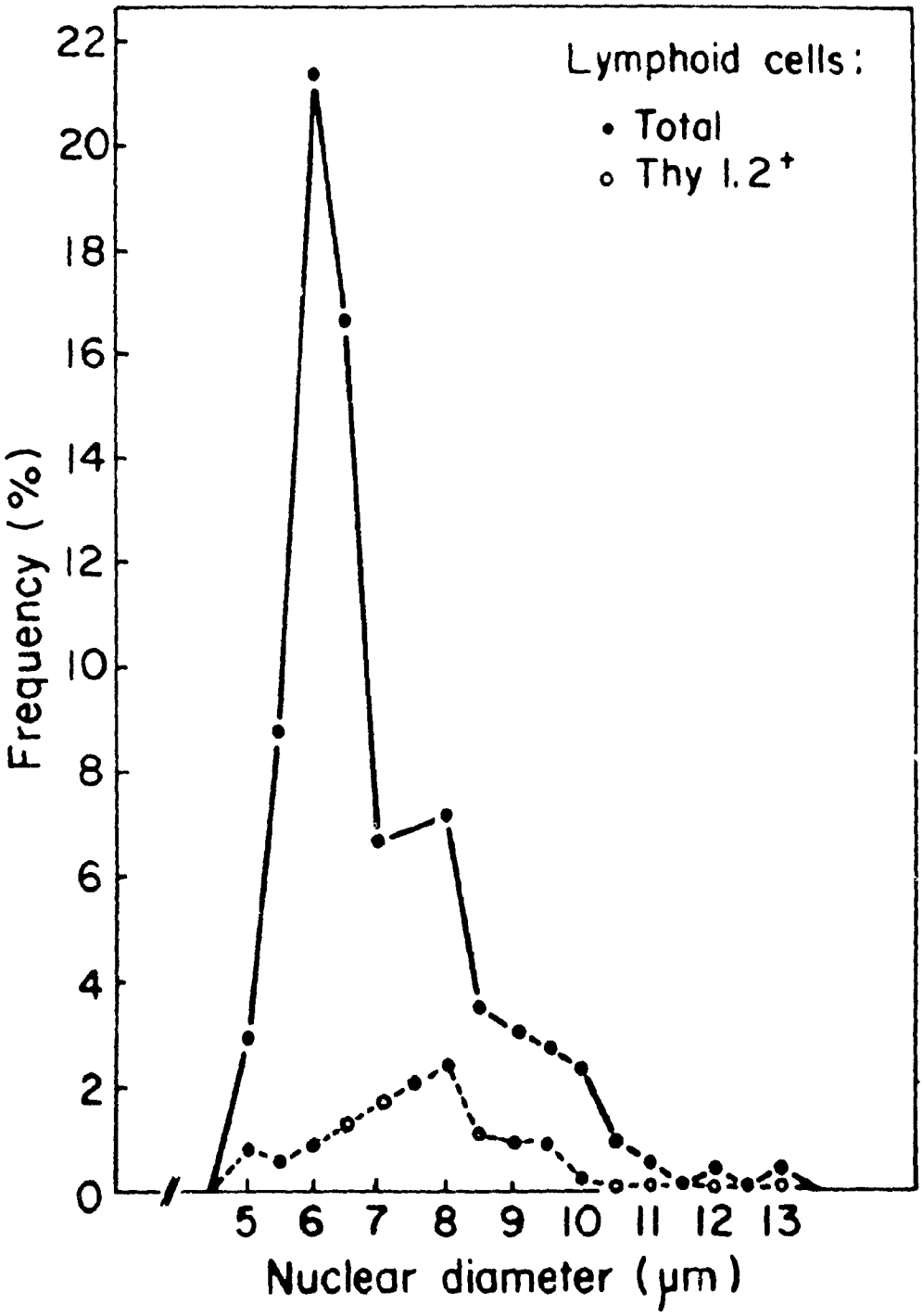

Lymphoid cells:
• Total
∘ Thy 1.2+
Frequency (%)
0
2
4
6
8
10
12
14
16
18
20
22
5
6
7
8
9
10
11
12
13
Nuclear diameter (μm)

Fig. 14. Grain count distribution of Thy1$^+$ small lymphocytes in the thymus, spleen and bone marrow of normal mice. Shaded columns represent highly labeled cells (>40 grains/cell).

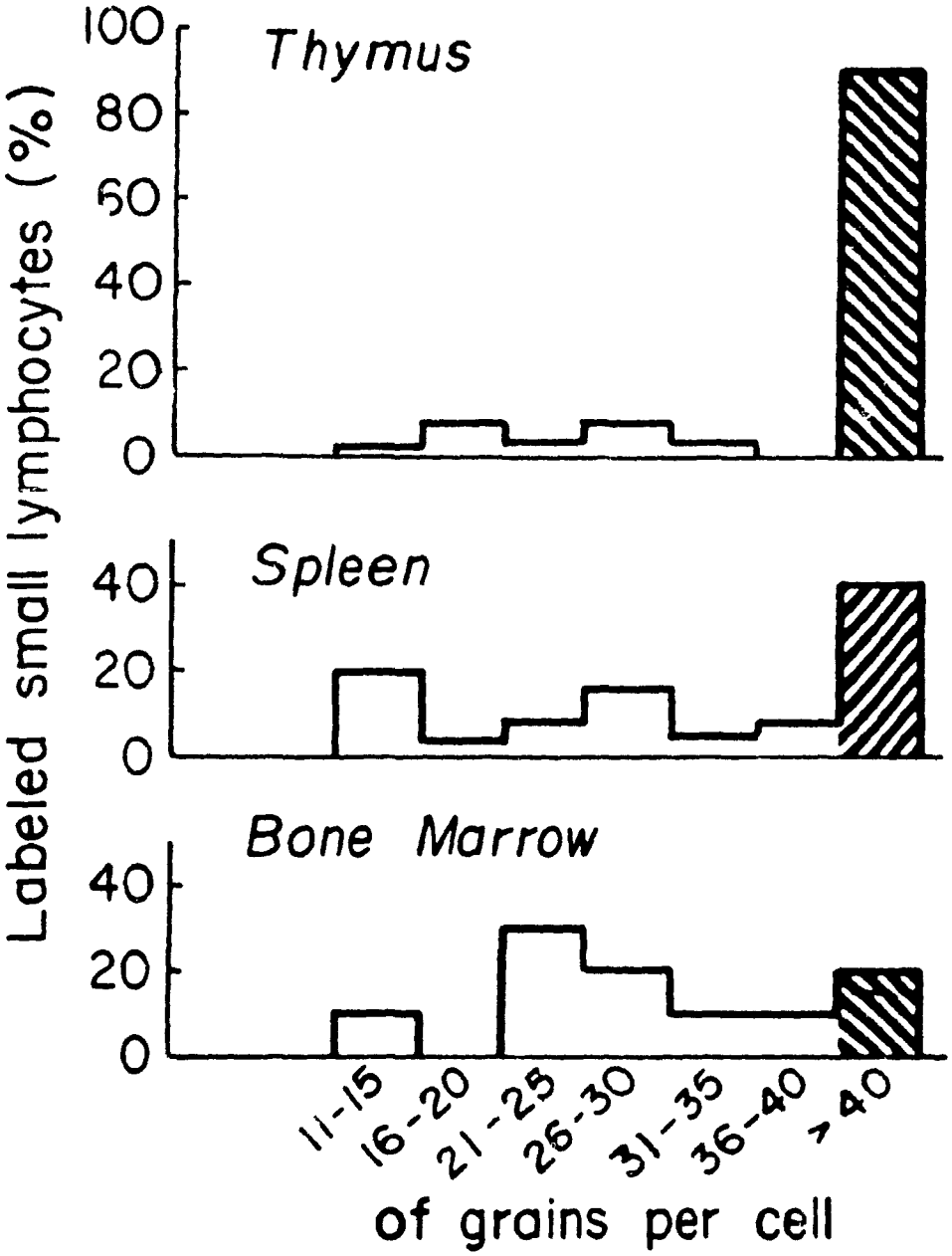

Thymus
Spleen
Bone Marrow
Labeled small lymphocytes (%)
100
80
60
40
20
0
40
20
0
40
20
0
11-15
16-20
21-25
26-30
31-35
36-40
>40
of grains per cell

Fig. 15. Size distribution of null cells lacking surface μ, cytoplasmic μ, and Thy1 among the total lymphoid cell population in the bone marrow of normal mice. This was derived by subtracting the profile of $Thy1^+$ cells (Fig. 13) from μ^- lympnoid population (Fig. 12a) in the bone marrow.

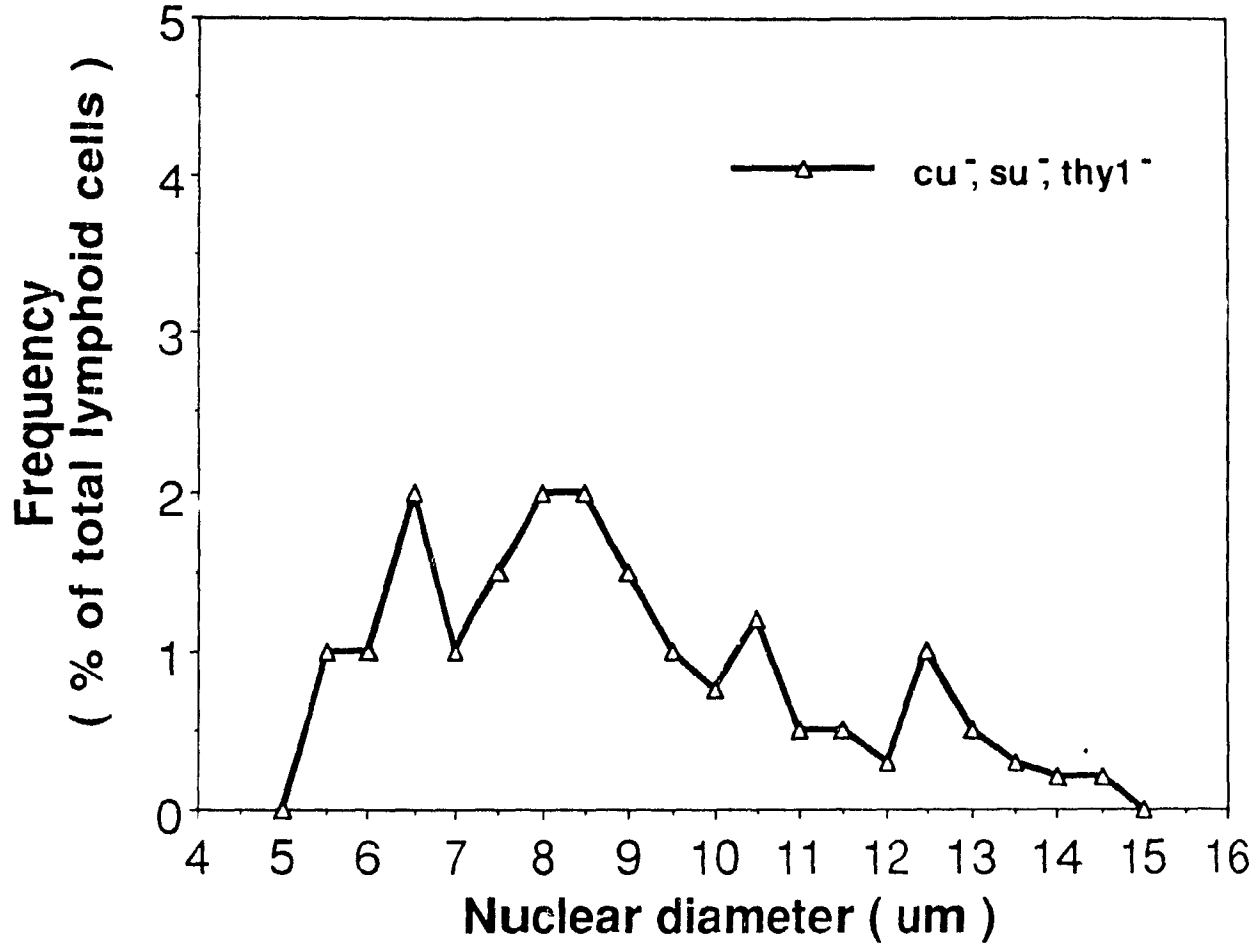

Frequency
(% of total lymphoid cells)
5
4
3
2
1
0
cu⁻; su⁻; thy1⁻
4 5 6 7 8 9 10 11 12 13 14 15 16
Nuclear diameter (um)

TABLE 1

Proportion of $s\mu^+$, $Thy1^+$, and Other Small Lymphocytes in the Bone Marrow, Spleen, and Thymus of Anti-IgM Treated and Control Mice[a]

	Proportion of small lymphocytes (%)					
	Anti-IgM treated mice			NRS controls[b]		
Tissue	$s\mu^+$	$Thy1^+$	Other[c]	$s\mu^+$	$Thy1^+$	Other[c]
Bone marrow	0	8	92	50	8	42
Spleen	0	92	8	52	43	5
Thymus	0	100	0	2	98	0

[a] Small lymphocytes were examined radioautographically after incubation with ^{125}I-anti-μ or with anti-Thy1 followed by ^{125}I-protein A (labeling threshold, >10 grains per cell).

[b] Control mice treated with normal rabbit serum.

[c] Cells lacking both surface μ chains and Thy1 antigen.

TABLE 2

Incidence[a] and Number of T Lineage Lymphoid Cells in Bone Marrow, Spleen, and Thymus

Tissue	Marker	Small lymphocytes		Large lymphoid cells	
		%	Cells/organ ($\times 10^5$)	%	Cells/organ ($\times 10^5$)
Bone marrow	Thy 1	8.3±0.4[a]	3.2±0.2	20 ±1.5	2.2±0.17
	L3T4	2.5±0.2	1.0±0.2	13.2±3.2	1.1±0.36
	Lyt 1	2.2±0.23	0.9±0.1	ND[b]	ND
	Lyt 2	1.6±0.37	0.6±0.13	ND	ND
	Lyt 1&2	0.37±0.3	0.1±0.08	ND	ND
Spleen	Thy 1	45.0±1.6	460±16.3		
	L3T4	20.1	205.3		
	Lyt 1	22.4±2.7	228.4±27.5	ND	
	Lyt 2	1.9±1.7	19.3±17.3		
	Lyt 1&2	5.0±1.2	57.0±12.2		
Thymus	Thy 1	98.6±0.6	623±10.5		
	L3T4	97.5	614		
	Lyt 1	6.8±3.5	42.8±20.1	ND	
	Lyt 2	4.1±2.4	25.8±13.2		
	Lyt 1&2	88.4±5.4	556.9±34.0		

[a] Mean ± standard error of the mean.

[b] ND: no data

TABLE 3

Incidence and Number of of Cells in Various Lymphoid Cell Subsets in the Bone Marrow of Normal[a] and Anti-IgM Treated[b] Mice

	Small lymphocytes (<8μ)				Large lymphoid (>8μ)			
	%[c]		Cells/femur ($x10^5$)		%		Cells/femur ($x10^5$)	
	Normal	Anti-IgM	Normal	Anti-IgM	Normal	Anti-IgM	Normal	Anti-IgM
B lineage								
cμ	30	56	12	10	40	40	4.6	2.2
sμ	50	0	20	0	0	0	0	0
Total μ[d]	80	56	32	10	40	40	4.6	2.2
T lineage								
Thy1[e]	8	8	3.2	1.5	20	ND	2.0	ND
Null cells								
$c\mu^-, s\mu^-, Thy1^-$	12	32	4.8	6.2	40	ND	3.4	ND

[a] The total nucleated cell count per femur in 8-10 week old C3H/HeJ mice was $16x10^6 \pm 0.41$ (mean ± standard error of the mean from 4-5 mice).

[b] The total nucleated cell count per femur in (C3H/HeJ x C57BL/6)F_1 mice was 12.1 x $10^6 \pm 0.57$ (mean ± standard error of the mean from 3-4 mice).

[c] Percent labeled of total small lymphocytes; their differential count represents 25% and 16% of total nucleated cells in normal and anti-IgM treated mice, respectively.

[d] Total identifiable lymphoid cells labeled by ^{125}I-anti-μ in fixed bone marrow smears detecting both cμ and sμ.

[e] Total lymphoid cells labeled by anti-Thy1.2, followed by ^{125}I-protein A.

RESULTS

SECTION II

This section was designed to reveal null small lymphocytes in the bone marrow by detecting surface markers on B and T lineage cells, as opposed to labeling intracellular μ and surface μ chains by ^{125}I-anti-μ in fixed marrow smears. This method has been used to examine the ontogenic development and turnover kinetics of null, 14.8$^+$, and Thy1$^+$ (including Thy1^{lo} and Thy1^{hi} cells) small lymphocytes in vivo.

1. Double immunofluorescence labeling of 14.8$^+$ and μ^+ B lineage cells in the bone marrow

Double immunofluorescence labeling showed that mAb 14.8 bound to all sμ^+ B lymphocytes and cμ^+ pre-B cells in the bone marrow (Table 4). Some (4.4%) 14.8$^+$ cells lacked both cμ and sμ, and were mostly large undifferentiated cells with little cytoplasm (Landreth et al, 1983; Park & Osmond, 1987). Thereafter, mAb 14.8 was used to label the surface of B lineage cells in radioautographic preparations, in combination with mAb anti-Thy1. This has allowed the detection of null small lymphocytes, defined as cells which remained unlabeled by both mAbs, ie. 14.8$^-$Thy1$^-$.

2. Subsets of 14.8^+, $Thy1^+$, and null small lymphocytes in the bone marrow as shown by radioautography

Radioautographic immunolabeling of bone marrow cell suspensions with either mAb 14.8 or mAb anti-Thy1, detected in each case with ^{125}I-anti-rat IgG, showed that of the total small lymphocyte population identified in hematologically stained radioautographic smears, 80% were 14.8^+ B lineage cells while 8% were labeled by anti-Thy1 (Table 5). By subtraction, at least 12% of the bone marrow small lymphocytes would thus remain as unlabeled null cells, lacking both the 14.8 determinant and the Thy1 antigen (14.8^-Thy1^-) (Table 5). After radioautographic double immunolabeling of bone marrow cell suspensions, using both mAb 14.8 and mAb anti-Thy1 together, a comparable proportion of small lymphocytes again remained unlabeled (Table 5). Such 14.8^-Thy1^- null small lymphocytes, morphologically indistinguishable from those labeled with mAbs 14.8 and anti-Thy1 (Fig. 16), formed a substantial population, totalling approximately 0.4×10^5 cells/femur, equivalent to 2-3% of all nucleated cells in the bone marrow (Table 5).

In addition to small lymphocytes, mAb 14.8 bound to only approximately half (46-50%) of the cells of large lymphoid and blast morphology. Such large 14.8^+ cells are known to be proliferating B cell precursors (Landreth et al, 1983; Park & Osmond, 1987). No cells of other lineages showed mAb 14.8 labeling. Control preparations in which radioiodinated mAb

14.8 was incubated with rat lymphoid cells or ^{125}I-anti-rat IgG incubated directly with mouse bone marrow and spleen cells revealed no labeled cells.

3. Ontogenic development of null, 14.8$^+$, and Thy1$^+$ small lymphocytes in the liver, spleen and bone marrow

In this series, mAbs 14.8 and anti-Thy1 were used to identify and enumerate 14.8$^-$Thy1$^-$ null small lymphocytes from hemopoietic tissues of mice at different ages (Fig. 17).

There was a rapid increase in the number of total nucleated cells in the organs examined, beginning early in fetal life (day 11 of gestation in liver; day 16 of gestation in spleen; 3 days after birth in bone marrow) and plateauing throughout adulthood (Fig. 17).

The first identifiable null small lymphocytes (14.8$^-$Thy1$^-$) were found in the fetal liver as early as day 11 of gestation (3×10^3 cells), even before the appearance of any 14.8$^+$ or Thy1$^+$ small lymphocytes (Fig. 18a). The number of these null small lymphocytes increased rapidly in numbers to reach maximum levels at around birth. 14.8$^+$ and Thy1$^+$ small lymphocytes appeared later at 16 days and 18 days of gestation, respectively (Fig. 18a).

In the spleen, the first detectable null small lymphocytes appeared slightly after those in the fetal liver (day 16 of gestation; 7.5×10^3 cells) their total numbers

leveling off by 10 days after birth (3-4 x 10^6 cells) (Fig. 18b). The rate of increase in the number of both 14.8^+ and $Thy1^+$ cells in the spleen paralleled that of the null small lymphocytes (Fig. 18b).

In the bone marrow, null small lymphocytes increased rapidly after birth. The 60-fold increase in the number of bone marrow null small lymphocytes between 3 days to 3 weeks after birth was paralleled by 14.8^+ and $Thy1^+$ small lymphocytes (Fig. 18c). The rate of increase was greatest between 3 days and 7 days after birth. All three subpopulations had reached a maximum size by 3 weeks of age (Fig. 18c).

4. Turnover kinetics of bone marrow lymphocyte populations determined by hydroxyurea treatment

Repeated administration of HU resulted in a rapid decrease in the total number of nucleated cells in the bone marrow during the 3 day injection period, falling from 14 X 10^6 to less than 1 x 10^6 cells per femur (Fig. 19). Time 0 represents untreated control mice. Bone marrow cellularity in saline-injected control mice remained unchanged for 42 hrs but declined thereafter. A similar delayed decline was also observed in the spleens of control mice.

a) Turnover of bone marrow small lymphocytes

The number of small lymphocytes derived from differential cytology of stained bone marrow smears fell rapidly during HU

treatment from 40 x 10^5 cells/femur to 5 X 10^5 cells/femur by 2 days (Fig. 20). Almost 90% of small lymphocytes were lost from the marrow by 3 days after HU (Fig. 20a). The disappearance of small lymphocytes from the bone marrow with time, corresponded with an exponential decline (Fig. 20b), having a half renewal time ($T_{\frac{1}{2}}$) of 21.5 hr (Table 6). The lack of any immediate deletion by HU, as shown by extrapolation of the data back to zero time, agrees with the concept that bone marrow small lymphocytes, though rapidly renewed from proliferating precursor cells, were not themselves in cell cycle.

b) Turnover of null, 14.8$^+$, and Thy1$^+$ small lymphocytes in bone marrow

Figure 21 shows the differential effects of HU treatment on the 3 subpopulations of bone marrow small lymphocytes. Null small lymphocytes, defined as small lymphocytes showing no radioautographic labeling after combined exposure to mAbs 14.8 and anti-Thy1, were unique in their exceptionally rapid rate of decay. Their numbers fell from 5 x 10^5 cells/femur to 0.1 x 10^5 cells/femur by 24 hr (Fig. 21a). The decay curve, expressed semilogarithmically (Fig. 21b), was not exactly linear over the entire 3 day period, raising the possibility of more than one kinetic component. A computer iterative analysis of the curve revealed that the great majority of the null small lymphocytes (94%) had remarkably short turnover

times in the bone marrow ($T_{\frac{1}{2}}$, 7.5 hr), while only a small fraction (6%) of null cells might be more slowly renewed ($T_{\frac{1}{2}}$, 96 hr). The number of small lymphocytes labeled by exposure to mAb 14.8 alone fell from 32 x 10^5 cells/femur to 4 x 10^5 cells/femur over the 3 day period of HU treatment (Fig. 21a), representing a rapid exponential renewal ($T_{\frac{1}{2}}$, 20.5 hr) (Fig. 21b). The small lymphocytes binding mAb anti-Thy1 declined only gradually in numbers (Fig. 21a) and had the slowest kinetics ($T_{\frac{1}{2}}$, 53 hr) (Fig. 21b).

c) Turnover of Thy1^{lo} and Thy1^{hi} small lymphocytes in the bone marrow

Subdividing the total Thy1$^+$ small lymphocytes into Thy1^{lo} and Thy1^{hi} cells, figure 22 represents the differential effects of HU treatment on these cell subpopulations in the bone marrow. During this period, the number of Thy1^{lo} cells in the bone marrow fell rapidly ($T_{\frac{1}{2}}$, 28 hr), whereas the Thy1^{hi} cells declined only slowly ($T_{\frac{1}{2}}$, 123 hr) after an initial lag period of 13 hrs.

d) Turnover of null small lymphocytes in the spleen

In the spleen 14.8$^-$Thy1$^-$ null cells accounted for 4.2% ± 0.14 of all small lymphocytes, 4.5 X 10^6 ± 0.32 cells per spleen. During HU treatment, these cells showed no fall in numbers for the first 6 hr, but then declined rapidly ($T_{\frac{1}{2}}$, 16.5 hr) (Fig. 23). In the saline-injected control mice, some

decline in the number of splenic null cells occurred after a longer initial lag period (Fig. 23).

5. Production rate of null small lymphocytes and other lymphocyte subsets in the bone marrow.

Table 6 summarizes the renewal times and production rates of the 3 small lymphocyte subsets in the bone marrow, derived from the foregoing data The respective turnover rates have been combined with the absolute number of cells per femur to calculate the total turnover of cells per femur and the turnover rate per whole-body marrow organ per day. The production rate of null small lymphocytes in the bone marrow was considerable, equivalent to almost one-third the production rate of 14.8^{+} small lymphocytes, and totalling 1.3 X 10^{7} cells per day in the whole animal. The production of $Thy1^{lo}$ cells represented approximately 2% of the total production of small lymphocytes and was one tenth that of the null small lymphocytes in the bone marrow.

Fig. 16. Radioautograph of two unlabeled null small lymphocytes and a heavily labeled large lymphoid cell in bone marrow after expos re to mAb 14.8 and anti-Thy1. The small lymphocytes have few or no grains, consistent with background, despite the high degree of radioautographic exposure (exposure time, 10 days) (x 1500).

Fig. 17. Number of nucleated cells in the liver (○), spleen (♦), and bone marrow (□) of prenatal and postnatal mice.

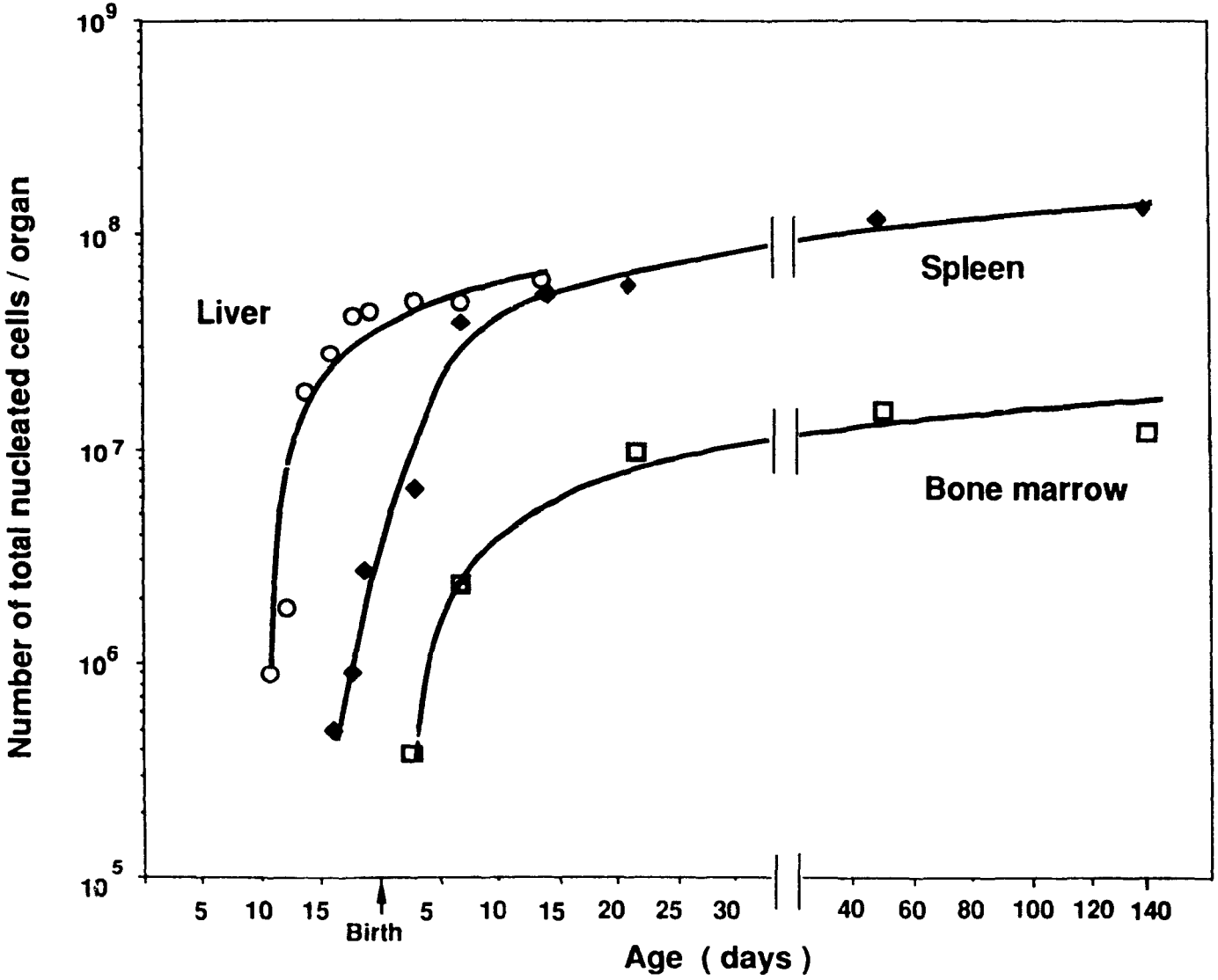

Number of total nucleated cells / organ
10^9
10^8
10^7
10^6
10^5
Liver
Spleen
Bone marrow
5
10
15
Birth
5
10
15
20
25
30
40
60
80
100
120
140
Age (days)

Fig. 18. Ontogenic development of null (△), 14.8^{+} (○), and $Thy1^{+}$ (□) small lymphocytes in the a) liver, b) spleen, and c) bone marrow of prenatal and postnatal mice.

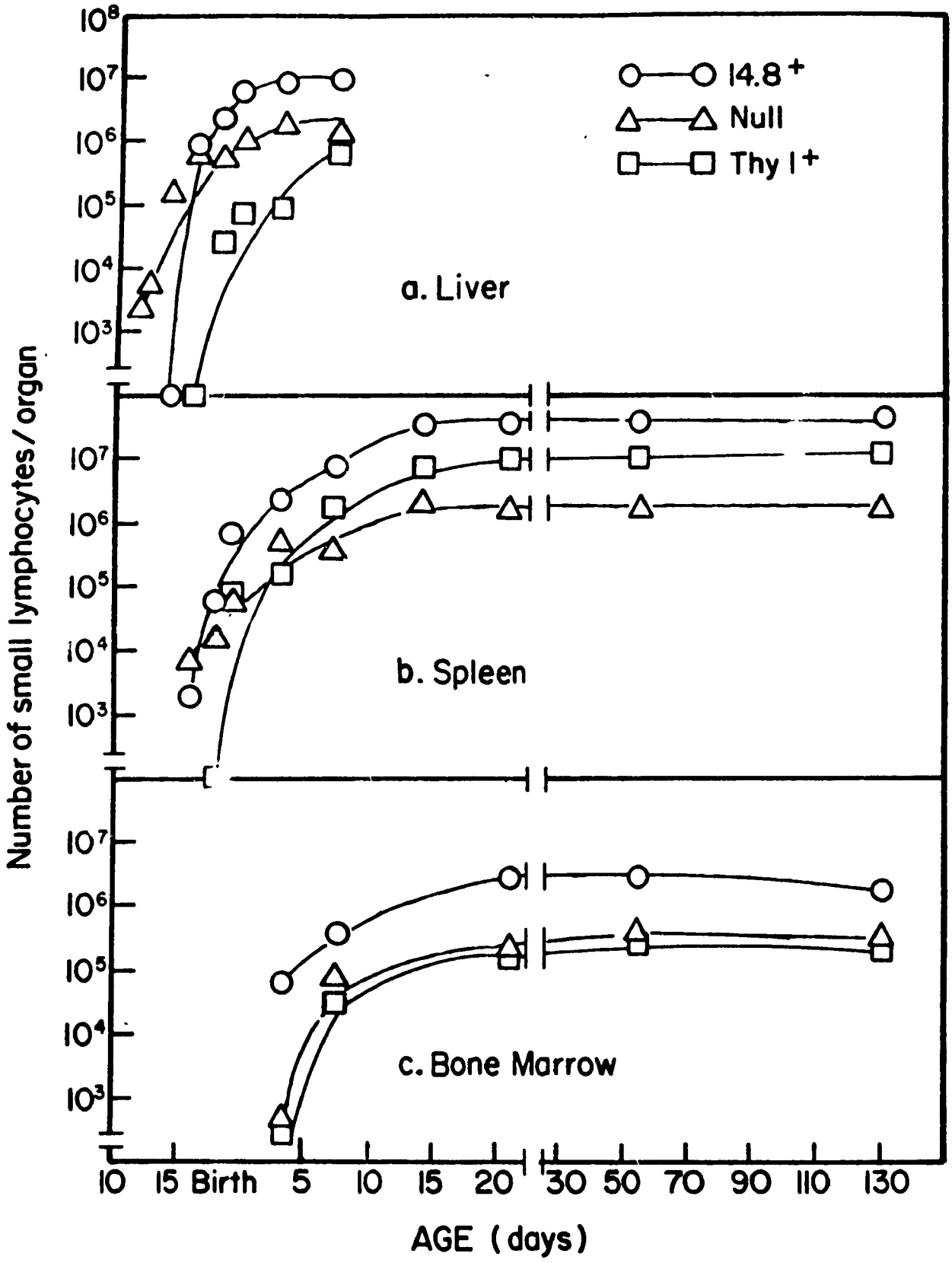

14.8+
Null
Thy 1+
a. Liver
b. Spleen
c. Bone Marrow
Number of small lymphocytes/organ
AGE (days)
10 15 Birth 5 10 15 20 30 50 70 90 110 130

Fig. 19. Number of nucleated cells in the bone marrow of 8-10 wk old C3H/HeJ mice treated with hydroxyurea (■), or saline controls (□). Time 0 represents normal untreated mice. The curves represent the best fit through individual values.

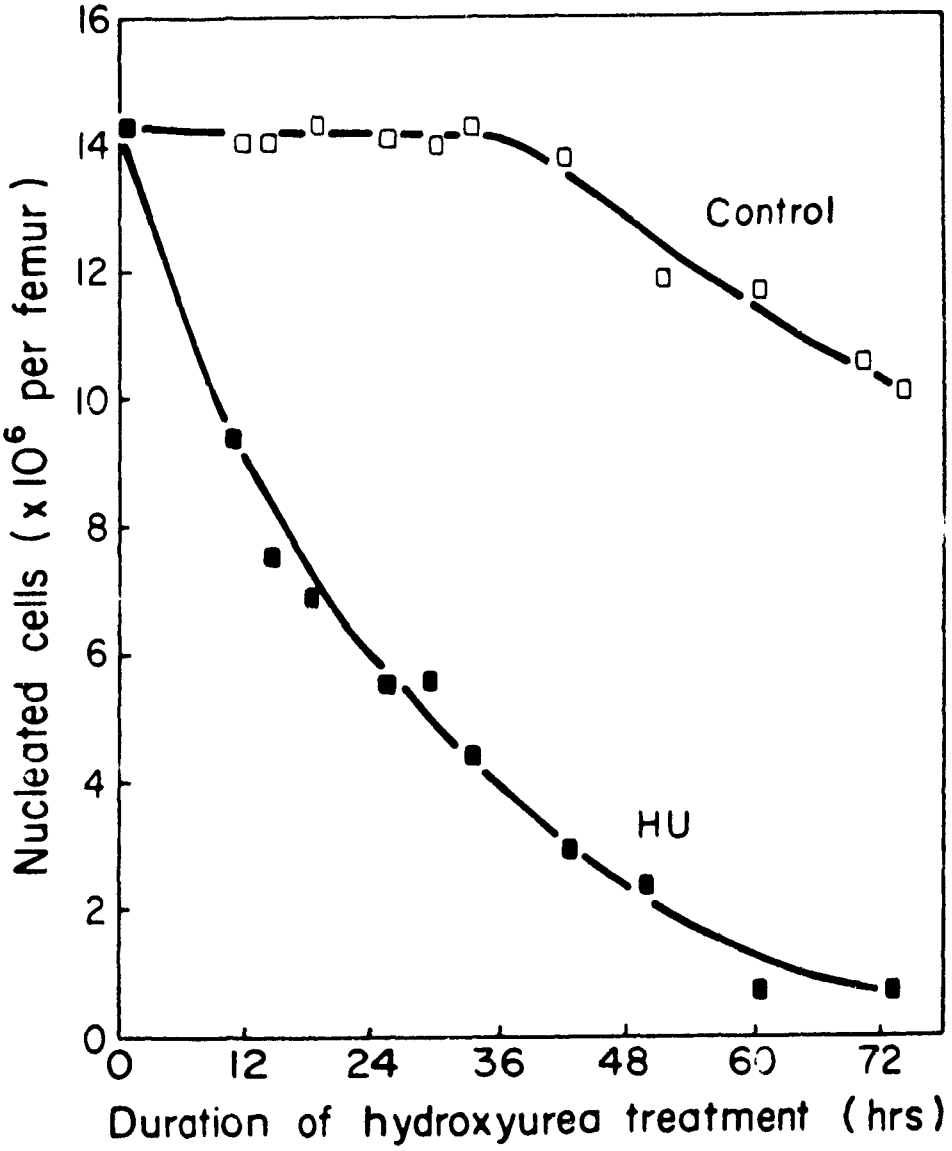

16
14
12
10
8
6
4
2
0
Nucleated cells (x 10^6 per femur)
Control
HU
0
12
24
36
48
60
72
Duration of hydroxyurea treatment (hrs)

Fig. 20a. (above) Number of small lymphocytes in bone marrow of hydroxyurea treated mice (•) and saline injected controls (o). Small lymphocytes were identified by differential cytology of MacNeal's stained preparations. Time 0 represents normal untreated mice. The curve represent the best fit through individual values.

b. (below) Turnover of small lymphocytes in bone marrow during hydroxyurea treatment expressed as a percentage of untreated mice. The regression line was calculated by the method of least squares from the individual values.

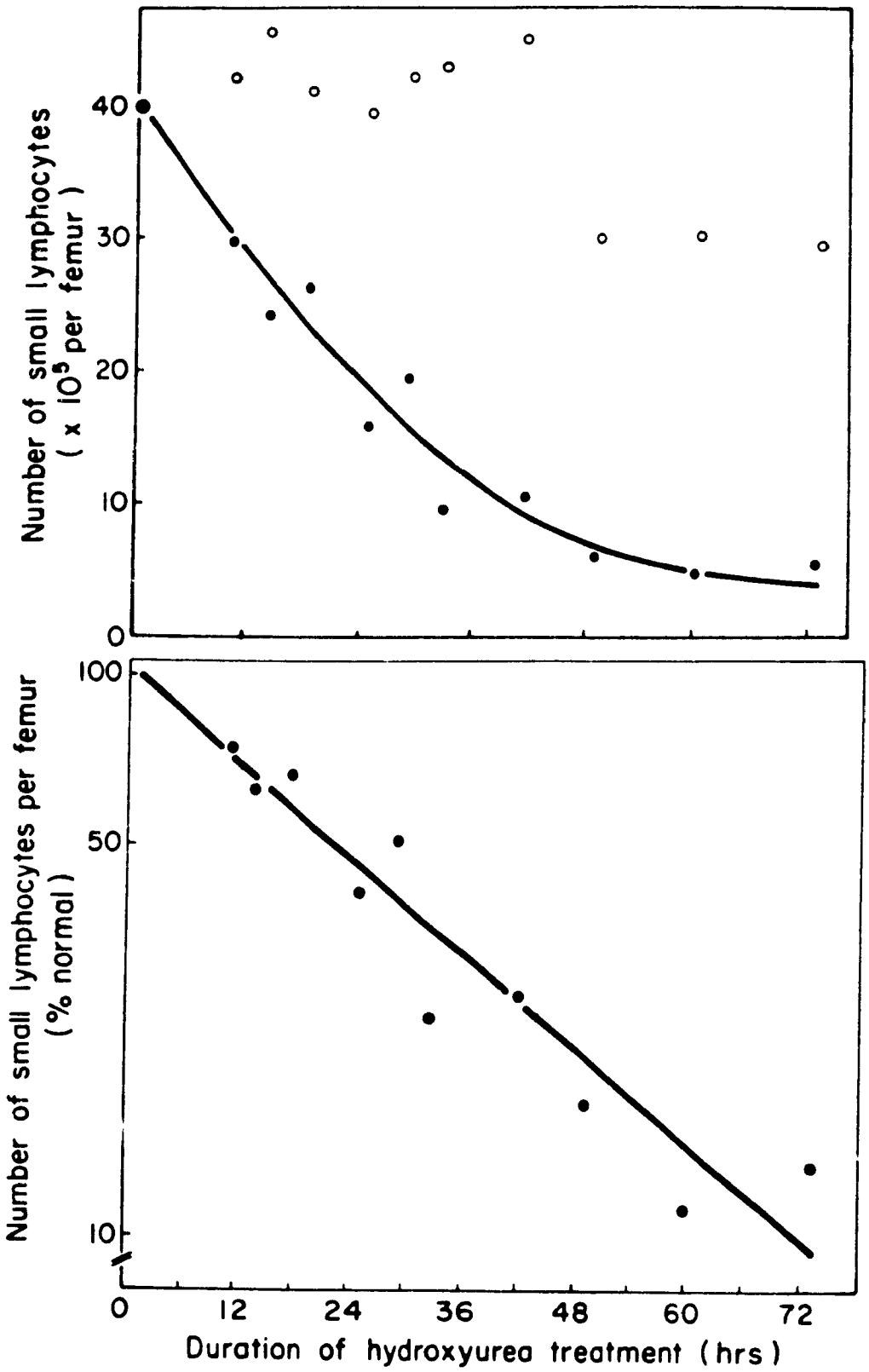

Number of small lymphocytes (x 10^3 per femur)
40
30
20
10
0
Number of small lymphocytes per femur (% normal)
100
50
10
0
12
24
36
48
60
72
Duration of hydroxyurea treatment (hrs)

Fig. 21a. (left) Number of 14.8^+ (●), $Thy1^+$ (■), and null (14.8^-Thy1^-) (▲) small lymphocytes in bone marrow during hydroxyurea treatment. Time 0 represents normal untreated mice. The curves represent the best fit through individual values.

b. (right) Turnover of 14.8^+ (●), $Thy1^+$ (■), and null (14.8^-Thy1^-) (▲) small lymphocytes in bone marrow during hydroxyurea treatment expressed as a percentage of normal untreated mice. The regression lines of 14.8^+ and $Thy1^+$ cells was calculated by the method of least squares from the individual values, whereas a polynomial regession line was performed for individual null values.

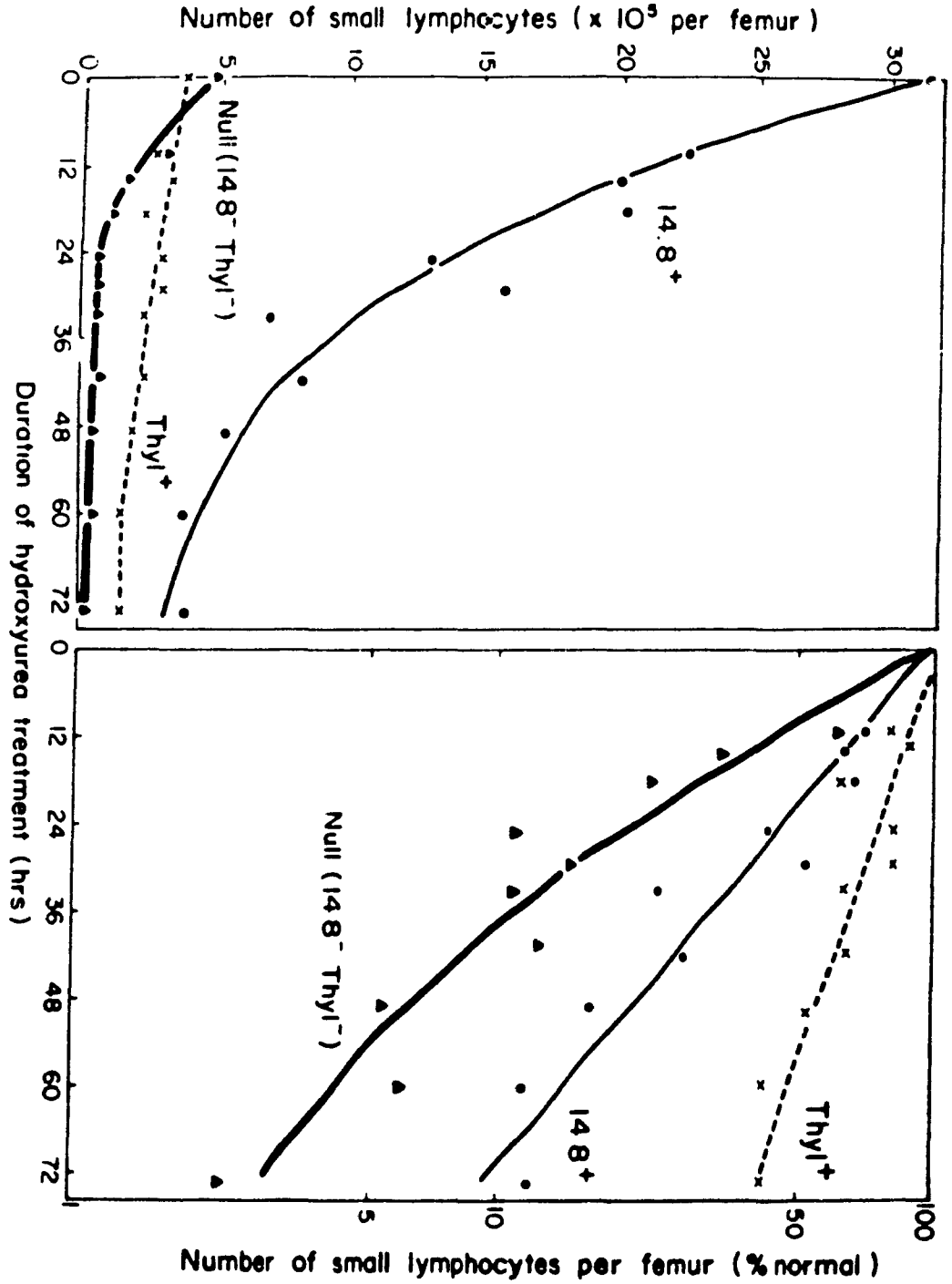

Number of small lymphocytes (x 10^5 per femur)
0
5
10
15
20
25
30
Null (14.8⁻ Thy1⁻)
14.8⁺
Thy1⁺
Duration of hydroxyurea treatment (hrs)
0
12
24
36
48
60
72
Null (14.8⁻ Thy1⁻)
14.8⁺
Thy1⁺
1
5
10
50
100
Number of small lymphocytes per femur (% normal)

Fig. 22. Turnover of $Thy1^{lo}$ (□) and $Thy1^{hi}$ (■) small lymphocytes in the bone marrow during hydroxyurea treatment expressed as a percentage of untreated mice. The regression lines were calculated by the method of least squares from the individual values.

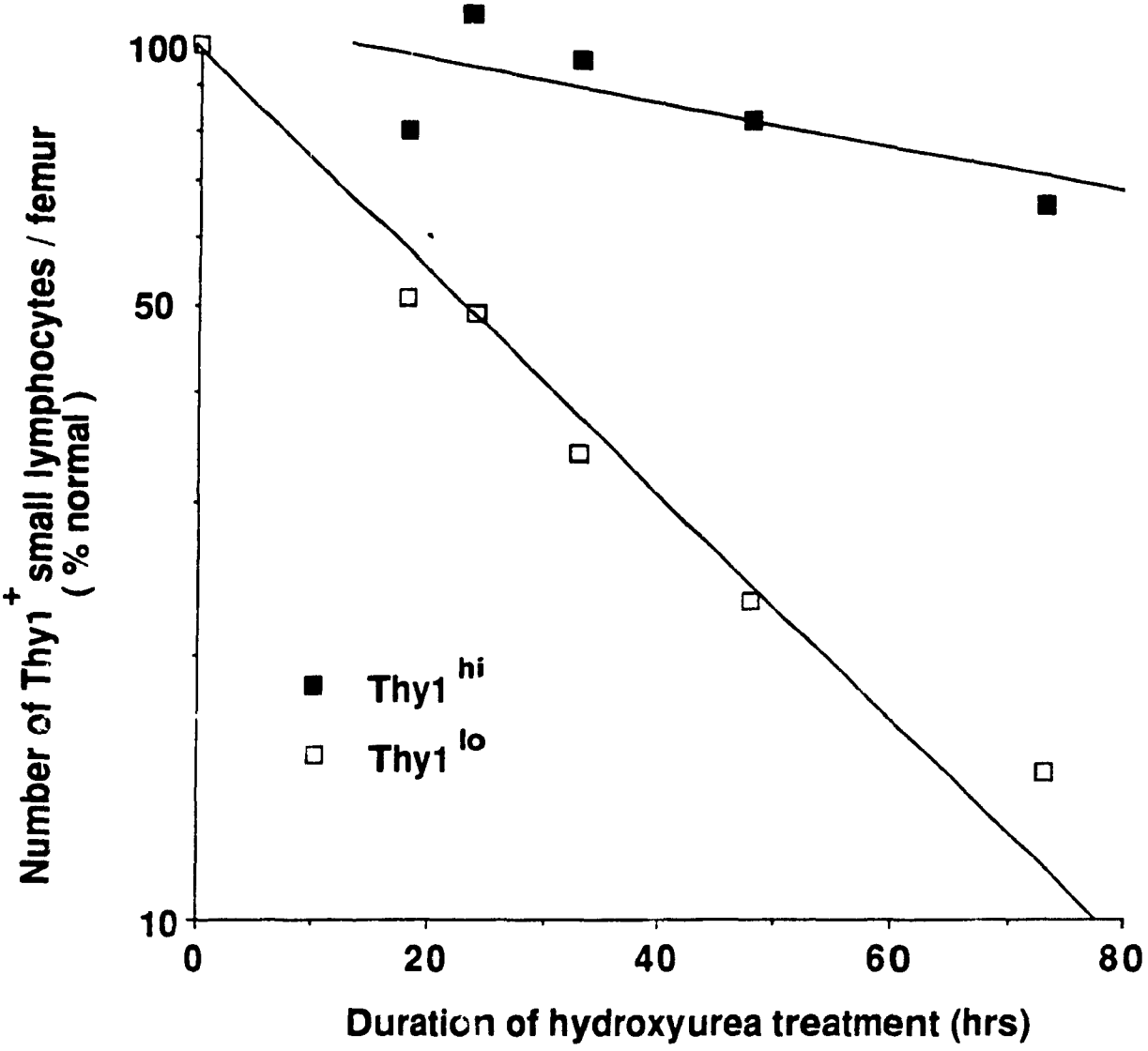

100
50
10
0
20
40
60
80
Number of Thy1+ small lymphocytes / femur
(% normal)
Thy1 hi
Thy1 lo
Duration of hydroxyurea treatment (hrs)

Fig. 23. Turnover of null (14.8$^-$Thy1$^-$) small lymphocytes in the spleen during hydroxyurea treatment (▲) and saline injected controls (△) expressed as a percentage of normal untreated mice. Time 0 represents untreated mice. The regession lines were calculated by the method of least squares fro~ the individual values.

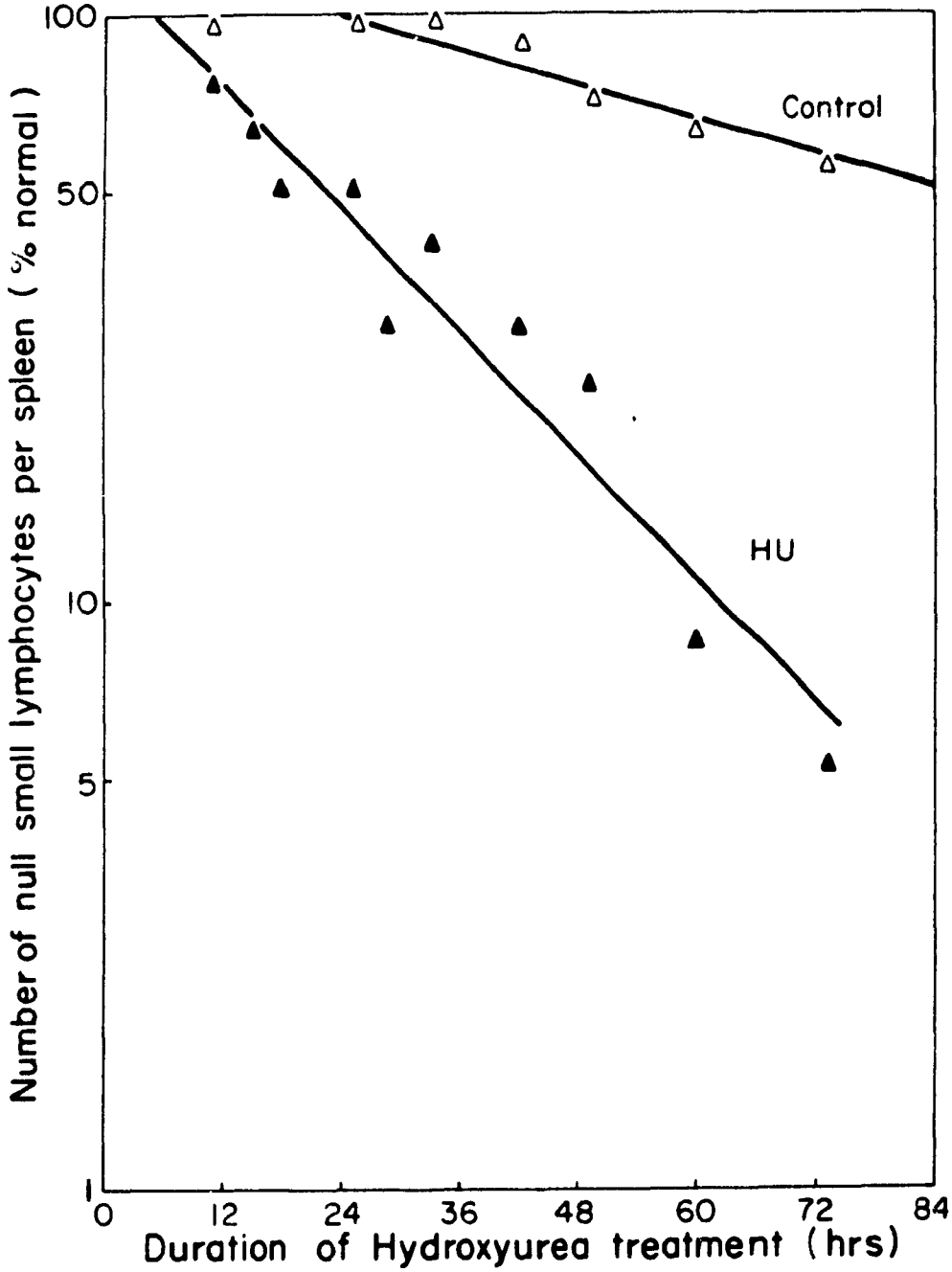

100
50
10
5
1
0
12
24
36
48
60
72
84
Control
HU
Number of null small lymphocytes per spleen (% normal)
Duration of Hydroxyurea treatment (hrs)

TABLE 4

Incidence of 14.8^+ and μ^+ B Lineage Cells in the Bone Marrow[a] using Double Immunofluorescence

Markers			Incidence	Absolute numbers/femur
14.8	cμ	sμ	(%)	(x 10^5)
+	-	-	4.4[b]	7.0
+	-	+	9.5[c]	15.2
+	+	+	18.5[b]	29.6
+	+	-	9.0[d]	14.4

[a] Proportion of labeled cells in relation to all nucleated cells in the bone marrow of 8-10 wk old C3H/HeJ mice.

[b] Double immunofluorescence labeling for surface binding of mAb 14.8 and for the presence of total μ chains (cμ and sμ).

[c] Double immunofluorescence labeling for surface binding of mAb 14.8 and surface μ chains.

[d] Derived from the subtraction $14.8^+s\mu^+$ from 14.8^+ total μ^+.

TABLE 5

Small Lymphocyte Subsets in Mouse Bone Marrow[a]

Cell marker	Incidence[b] (%)	Cells/femur[b] ($\times 10^5$)
Combined antibody labeling[c]		
Null (14.8^-Thy1^-)	14.3±1.7	5.6±0.4
Single antibody labeling[d]		
mAb 14.8^+	79.5±1.2	32.0±0.6
$Thy1^+$	8.4±0.6	3.2±0.2
Null (14.8^-Thy1^-)	12.1±0.6	4.2±0.2

[a] Proportion of marker-bearing small lymphocytes relative to all small lymphocytes in the boneo marrow of 8-10 wk old C3H/HeJ

[b] Mean ± standard error of the mean; n=11.

[c] Cells were exposed simultaneously to both mAb 14.8 and mAb anti-Thy1. The incidence of null (14.8^-Thy1^-) small lymphocytes was measured directly as the proportion of small lymphocytes which remained unlabeled.

[d] Cells were exposed to either mAb 14.8 or mAb anti-Thy1. The incidence of null (14.8^-Thy1^-) cells was derived by subtracting the combined incidences of labeled 14.8^+ and $Thy1^+$ small lymphocytes from the total population of small lymphocytes.

TABLE 6

Renewal Of Small Lymphocyte Subsets in Mouse Bone Marrow [a]

Small lymphocyte	$T_{1/2}$ (hr)	Renewal rate [b] (%/hr)	Cell turnover ($\times 10^5$) /hr/femur [c]	/day/whole body [d]
Null [e]	7.5	9.2	0.34	130
14.8^{+} [f]	20.5	3.4	1.06	401
$Thy1^{lo}$ [g]	28	2.5	0.032	12
$Thy1^{hi}$ [h]	123	0.5	0.008	3
Total [i]	21.5	3.2	1.27	483

[a] 8-10 wk old C3H/HeJ mice

[b] $\ln 2 / T_{1/2}$ = Renewal rate (%/hr)

[c] Renewal rate x total number of cells/femur

[d] Cell turnover/hr/femur x 24 hr x 15.8 (factor to convert one femoral bone marrow to whole body bone marrow (Berner et al, 1981; Opstelten & Osmond, 1983)

[e] Small lymphocytes remaining unlabeled after simultaneous exposure to mAb 14.8 and anti-Thy1

[f] Small lymphocytes labeled by mAb 14.8

[g] Small lymphocytes labeled (11-40 grains/cell) by mAb anti-Thy1.2

[h] Small lymphocytes labeled (>40/grains/cell) by mAb anti-Thy1.2

[i] Total small lymphocytes identified by differential cytology of MacNeal's tetrachrome stained preparations

RESULTS

SECTION III

Section III was designed to assay NK activity in the bone marrow and to examine certain cell surface markers on null small lymphocytes, testing the possibility that these cells may represent NK lineage cells.

1. Natural killer cell cytotoxic activity in bone marrow and spleen: effect of interferon

To determine whether any mature functional NK cells could be detected in young (4 week old) and adult (8-10 week old) C3H/HeJ mice, the NK cell cytotoxic activities of both bone marrow and spleen cells were tested (Fig. 24). The effector to target cell ratios (E:T) used were 5:1, 10:1 and 20:1. In figure 24, each line represents pooled cells from 3-5 animals. The cytotoxic index of the bone marrow cells was relatively high in young animals but decreased with age, whereas the spleen NK cell activity increased with age.

The NK cell activity of adult bone marrow cells was assayed before and after fractionation on continuous sucrose density gradients, with or without αIFN boosting in vitro. Unfractionated bone marrow cells had low NK activity, which was slightly increased by αIFN. However, when lymphocyte-rich fractions from bone marrow cell suspensions were exposed to

αIFN, NK activity increased two-fold (at 20:1 ET ratio) over untreated whole bone marrow cells (Fig. 25).

2. Incidence and number of NK1.1$^+$ small lymphocytes in the bone marrow and spleen

The numbers of NK1.1$^+$ small lymphocytes in the bone marrow of young (4 wk) and mature (10 wk) C57BL/6 mice, determined radioautographically after exposing bone marrow cells to anti-NK1.1 and ^{125}I-anti-mouse Igγ, are detailed in Table 7. A distinct subset of bone marrow small lymphocytes (3-5%) showed well-marked NK1.1 labeling, increasing in incidence but showing a similar total number of cells per femur with increasing age. Similar results were obtained for 10 wk old (C3H/HeJ x C57BL/6)F1 hybrid mice (data not shown).

Cells expressing the NK1.1 marker were almost all small lymphocytes (<8μm diameter) containing few, if any, cytoplasmic granules. No other cells showed NK1.1 labeling except for some medium-sized lymphoid cells (9-10μm diameter) and light labeling on a few monocytes and granulocytes. In control experiments, either exposing C57BL/6 strain cells to radiolabeled anti-mouse Igγ alone or using anti-NK1.1 antibody with cells from C3H/HeJ mice, an NK1.1$^-$ strain, no labeling of bone marrow small lymphocytes (<0.2%) was seen.

In the spleen, NK1.1$^+$ cells formed a slightly lower proportion of the total small lymphocytes than in the bone

marrow, but unlike the bone marrow they increased considerably in numbers with increasing age (Table 7).

The expression of other cell surface markers known to characterize mature NK cells, among other cells, is summarized in Table 7. Asialo GM1 was detected on about the same proportion of bone marrow small lymphocytes as NK1.1. The difference in the absolute number of spleen small lymphocytes bearing asialo GM1 with those expressing NK1.1 could be attributable to the strain differences in spleen cellularities.

3. Double labeling of null small lymphocytes expressing NK cell related markers

Double labeling techniques revealed null small lymphocytes by radioautography while simultaneously detecting a) the NK1.1 marker using avidin-biotin-immunoperoxidase complexes, b) tumor cell binding properties, and c) Fc receptors by erythrocyte rosetting.

a) NK1.1:

When immunoperoxidase labeling of NK1.1 was combined with radioautographic labeling by mAbs 14.8 and anti-Thy1 to reveal null cells, almost 10% of the 14.8$^-$Thy1$^-$ null small lymphocyte population in the bone marrow clearly expressed NK1.1 (Table 8 and 10) giving a granular peroxidase reaction on the cell surface (fig. 26), representing approximately 1/5 of the

NK1.1$^+$ cells in the bone marrow. Separate experiments, combining immunoperoxidase labeling with radioautographic labeling by either mAb 14.8 or anti-Thy1 alone, demonstrated that many (44%) of the remaining NK1.1$^+$ small lymphocytes were within the Thy1$^+$ population. Approximately 16% of the total Thy1$^+$ small lymphocyte population expressed NK1.1. Of these cells, 6.3% were Thy1^{lo} and 9.6% were Thy1^{hi} cells (Table 8). Of the few 14.8$^+$ cells expressing NK1.1 most had low grain count densities. When the primary antibody anti-NK1.1 was omitted in control experiments, few (0.3%) cells were labeled.

b) Target cell binding:

Table 9 and 10 shows the incidence of small lymphocyte binding to tumor target cells. Many (13-17%) small lymphocytes in both bone marrow and spleen showed a capacity to bind spontaneously to the surface of YAC.1 lymphoma cells *in vitro* (Fig.27). Double labeling demonstrated that one-third of the null small lymphocytes in the bone marrow and half of those in the spleen bind the YAC tumor cells, forming close cell contacts. Separate experiments, using either mAbs 14.8 or anti-Thy1 alone, showed that these labeled cells also bind tumor cells, but to a lesser degree. A further experiment combining radioautographic labeling of NK1.1 together with tumor cell binding revealed that these two properties are coexpressed on 1.4% of bone marrow small lymphocytes, representing 25% of the NK1.1$^+$ cells and 12% of the tumor

cell-binding small lymphocytes, respectively.

In addition, some non-lymphoid cells also bound to the YAC lymphoma cells. Such cells which included granulocytes, monocytes and erythroblasts represent either non-NK cell-related binding or non-specific adherence. In age-matched A/SN control mice, a strain previously reported to have low NK activity (Kiessling et al, 1975), only 1-2% of the small lymphocytes in both bone marrow and spleen bound to tumor target cells.

c) Fc receptors:

Table 10 depicts the proportion of small lymphocyte subsets in the bone marrow bearing Fc receptors (fig.27). More than a quarter of all small lymphocytes in the bone marrow formed Fc receptor rosettes with antibody-coated erythrocytes (Table 10). Double labeling revealed that 40% of the $14.8^{-}Thy1^{-}$ null small lymphocytes in the bone marrow express Fc receptors, while fewer (19.5%) 14.8^{+} and none of the $Thy1^{+}$ small lymphocytes formed rosettes for Fc receptors (Fig. 28, Table 10). Control preparations of bone marrow cells mixed with uncoated sheep erythrocytes showed low incidences (2%) of small rosettes (4-5 attached erythrocytes).

4. Depletion of NK1.1$^+$ small lymphocytes and null small lymphocytes by hydroxyurea

The effect of HU treatment for periods of 12-18 hr was examined to compare the dynamics of NK1.1$^+$ small lymphocytes and 14.8$^-$Thy1$^-$ null small lymphocytes separately in both the bone marrow and spleen (Table 11). In the bone marrow, the null small lymphocytes, in accord with the previous observations (Fig. 21b), were rapidly affected by HU treatment, being severely depleted at 12-18 hr compared with control (PBS treated) mice, which showed no change from normal values. NK1.1$^+$ cells, in contrast, became depleted only after a delay. They maintained relatively normal numbers for 12 hr but had subsequently declined by 18 hr. In the spleen both the NK1.1$^+$ and null small lymphocytes showed an initial lag in HU effect; they were still present in normal numbers after 12 hr but were depressed by 18 hr. In both the bone marrow and spleen after 18 hr HU treatment, the reduction of NK1.1$^+$ small lymphocytes was less severe than that of null small lymphocytes.

Fig. 24. Natural killer cell activity in the bone marrow and spleen of young (4 week old) and adult (8-10 week old) C3H/HeJ mice. Femurs and spleens were pooled from 3 to 5 mice.

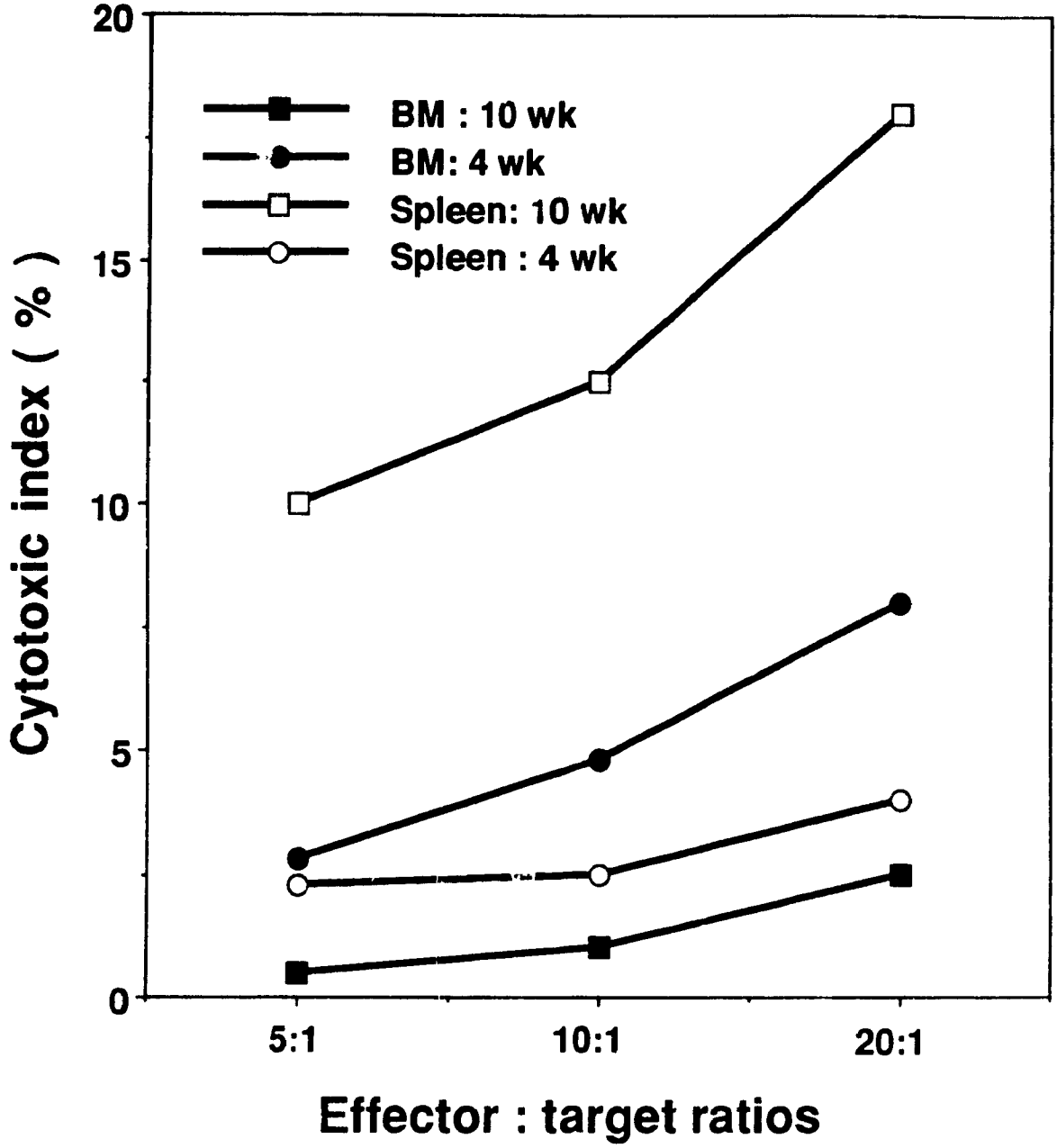

20
15
10
5
0
Cytotoxic index (%)
BM : 10 wk
BM: 4 wk
Spleen: 10 wk
Spleen : 4 wk
5:1
10:1
20:1
Effector : target ratios

Fig. 25. Natural killer cell activity of bone marrow cell suspensions and their lymphoid cell-rich fractions of bone marrow cells, with and without prior in vitro exposure to αIFN. Cells were pooled from 8-10 mice.

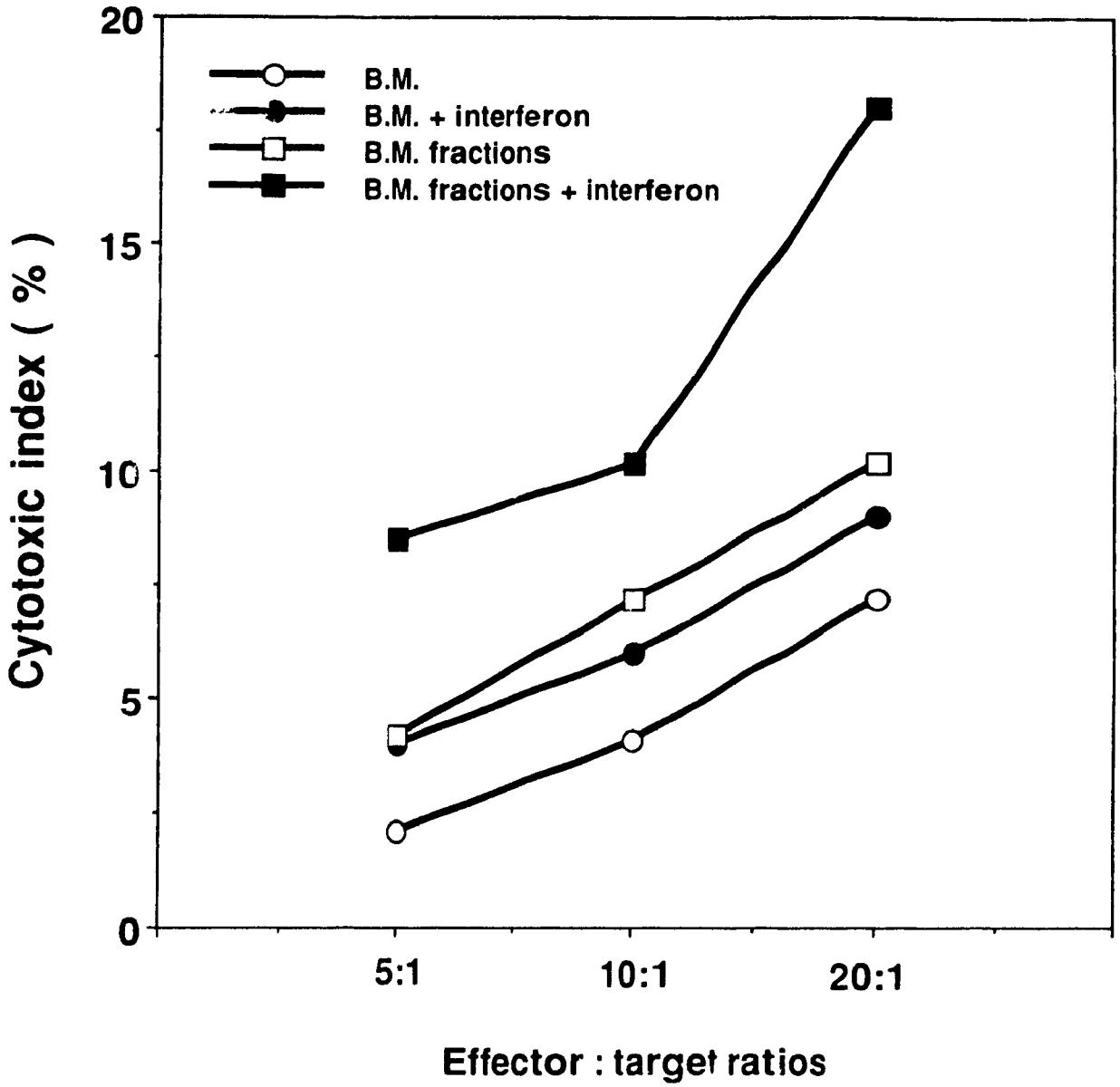

20
15
10
5
0
B.M.
B.M. + interferon
B.M. fractions
B.M. fractions + interferon
Cytotoxic index (%)
5:1
10:1
20:1
Effector : target ratios

Fig. 26. Photomicrograph of combined radioautography and immunoperoxidase labeling of bone marrow cells. A null small lymphocyte lacking radioautographic labeling for mAb 14.8 and Thy1 expresses the NK1.1 marker, as shown by the brown DAB reaction product on the cell surface. The large myeloid cell is devoid of any peroxidase labeling (x 1500).

Fig. 27. Photomicrograph of combined radioautography and tumor cell binding of bone marrow cells.

a. (left) an unlabeled null small lymphocyte is firmly bound to a large YAC lymphoma cell. Note the unstained Golgi region of the null cell oriented towards the tumor cells (x 1500).

b. (right) a labeled small lymphocyte is firmly bound to a large YAC lymphoma cell (x 1500).

Fig. 28. Photomicrograph of combined radioautography and erythrocyte rosetting of bone marrow cells.

a. (left) an unlabeled null small lymphocyte surrounded by antibody coated sheep erythrocytes, detecting Fc receptors (X 1500).

b. (right) a labeled small lymphocyte surrounded by antibody coated sheep erythrocytes, detecting Fc receptors (X 1500).

Fig. 29. Incidence of Fc receptors on subsets of null, 14.8^+, and $Thy1^+$ small lymphocytes in the bone marrow. Cells were pooled from 3-5 mice.

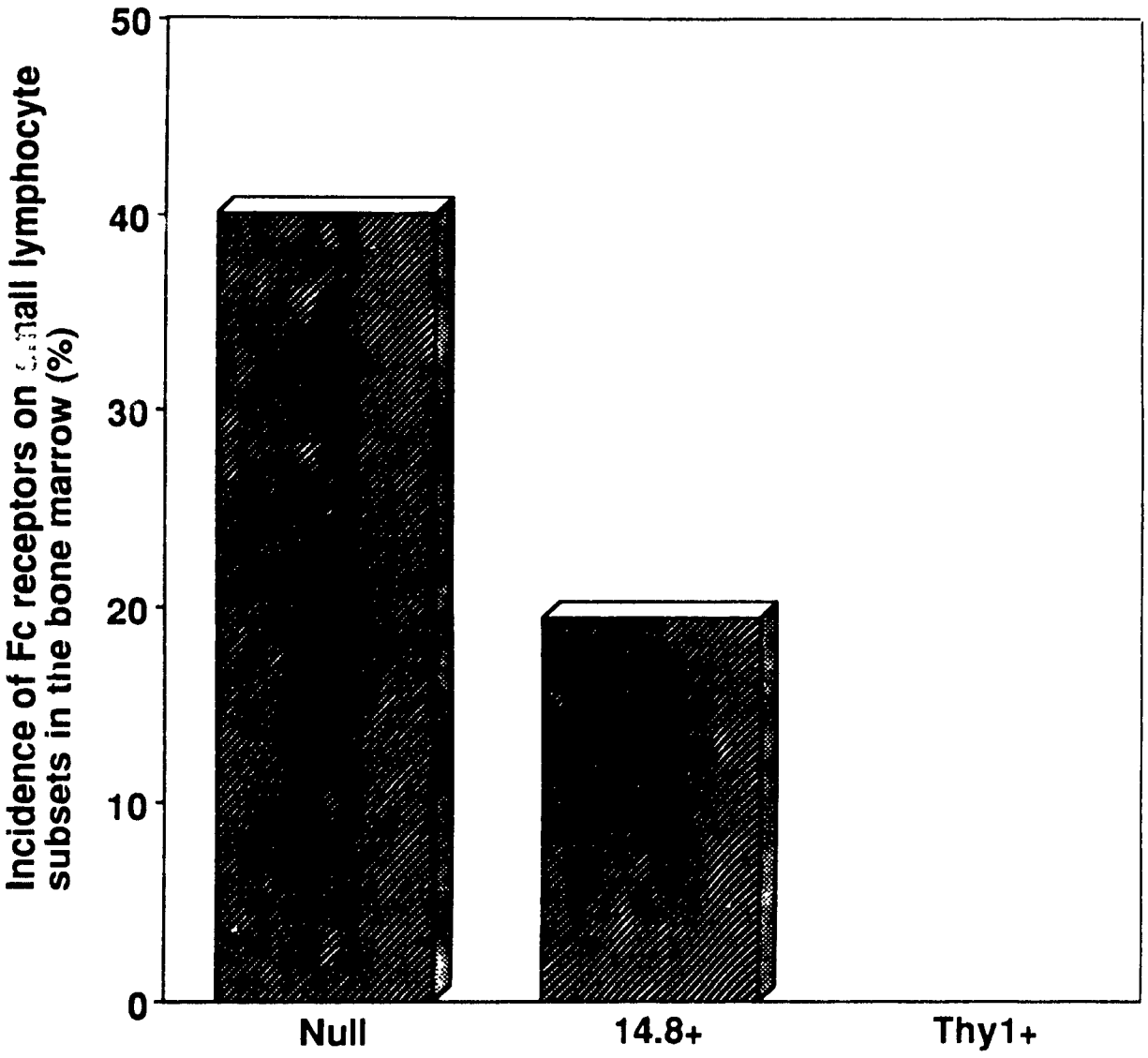

Incidence of Fc receptors on small lymphocyte subsets in the bone marrow (%)
50
40
30
20
10
0
Null
14.8+
Thy1+

TABLE 7

Expression of NK1.1 and Asialo GM1 by Small Lymphocytes in Bone Marrow and Spleen of Young Adult Mice

Marker	Organ	Incidence (%)		Number ($x10^5$)[a]	
		4 wk	10 wk	4 wk	10 wk
NK1.1[b]	Bone marrow	3.0	5.6±0.7	1.8	1.7±0.08
	Spleen	2.2	4.4±0.25	12.9	31.6±0.9
Asialo GM1[c]	Bone marrow	N.D.	5.0±0.9	N.D.	2.0±0.06
	Spleen	N.D.	5.4±1.1	N.D.	58.3±9.8

[a] Number of cells per femur and per spleen.

[b] Values derived from cells pooled from a group of 4 C57BL/6 mice aged 4 wk, and from 4 groups of 3-5 10 wk old C57BL/6 mice (mean ± standard error of the mean.

[c] Values derived from cells pooled from 3 groups of 3-5 10 wk old C3H/HeJ mice.

TABLE 8

Expression of NK1.1 on Various Small Lymphocyte Subpopulations in the Bone Marrow [a]

Population of small lymphocytes	NK1.1 bearing-cells (%) [b]
Null (14.8^- $Thy1^-$)	9.5
14.8^+	1.5
$Thy1^{lo}$ [c]	6.3
$Thy1^{hi}$ [d]	9.6

[a] Immunoperoxidase labeling of NK1.1 together with radioautographic labeling by mAbs 14.8 and anti-Thy1 to reveal 14.8^+, $Thy1^+$, and null lymphocytes

[b] Values derived from pooled cells from 4 C57BL/6 mice at 10 wk of age

[c] Small lymphocytes bearing low density Thy1 ($Thy1^{lo}$; 11-40 grains\cell)

[d] Small lymphocytes bearing high density Thy1 ($Thy1^{hi}$; >40 grains\cell)

TABLE 9

Incidence of Tumor Cell Binding on Small Lymphocyte Subsets in the Bone Marrow and Spleen

Tissue	Subset	Incidence of (%)[b]	
		Small lymphocyte subpopulation binding the tumor target cell[a]	Tumor cell binding capacity among:
Bone marrow	total	17.2 ± 0.87[c]	–
	null (14.8$^-$ Thy1$^-$)	33[d]	22
	Thy1$^+$	27[e]	15
	14.8$^+$	16	63
	sμ$^+$	5	16
	NK1.1$^+$	25	12
Spleen	total	13.6 ± 1.8	–
	null (14.8$^-$ Thy1$^-$)	53	37
	Thy1$^+$	30	32
	14.8$^+$	9	31
	sμ$^+$	7	38
	NK1.1	ND[f]	ND

[a] YAC.1 lymphoma was used as the target cell

[b] Values derived from pooled cells from four 10 week old C3H/HeJ mice.

[c] Mean ± standard error of the mean; n=8

[d] Incidence of small lymphocytes devoid of radioautographic grains forming close contacts with large YAC lymphoma cells

[e] Incidence of labeled small lymphocytes forming close cell contacts with large YAC lymphoma cells.

[f] ND: do data

TABLE 10

Summary of the Expression of NK1.1, Asialo GM1, Tumor Cell Binding Capacity and Fc Receptors by Null Small Lymphocytes in Bone Marrow

Cell marker	Incidence of marker-bearing cells (%) among:	
	Total small lymphocytes	Null small lymphocytes
NK1.1[a]	5.6±0.7	9.5
Asialo GM1[b]	5.1±0.9	ND[e]
Tumor cell binding[c]	17.2±0.87	33.2
Fc receptors[d]	28.6	40.1

[a] Immunoperoxidase labeling of NK1.1 together with radioautographic labeling by mAbs 14.8 and anti Thy1 to reveal null lymphocytes in 10 wk old C57BL/6 mice (Mean ± SEM, 4 groups of 3-5 mice).

[b] Cell suspensions were incubated with rabbit anti-asialo GM1, detected by ^{125}I-anti-rabbit IgG and radioautography in 10 wk old C3H/HeJ mice (Mean ± SEM, 3 groups of 3-5 mice).

[c] Tumor cell (YAC 1 lymphoma) binding together with radioautographic labeling by mAbs 14.8 and anti-Thy1 to reveal null lymphocytes in 10 wk old C3H/HeJ mice (Mean ± SEM, 4 groups of 3-5 mice).

[d] Rosetting with antibody-coated SRBC to detect FcR together with radioautographic labeling by mAbs 14.8 and anti-Thy1 to reveal null lymphocytes in 10 wk old C3H/HeJ mice.

[e] ND: no data.

TABLE 11

Effects of Hydroxyurea Treatment on Numbers of NK1.1$^+$ Small Lymphocytes and 14.8$^-$Thy1$^-$ Null Small Lymphocytes in the Bone Marrow and Spleen[a]

Organ	Cell population	Duration of HU treatment (hrs)	Incidence (%)	Number (x 10^5)	(% of control
Bone marrow	NK1.1$^+$[c]	0[b]	5.5	3.2	100
		12	9.0	3.8	119
		18	5.5	2.6	81
	Null[d]	0	12.5	7.3	100
		12	12.5	5.0	68
		18	9.0	4.3	59
Spleen	NK1.1$^+$	0	6.6	49.8	100
		12	6.4	49.9	100
		18	5.5	41.9	84
	Null	0	7.5	58.4	100
		12	7.7	60.0	103
		18	4.6	35.5	61

[a] Groups of five 10 wk-old C57BL/6 mice were given 2 i.p. injections of HU, the first at the beginning of the experiment and the second either at 11 hr in mice sampled at 12 hr or at 12 hr in mice sampled at 18 hr.

[b] Mean values from 2 control groups of five 10 wk-old C57BL/6 mice given 2 i.p.injections of PBS at the beginning of the experiment and at 11-12 hr, and sampled at 12 hr and 18 hr, respectively.

[c] Small lymphocytes radioautographically labeled by exposure to anti-NK1.1 and ^{125}I anti-mouse IgG.

[d] Small lymphocytes remaining radioautographically unlabeled after exposure to both mAbs 14.8 and anti-Thy1.2 plus ^{125}I-anti-rat IgG.

SECTION IV

This section quantitates the response of the null, NK1.1^{+}, and Thy1^{lo} small lymphocytes in the bone marrow of mice bearing two dissimilar tumors, the non-metastatic Ehrlich ascites (EA) tumor and the Lewis lung carcinoma (LLc), a highly metastactic solid tumor, in order to determine the potential significance of null cells in the bone marrow and to what extent these cells may belong to the NK lineage.

1. Bone marrow cellularity in Ehrlich ascites tumor-bearing mice

Figure 30 illustrates the number of total nucleated cells and small lymphocytes in the bone marrow of C3H/HeJ mice bearing an Ehrlich ascites (EA) tumor during 13 days of tumor growth. An intraperitoneal injection of (10^6) EA tumor cells resulted in the development of an extensive ascites tumor (3-5ml). The total number of small lymphocytes increased from 38 ± 0.9 x 10^5 cells/femur at 0-4 days to 61 ± 1.5 x 10^5 cells/femur at 5-7 days, returning to normal by 11-13 days. These changes contributed to an overall increase in bone marrow cellularity from 148 ± 5.4 x 10^5 nucleated cells/femur at 0-4 days to reach maximal values of 185 ± 4.2 x 10^5 nucleated cells/femur at 9-11 days.

2. Number of null, 14.8$^+$ and Thy1$^+$ small lymphocytes in the bone marrow of Ehrlich ascites tumor-bearing mice

By means of surface labeling and radioautography the total marrow small lymphocyte population of C3H/HeJ mice was subdivided, distinguishing null small lymphocytes from those bearing 14.8 and Thy1 markers (Fig. 31). Increases in the numbers of all three small lymphocyte subpopulations in the bone marrow were shown during the growth of the EA tumor (Fig. 31). Expressing the same data as a percentage of normal (non-tumor-bearing) values, null small lymphocytes showed the greatest increase (fig. 32). Null cells increased sharply to more than 3 times normal values between 5 and 6 days of EA tumor growth, followed by a return to normal levels by day 13 (Fig. 32).

Thy1$^+$ cells showed a more protracted effect, maintaining a 2-fold increase in numbers from 4 days onwards. Individual Thy1$^+$ cells express a range of Thy1 density, as revealed by radioautographic grain counts (section I of thesis), distinguising low intensities of Thy1 labeling (Thy1^{lo} cells) from the typical mature T lymphocytes having high intensities of Thy1 (Thy1^{hi} cells). Normally, Thy1^{lo} cells account for approximately 80% of the total Thy1$^+$ cells in the bone marrow. During EA tumor growth, the Thy1^{lo} cells were increased in number from 5 days to 9 days and then returned to normal values by 13 days (table 12). The Thy1^{hi} cells, in contrast,

were elevated from 5 days to the end of the experimental period. The 14.8^+ cells were increased approximately 1.5-fold for a limited period (5-9 days).

3. Bone marrow cellularity in Lewis lung carcinoma bearing mice : effect of indomethacin.

Subcutaneous growth of 10^6 LLc cells in C57Bl/6 mice gave rise to a solid tumor at the site of implantation, measuring approximately 2.0-2.5 cm in diameter by the end of the 32 day experimental period. During this period, the total number of small lymphocytes in the bone marrow increased slightly from 60 x 10^5 cells/femur at 0 days to almost 70 x 10^5 cells/femur at 5-16 days, then returning below normal values. Bone marrow cellularity also increased from 200 x 10^5 nucleated cells/femur at 0 days to 237 x 10^5 nucleated cells/femur at 5-16 days (fig. 33).

Following indomethacin treatment, the response of small lymphocyte numbers and bone marrow cellularity was augmented significantly, (82 x 10^5 cells/femur and 294 x 10^5 nucleated cells/femur respectively), and peaked at an earlier time interval (9 days) (fig. 33).

4. Number of null, 14.8^+ and $Thy1^+$ small lymphocytes in the bone marrow of Lewis lung carcinoma bearing mice: effect of indomethacin

The rise and fall of the number of null small lymphocytes

observed in the bone marrow of untreated LLc tumor-bearing C57BL/6 mice (Fig. 34) was similar to profiles obtained in EA tumor-bearing mice (Fig 32). Null small lymphocytes reached 4 times control values by day 9-16 days of tumor growth and returned towards normal levels. $Thy1^+$ small lymphocytes remained moderately elevated (1.5-2.5 fold) from 7 days onwards, whereas 14.8^+ cells remained slightly below normal values throughout.

Indomethacin treatment appeared to enhance the response of the null lymphocyte population (fig. 34). The initial increase in number of null small lymphocytes continued to rise to reach 6-fold normal levels at 9 days. Thereafter, however, the enhancement was not maintained. The number of null small lymphocytes showed a sharp decline which, from 16-32 days, resembled that in untreated LLc tumor-bearing mice. The patterns of response of $Thy1^+$ and 14.8^+ cells were not altered by indomethacin treatment. Once these mice were given indomethacin, the overall profile of all three subpopulations was similar to that of the untreated tumor bearing mice, the null cells reaching 600% of normal values (Fig. 34).

5. <u>Spleen cellularity in Lewis lung carcinoma bearing mice: effect of indomethacin.</u>

The number of small lymphocytes and total nucleated cells in the spleen of LLc tumor-bearing C57Bl/6 mice increased approximately 2-fold at 7-14 days relative to normal values

(fig. 35). After indomethacin treatment, spleen cell numbers of both small lymphocytes and total nucleated cells rose rapidly to peak at almost 3 times normal values by 5 days and returned to the level of untreated tumor-bearing spleen (control) values at 14-16 days. The effect of indomethacin was observed earlier in the spleen than in the bone marrow.

6. Number of null, 14.8^+ and $Thy1^+$ small lymphocytes in the spleen of Lewis lung carcinoma bearing mice: effect of indomethacin

The response of splenic small lymphocytes subpopulations in untreated LLc tumor-bearing mice was similar to that obtained in the bone marrow (fig. 36). As in the bone marrow, null small lymphocytes in the spleen showed the greatest proportionate increase in population size relative to $Thy1^+$ and 14.8^+ cells. The null lymphocytes which normally comprised 4.2 ± 0.14 % of splenic lymphocytes, representing $4.5 \pm 0.32 \times 10^6$ cells/spleen, were increased 3-5 fold in absolute numbers at 7-14 days in LLc tumor-bearing mice. All three subpopulations returned to slightly below normal values. When such mice were treated with indomethacin, the null lymphocyte response in the spleen was considerably enhanced, increasing the number of null cells 10-fold during the first 5 days of tumor growth. By 16 days of treatment, however, the number of null, 14.8^+ and $Thy1^+$ small lymphocytes in the spleen remained 2 to 3-fold higher than those in untreated LLc tumor-

bearing mice.

7. NK1.1 expression by null small lymphocytes in the bone marrow during LLc growth

To determine whether the bone marrow null small lymphocytes which responded to tumor growth included NK cells, double labeling experiments were performed by combining radioautography and immunoperoxidase to detect the expression of the NK1.1 marker on null small lymphocytes. Normally, approximately one tenth of $14.8^{-}Thy1^{-}$ null small lymphocytes in bone marrow expressed NK1.1 (table 13). During LLc tumor growth, however, the proportion of null lymphocytes expressing NK1.1 increased to 35% at 9 days (table 13). Consequently, whereas the total number of null small lymphocytes in the bone marrow increased 4-fold in number in the first 9 days of LLc tumor growth (fig. 34), the $NK1.1^{+}$ fraction of null cells increased 10-30 fold in the same time period (table 13).

Fig. 30. Number of total nucleated cells (□) and small lymphocytes (o) in the bone marrow of Ehrlich ascites tumor-bearing C3H/HeJ mice. Time 0 represents normal untreated mice. Vertical bar represents standard error of the mean.

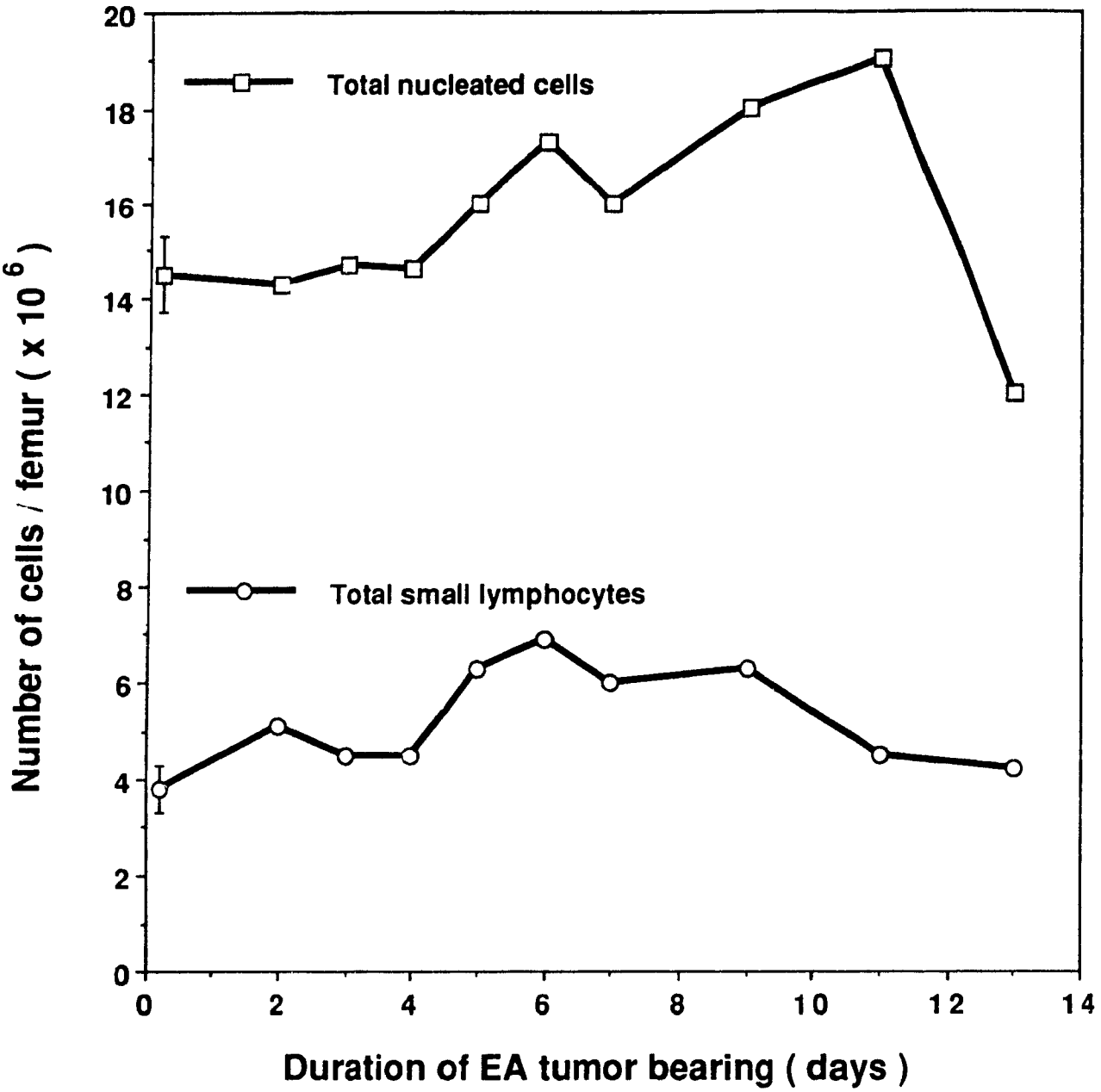

Total nucleated cells
Total small lymphocytes
Number of cells / femur (x 10 6)
Duration of EA tumor bearing (days)
0
2
4
6
8
10
12
14
16
18
20

Fig. 31. Number of null (△), 14.8$^+$ (o) and Thy1$^+$ (□) small lymphocytes in the bone marrow of Ehrlich ascites tumor bearing C3H/HeJ mice. Time 0 represents normal untreated mice.

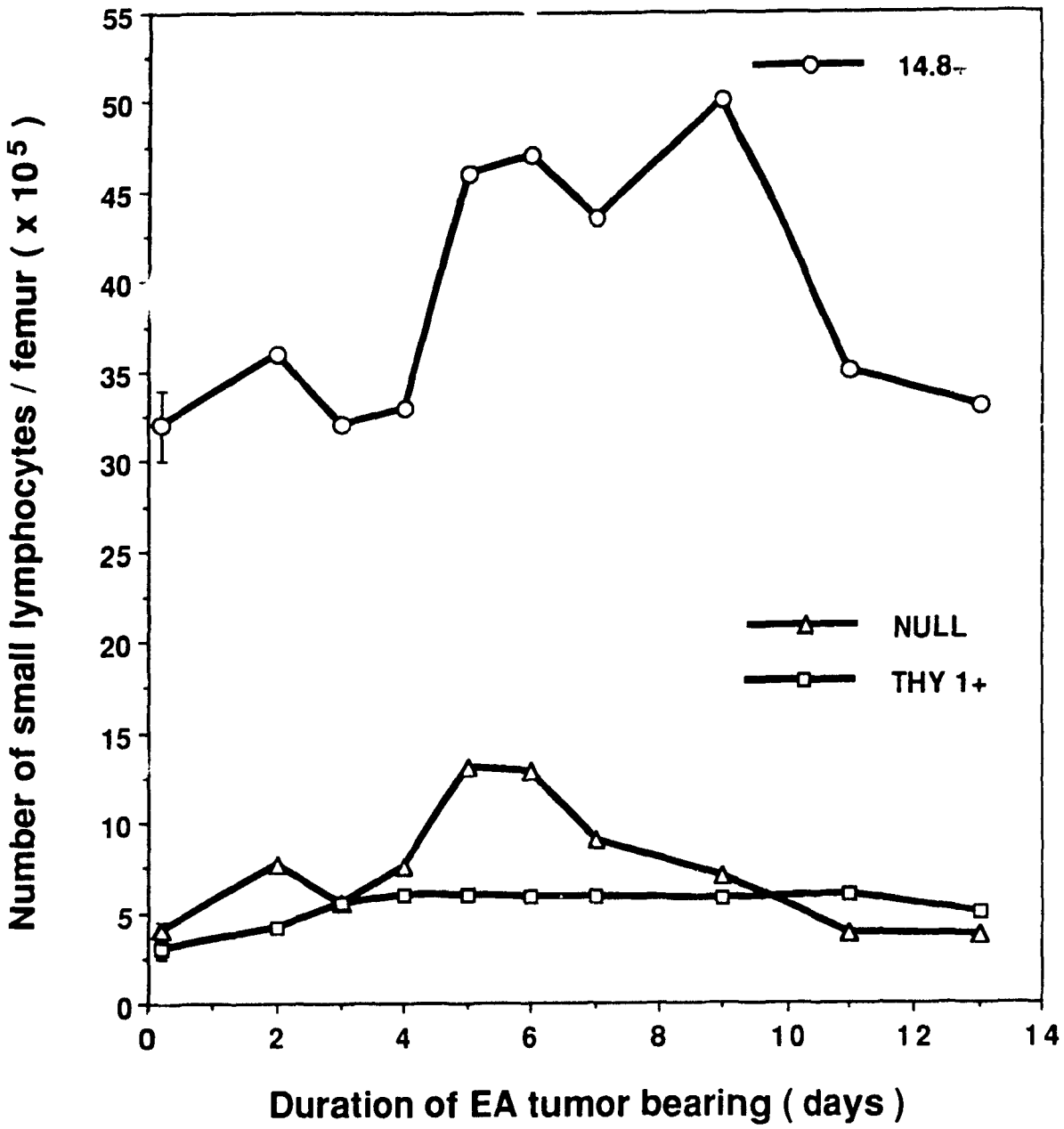

Number of small lymphocytes / femur (x 10^5)
55
50
45
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
Duration of EA tumor bearing (days)
14.8+
NULL
THY 1+

Fig. 32. Number of null (△), 14.8$^+$ (○) and Thy1$^+$ (□) small lymphocytes in the bone marrow of Ehrlich ascites tumor bearing C3H/HeJ mice, shown as a percentage of normal control values (n=1) at 100%.

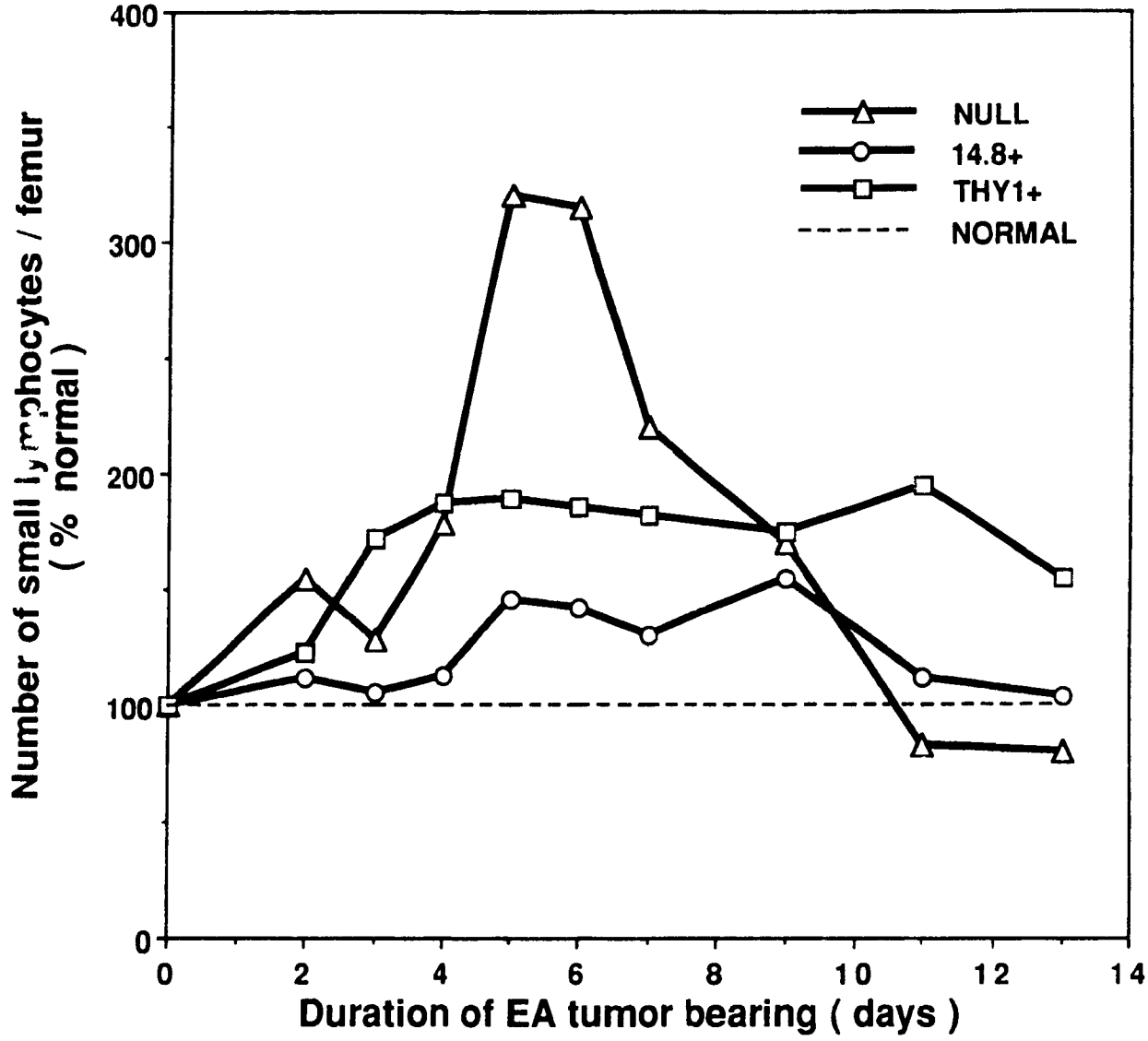

400
300
200
100
0
0
2
4
6
8
10
12
14
NULL
14.8+
THY1+
NORMAL
Number of small lymphocytes / femur
(% normal)
Duration of EA tumor bearing (days)

Fig. 33. Number of total nucleated cells (■, +indomethacin; □, -indomethacin) and small lymphocytes (●, +indomethacin; ○, -indomethacin) in the bone marrow of Lewis lung carcinoma bearing C57BL/6 mice. Time 0 represents normal untreated mice.

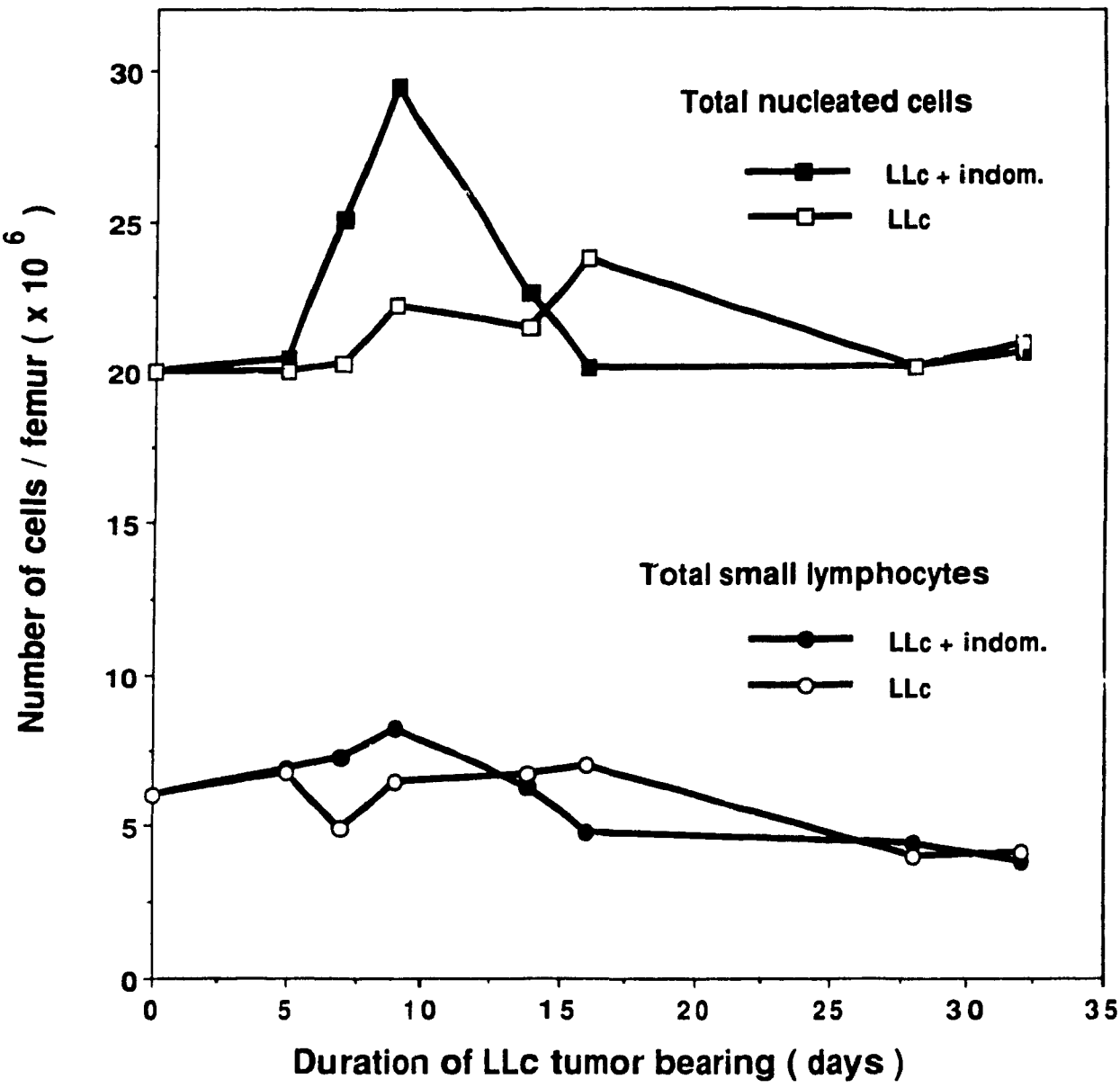

Total nucleated cells
LLc + indom.
LLc
Total small lymphocytes
LLc + indom.
LLc
Number of cells / femur (x 10 6)
Duration of LLc tumor bearing (days)
0
5
10
15
20
25
30
35

Fig. 34. Number of null (△), 14.8$^+$ (o) and Thy1$^+$ (□) small lymphocytes in the bone marrow of Lewis lung carcinoma bearing C57BL/6 mice, shown as a percentage of normal control values at 100%. (Left) untreated tumor-bearing mice; (Right) indomethacin treated tumor-bearing mice.

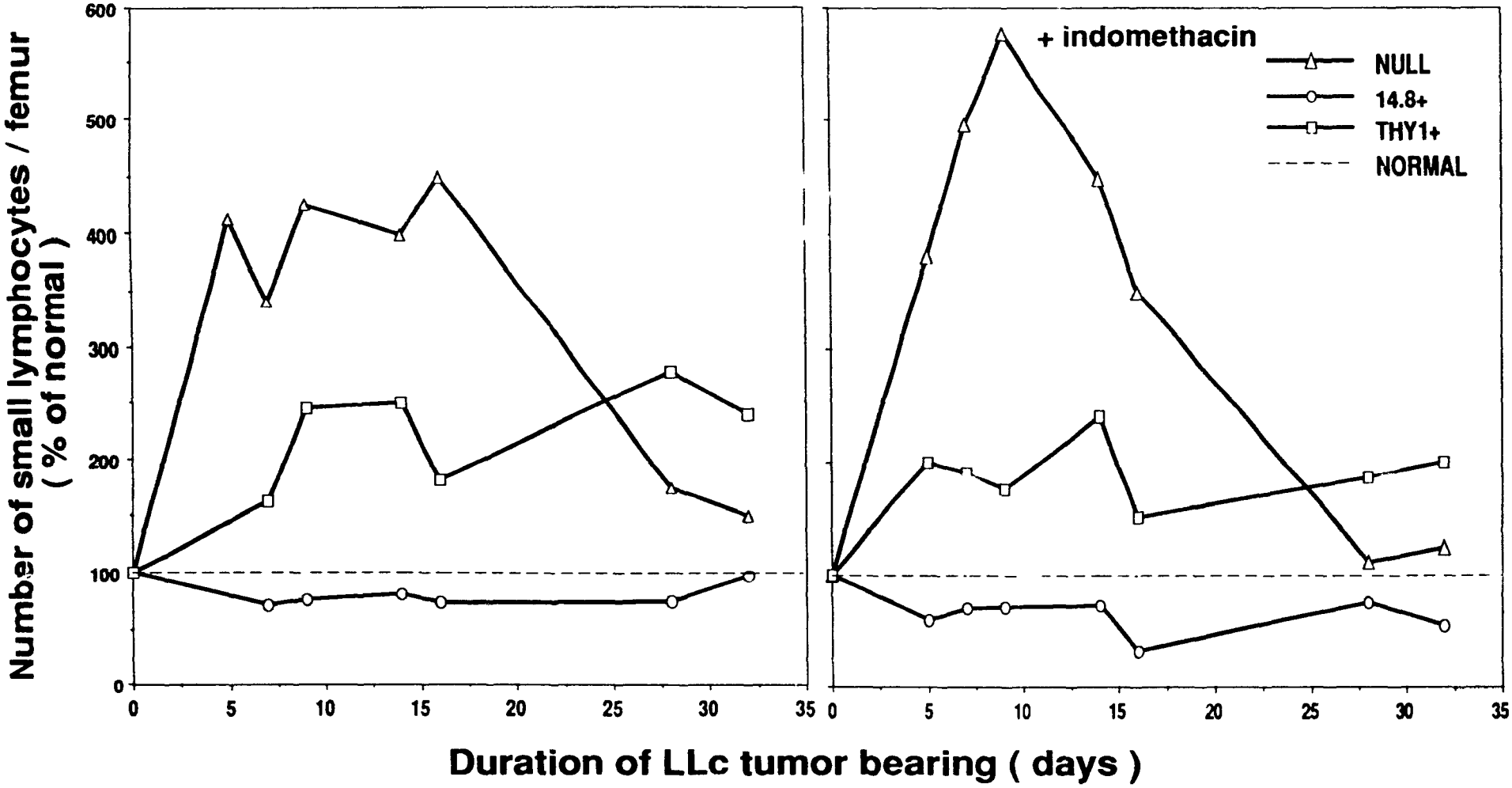

Number of small lymphocytes / femur
(% of normal)
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
+ indomethacin
NULL
14.8+
THY1+
NORMAL
Duration of LLc tumor bearing (days)

Fig. 35. Number of total nucleated cells (■, +indomethacin; □, -indomethacin) and small lymphocytes (●, + indomethacin; ○, -indomethacin) in the spleen of Lewis lung carcinoma bearing C57BL/6 mice. Time 0 represents normal untreated mice.

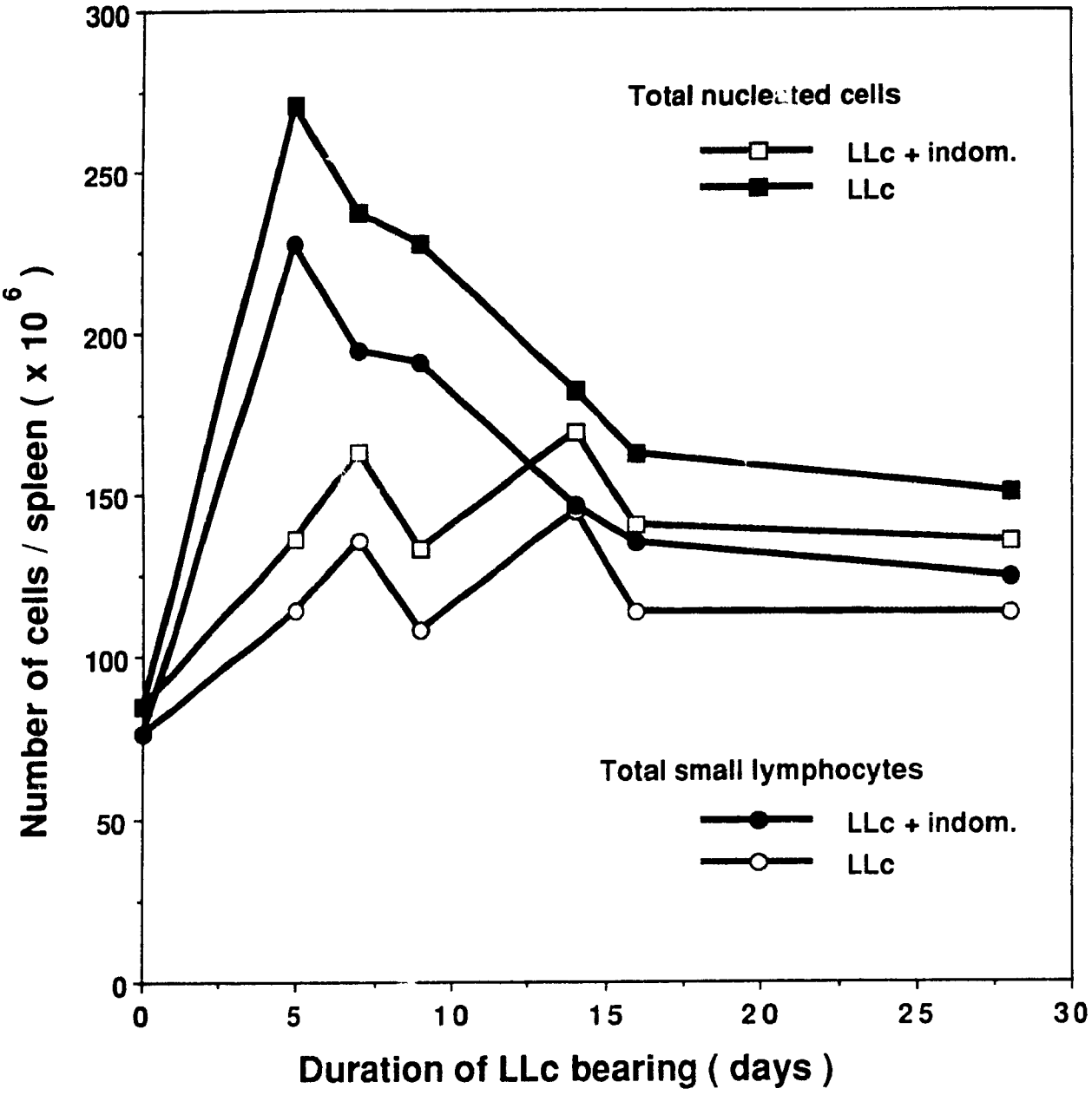

Total nucleated cells
LLc + indom.
LLc
Total small lymphocytes
LLc + indom.
LLc
Number of cells / spleen (x 10 6)
Duration of LLc bearing (days)
300
250
200
150
100
50
0
0
5
10
15
20
25
30

Fig. 36. Number of null (△), 14.8$^+$ (○) and Thy1$^+$ (□) small lymphocytes in the spleen of Lewis lung carcinoma bearing C57BL/6 mice, shown as a percentage of normal control values at 100%. (Left) untreated tumor-bearing mice; (right) indomethacin treated tumor-bearing mice.

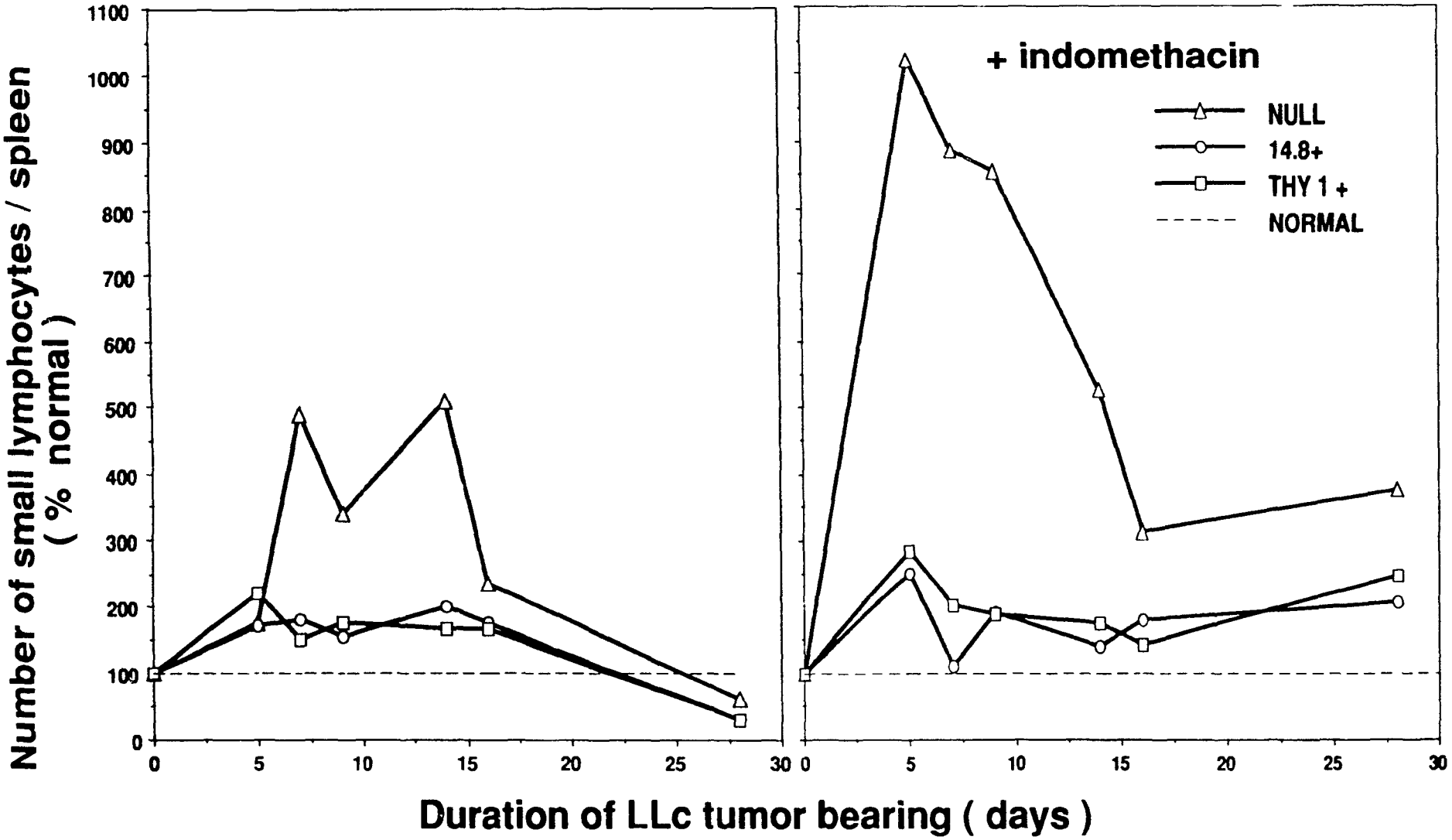

Number of small lymphocytes / spleen
(% normal)
1100
1000
900
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
+ indomethacin
NULL
14.8+
THY 1 +
NORMAL
Duration of LLc tumor bearing (days)

Table 12

Number of $Thy1^{lo}$ and $Thy1^{hi}$ small lymphocytes in the bone marrow of EA tumor-bearing mice

Duration of EA tumor-bearing (days)	Number of small lymphocytes/femur (% normal)	
	$Thy1^{lo}$ [a]	$Thy1^{hi}$ [b]
0	100	100
5	187	211
7	161	250
9	166	218
11	157	225
13	102	227

[a] small lymphocytes bearing low intensity Thy1; 11-40 grains/cell

[b] small lymphocytes bearing high intensity Thy1; >40 grains/cell

TABLE 13

Expression of NK1.1 by Null Small Lymphocytes in the Bone Marrow during the Growth of Lewis Lung Carcinoma (LLc)

Duration of LLc tumor-bearing (days)	Proportion of null small lymphocytes expressing NK1.1[a] (%)	Number of $NK1.1^+$ null small lymphocytes (cells/femur x 10^{-3})
0	9.5	0.6
5	22.7	6.5
7	21.3	5.0
9	35.3	18.1
14	21.6	6.0
16	18.5	5.8
28	14.0	1.7

[a] A double labeling procedure was used to reveal null (14.8^- $Thy1^-$) small lymphocytes by radioautography while detecting the expression of NK1.1 by immunoperoxidase

DISCUSSION

The radioautographic immunolabeling methods developed in the present work, revealed the existence in the bone marrow of a population of lymphocytes which cannot be assigned to either the B cell or T cell lineage on the basis of μ chains (cμ or sμ), the mAb 14.8 determinant, B220, and the T-cell surface marker, Thy1, and have therefore been termed null lymphocytes. Studies of population dynamics have shown that null small lymphocytes are in a state of rapid renewal, generated from proliferating precursor cells in the bone marrow. Their high rate of production is almost one third as great as that of primary B lymphocytes suggesting that null cells could make an important functional contribution to the immune system. Some of these newly-formed small lymphocytes in the bone marrow express the natural killer cell marker, NK1.1, as well as other NK cell-associated characteristics. Mouse bone marrow also contains a population of small lymphocytes which express Thy1 in low intensity (Thy1^{lo} cells). Like null cells, these Thy1^{lo} cells are rapidly renewed and in some cases express NK1.1. When mice were given two dissimilar tumors, there were marked increases of limited duration in the numbers of null, NK1.1^{+} and Thy1^{lo} small lymphocytes in the bone marrow. The effect on null cells was augmented in mice given indomethacin. These findings correlated with those on the functional activity of NK cells in tumor-bearing mice, in

accord with the view that the production of null and Thy1^{lo} small lymphocytes in the bone marrow represent the genesis of cells of the NK cell lineage.

Radioautographic labeling of cytoplasmic μ chain in pre-B cells

Techniques were first developed to determine the incidence and size distribution of $c\mu^+s\mu^-$ pre-B cells among cells identified by conventional hematological staining as being small lymphocytes and large lymphoid cells. Following the procedure commonly used for immunofluorescence labeling of $c\mu$ (Raff et al, 1976), cell smears were fixed to allow the penetration of the radiolabeled anti-μ antibody. A tendency of such fixed preparations to bind serum components nonspecifically, as revealed by the sensitive technique of radioautography under strong radioautographic exposure conditions, was largely overcome by pre-exposing the fixed cells to normal goat serum. The specificity of labeling for μ chains was demonstrated by competitive displacement and by the lack of labeling of the μ^- cells in the thymus of normal mice or in the spleen and thymus of anti-IgM treated mice. In addition, an absence of Fc receptor binding was indicated by the lack of labeling of cells known to bear Fc receptors but to lack $s\mu$. Thus, both in normal mice and in anti-IgM treated mice there was no clear-cut labeling of either mature granulocytes or thymocytes, despite the well-marked expression

of Fc receptors detected by rosetting techniques in our laboratory on a large majority of marrow granulocytes (75%) and a substantial subset of thymocytes (10-15%) (Yang & Osmond, 1979). The radioautographic technique developed in the present work may also be useful to detect other intracellular components of individual cells in stained cell preparations using appropriate antibodies.

The present study demonstrates that many cells having the typical morphology of small lymphocytes and large lymphoid cells are $c\mu^{+}s\mu^{-}$ pre-B cells. A similar morphology has been demonstrated by the staining of small numbers of individual cells demonstrated to contain cytoplasmic μ chains by immunofluorescence (Owen et al, 1977) and by separating presumptive pre-B cells with a monoclonal antibody (Landreth et al, 1982, 1983).

The size distribution of the lymphoid cells in the bone marrow (Fig. 9) accords with previous work on smears, a cell diameter of 8 μm being the boundary between predominantly noncycling small cells and rapidly dividing large lymphoid cells (Osmond & Nossal, 1974a; Miller & Osmond, 1975). In the cytocentrifuge preparations widely used for conventional immunofluorescence studies of $c\mu^{+}s\mu^{-}$ pre-B cells, the size profile is shifted about 2 μm larger, 10 μm diameter being the boundary between cycling and nondividing cells (Yang et al, 1978; Opstelten & Osmond, 1983; Osmond & Owen, 1984; Osmond & Park, 1987). With this proviso, the radioautographic cell

size profile of $c\mu^+s\mu^-$ cells resembles that determined by cell measurements in immunofluorescence preparations (Osmond & Owen, 1984). The $c\mu^+s\mu^-$ cell size distribution in radioautographs was determined in two ways, first by subtraction of $s\mu^+$ cells from total μ^+ cells in normal mice, and second by the total μ-chain labeling in mice depleted of all $s\mu^+$ cells by the in vivo administration of anti-IgM. The profiles obtained from both approaches are similar, though large $c\mu^+s\mu^-$ cells are relatively more prominent than usual in anti-IgM treated mice. This is consistent with an accumulation of slowly cycling large $c\mu^+s\mu^-$ cells in anti-IgM treated mice, as seen by immunofluorescence and mitotic arrest techniques (Opstelten & Osmond, 1983; Park & Osmond, 1987).

Heterogeneity of lymphoid cells in bone marrow

Of the large lymphoid cells in the bone marrow, originally described as transitional cells (Yoffey et al, 1965; Rosse, 1976), many have cytoplasmic μ chains and are thus now identified as proliferating pre-B cells. A majority (60%) of the large lymphoid cells are totally μ-negative, however. This is consistent with the known heterogeneity of this morphological cell category (Miller & Osmond, 1975; Rosse, 1976). While some of these μ-negative large lymphoid cells might be pro-B cells (early B-lymphocyte precursors before μ chain expression) they may also include precursors of other lymphocyte lineages or of nonlymphoid hemopoietic cells. Some

hemopoietic progenitor cells have the morphology of large lymphoid (transitional) cells (Moore et al, 1972; Yoffey, 1973; Trentin, 1973).

The small $c\mu^+s\mu^-$ pre-B cells have now been identified in conventionally stained smears as typical small lymphocytes, morphologically indistinguishable from mature B or T lymphocytes. Together with the $s\mu^+$ small lymphocytes these B lineage cells account for approximately 80% of the total small lymphocyte population in murine bone marrow.

Of the μ-negative small lymphocytes and large lymphoid cells in the bone marrow, some show the Thy1 marker characteristic of all mature T lymphocytes. However, the incidence of $Thy1^+$ small lymphocytes exceeds (2-fold) the combined incidences of $L3T4^+$ helper T cells and $Lyt2^+$ cytotoxic/suppressor T cells, suggesting that not all Thy1-bearing cells in the bone marrow necessarily belong to the T lineage (see section III). The presence of L3T4 on some large lymphoid cells in the bone marrow may correspond to a population of primitive hemopoietic stem cells, previously shown to express the L3T4 marker (Fredrickson & Basch, 1989). The expression of Lyt1 on some small lymphocytes in the bone marrow may correspond to either helper T cells which also display the L3T4 marker or a separate lineage of B cells bearing Lyt1, reported to originate from the peritoneal cavity and to produce autoantibodies (Hardy & Hayakawa, 1986).

The wide range of densities of the Thy1 antigen expressed

by individual small lymphocytes and large lymphoid cells in the bone marrow, as compared to those in the spleen and thymus, suggest that $Thy1^+$ lymphoid cells in the bone marrow are heterogeneous. The $Thy1^+$ cells may represent different cell lineages or maturational stages, including $Thy1^{lo}$ Lin^- hemopoietic stem cells (Muller-Sieburg et al, 1986; Sprangrude et al, 1988a), recirculating activated T cells (Rosse, 1976; Scollay et al, 1984) and NK cells bearing Thy1 (Herberman et al, 1978; Hurme & Sihvola, 1984; Hackett et al, 1986).

A substantial subset of marrow small lymphocytes lacks both detectable μ chains and Thy1 antigen. Previous work based on cell surface markers alone has shown the presence of many apparently "null" small lymphocytes in the marrow, lacking both B and T lineage surface markers (Osmond & Nossal, 1974a; Ryser & Vassalli, 1974; Press et al, 1977; Yang et al, 1978). The proportion of these cells that might be immature B cells was unknown, however. Kinetic data would be consistent with the view that all newly formed small lymphocytes in the marrow are B lymphocytes (Osmond & Nossal, 1974b; Yang et al, 1978). If so, however, the null small lymphocytes would all be expected to be post mitotic pre-B cells bearing cytoplasmic μ chains. The present finding of null small lymphocytes lacking detectable $c\mu$, suggests the presence of another lymphocyte lineage.

$c\mu^-s\mu^-$Thy1$^-$ null small lymphocyte population in bone marrow

Estimates of the magnitude of the population of $c\mu^-s\mu^-$ Thy1$^-$ null small lymphocytes in the marrow depend for their accuracy upon the sensitivity of the present technique for radiolabeling μ chains. An overestimate of the population of null cells would result from a lack of sensitivity of the technique in detecting the low concentrations of μ chains, found in the cytoplasm of pre-B cells and the surface membrane of immature B lymphocytes in the bone marrow. However, the observed incidences of bone marrow cells labeled for $s\mu$ and $c\mu$, (15% and 12% of the total nucleated marrow cells, respectively), accord with the highest reported values obtained by other techniques. It is also important to exclude the possibility that the labeling might represent either nonspecific binding or Fc-receptor binding, as already noted. With these provisos, the present work appears to provide a sensitive detection of μ chains and indicates the presence of $c\mu^-s\mu^-$Thy1$^-$ null population equivalent to approximately one tenth of the marrow small lymphocytes, (2-3% of all marrow nucleated cells). In view of the particularly rapid turnover of the $s\mu^-$ population of small lymphocytes of which they form a part (Chan & Osmond, 1979), these findings suggest a substantial continuous production of $c\mu^-s\mu^-$ Thy1 null small lymphocytes in the bone marrow.

Radioautographic identification of null small lymphocytes in the bone marrow using cell surface criteria ($B220^-Thy1^-$)

To ensure complete labeling of all B lineage cells in the bone marrow, including early B lymphocyte progenitors which do not express the lineage specific μ chains, null cells are now defined as small lymphocytes lacking two cell surface markers, the B220 glycoprotein (detected by mAb 14.8) and Thy1, in radioautographic preparations. Labeling cell surface components rather than intracellular markers would facilitate the fractionation of these lymphocyte subpopulations in any future work.

The proportion of bone marrow small lymphocytes binding mAb 14.8 in the the present work accords with that of post-mitotic pre-B cells and B lymphocytes as shown by the radioautographic labeling of lineage-specific μ chains. The incidence of 14.8^-Thy1^- null cells in the bone marrow corresponds well with values based on radioautographic labeling of μ chains and Thy1. Similar values have been obtained using a variety of strains and ages of mice, attesting to the consistent population size of null small lymphocytes. These results emphasise the magnitude of the null small lymphocyte population, which represents 12-14% of all bone marrow small lymphocytes, 2-3% of total nucleated cells and 0.5×10^6 cells per femur in adult mice.

Population dynamics of null small lymphocytes in bone marrow and spleen

Small lymphocytes are post-mitotic cells not in cell cycle, most of which in the bone marrow are recently derived from proliferating precursors (Miller & Osmond, 1975; Osmond, 1987). Administration of HU kills DNA-synthesising cells but does not affect non-dividing small lymphocytes (Rusthoven & Phillips, 1980; Miller, 1982; Rocha et al, 1983; Freitas et al, 1986). A decline in number of non-dividing cells during HU treatment thus reflects the rate at which the cells normally would have been renewed from dividing precursors and gives the turnover rate of the non-dividing cell compartment (Rusthoven & Phillips, 1980; Freitas et al, 1986; Osmond, 1987). The turnover and production rate of the total small lymphocyte population and of the 14.8^{+} small lymphocytes in mouse bone marrow determined by HU deletion in the present study correspond closely with the values previously determined by radioautographic ^{3}H-thymidine labeling combined with the labeling of either $c\mu$ or $s\mu$ (Osmond & Nossal, 1974b; Miller & Osmond, 1975; Yang et al, 1978; Landreth et al, 1981; Opstelten & Osmond, 1983). These findings confirm the rapid renewal of small lymphocytes in the bone marrow and suggest that the cellular dynamics revealed by the HU deletion technique in the present work represent normal steady state cell kinetics.

The population dynamics of $14.8^{+}Thy1^{-}$ null small

lymphocytes in the bone marrow are now shown to be distinctively different from those of the 14.8^+ and $Thy1^+$ subsets. Their exceptionally rapid renewal rate indicates that the newly-formed null cells have a short transit time within the bone marrow, combined with the substantial population size already noted, the rapid turnover of null cells reveal a large total production rate, calculated to total 1.3×10^7 cells per day. This continuous genesis of null cells could make a considerable contribution to the cell populations of the peripheral immune system.

Many newly-formed small lymphocytes have been shown by in vivo labeling techniques to migrate in large numbers from the bone marrow to the peripheral lymphoid tissues, notably the spleen (Brahim & Osmond, 1970, 1976). The present observation of an initial lag period before null cells begin to decline in the spleen during HU treatment would be consistent with an extrasplenic origin of these cells from the bone marrow. The lag period would represent the minimum length of time between the DNA synthetic phase of the immediate dividing precursor in the bone marrow and the migration of the newly-formed cell into the spleen. Thereafter, the rapid decay in number of splenic null cells during HU treatment ($T_{\frac{1}{2}}$,16.5 hr) suggests that these cells have only a short total life span in the peripheral lymphoid tissues. The large-scale production of null small lymphocytes in the bone marrow would thus maintain a dynamic population of

rapidly renewing null cells in the peripheral lymphoid tissues.

Natural killer cell-associated properties of null small lymphocytes in bone marrow

The distinctive population dynamics of null small lymphocytes in the bone marrow, together with their lack of B and T cell lineage markers suggest that these cells represent a functionally separate lineage of cells. The demonstration of NK cell-associated surface markers on some of the bone marrow null small lymphocytes, using double labeling techniques at the single cell level in the present work, now suggests an affiliation with the NK cell lineage.

NK cells in the spleen are known to be bone marrow-derived (Haller & Wigzell, 1977; Haller et al, 1977; Kumar et al, 1979), and bone marrow has been shown to contain immature NK cells which can develop into functional NK cells (Herberman et al, 1979; Hackett et al, 1986; Pollack & Rosse, 1987). The immature NK cells in the bone marrow appear to be small cells with few or no granules, which subsequently enlarge and develop cytoplasmic granules as they become functional (Abo et al, 1983; Davis et al, 1987; Pollack & Rosse, 1987). Although some studies have shown that murine NK cells are large granular lymphocytes (LGL), such cells have not been detectable in hematologically stained preparations of the mouse bone marrow either in the present work or in published

reports (Silvennoinen et al, 1986; Pollack & Rosse, 1987; our own observations).

Mature NK cells in mice are characterized by the expression of NK1.1, asialo GM1, tumor cell binding capacity and FcR, as indicated by cell enrichment and deletion studies (Roder et al, 1978; Kasai et al, 1980; Herberman, 1982; Koo & Peppard, 1984; Hackett et al, 1986; Koo et al, 1986; Pollack & Rosse, 1987). A subset of the null small lymphocytes in the bone marrow expressed these various surface properties in the current work, suggesting that these cells may represent a population of NK lineage cells. Using functional assays, the bone marrow has been shown to contain immature NK cells which can respond to IFN and develop rapidly into fully effective NK cells (fig. 23) (Djeu et al, 1979; Herberman et al, 1979; Kumar et al, 1979; Miller, 1982; Koo et al, 1986; Hackett et al, 1986; Davis et al, 1987). Many of the bone marrow null small lymphocytes express FcR, fewer bind to tumor (YAC) cells and (10%) express NK1.1 (Table IV). Asialo GM1 is found on the same proportion of bone marrow small lymphocytes as NK1.1. The present findings raise the possibility that the null small lymphocytes in the bone marrow represent a late stage of NK cell development. The cells would lack NK cell markers when first formed from dividing precursors, subsequently progressively expressing FcR, tumor cell-binding capacity and NK1.1 together with asialo GM1. Such a post-mitotic maturation process would resemble the $sIgM^-$ to $sIgM^+$

development of newly-formed lymphocytes of the B lineage which is accompanied by progressive expression of B lineage-associated surface properties (Osmond & Nossal, 1974a,b; Yang et al, 1978; Osmond, 1987). Such a hypothetical model of NK cell genesis in the bone marrow can be examined in the light of existing data, as follows.

Of the 4 NK cell-associated surface markers used in the present study, NK1.1 appears to characterize all functionally mature NK cells (Glimcher et al, 1977; Koo & Peppard, 1984; Hackett et al, 1986; Koo et al, 1986). NK1.1 expression thus provides evidence for the NK cell identity of bone marrow null small lymphocytes. The total incidence of NK1.1$^+$ cells in the bone marrow was consistent in 4 separate sets of experiments. The double labeling technique provided clear visualization of peroxidase-labeled NK1.1 on null small lymphocytes revealed by their lack of radioautographic labeling for B and T cell lineage markers. A quarter of all the NK1.1$^+$ cells in the bone marrow bound to YAC tumor cells, consistent with findings of Pollack and Rosse (1987). In other tissues, asialo GM1 is present on functional NK cells (Kasai et al, 1980; Beck et al, 1982) and on almost all NK1.1$^+$ cells (Hackett et al, 1986). The coincidence of the various NK cell-associated markers on individual null cells remains to be verified.

Population dynamics and development of NK1.1$^+$ and null small lymphocytes

Kinetic studies fully accord with the hypothesis that much of the functional NK cells present in the bone marrow and spleen reside within the population of null small lymphocytes (Miller, 1982; Pollack & Rosse, 1987). By assaying the ability of bone marrow and spleen cells to lyse YAC lymphoma cells during HU treatment, Miller (1982) has shown that although functional NK cells are not themselves in cell cycle, they exist in a state of rapid renewal in the bone marrow ($T_{\frac{1}{2}}$, 7.5 hr) and spleen ($T_{\frac{1}{2}}$, 20.5 hr). In the spleen they are deleted by HU only after an initial lag period of 24 hours (Miller, 1982). These kinetic parameters all correlate well with those observed for the null small lymphocyte populations in the bone marrow and spleen using the same HU deletion regimen in the present work. If, as proposed, the NK1.1 marker is expressed on newly-formed null small lymphocytes only after a post-mitotic maturation period, a delay would be expected in their deletion compared with the null cell population as a whole. This prediction is borne out by the present HU deletion results from NK1.1$^+$ cells. Whereas in the bone marrow the null small lymphocytes decline rapidly, the NK1.1$^+$ small lymphocytes do so only after a lag period of at least 18 hours. This would be consistent with the observed incidence of NK1.1$^+$ cells and the turnover of null small lymphocytes if the NK1.1$^+$ null cells are proposed to represent

the oldest subset of null small lymphocytes in the bone marrow. The preliminary kinetic data for NK1.1$^+$ cells suggest that the NK1.1 marker first appears only after a distinct lag period on newly-formed bone marrow null small lymphocytes, which thus undergo an NK1.1$^-$ to NK1.1$^+$ transition during a post-mitotic maturation period.

The rapid postnatal increase in null small lymphocytes after birth and the presence of NK1.1$^+$ cells in the bone marrow of young mice are consistent with the early development of NK cell function previously noted in the bone marrow (Miller et al, 1981). The present data reveal that the number of null small lymphocytes and NK1.1$^+$ cells are maintained in the bone marrow of adult mice. The NK cell function of whole bone marrow cell suspensions has been found to decline with age (Miller et al, 1981). The NK1.1$^+$ cells themselves, however, when separated from the bone marrow of adult mice, remain highly lytic (Hackett et al, 1986). Thus, the bone marrow of adult mice may continue to produce NK cells whose functional potentials are normally inhibited within the bone marrow, possibly by a suppressor cell mechanism (Savary & Lotzova, 1978; Hackett et al, 1986).

The NK1.1$^+$ cells in the bone marrow are mostly small lymphocytes (<8μm diameter in smears), while some are marginally larger lymphoid cells (9-10μm diameter in smears). This suggests that newly-formed NK cells form part of the small lymphocyte population of the bone marrow and lymphoid

tissues, morphologically indistinguishable from resting B and T lymphocytes, subsequently enlarging and developing cytoplasmic granules as they become functional (Abo et al, 1983; Davis et al, 1987).

Bone marrow small lymphocytes, including immature B cells, are markedly sensitive to stress. This accounts for the fall in numbers of total nucleated cells and of small lymphocytes in the bone marrow of saline-injected control mice. The delayed decline in null small lymphocytes now observed in the spleen of saline-injected mice suggests that these cells also are stress-sensitive, consistent with the susceptibility of NK cell function to stress in vivo (Pollock et al, 1987).

Expression of natural killer cell-associated surface markers on other lymphocyte subpopulations in the bone marrow: potentials of Thy1$^+$ cells

The NK cell-associated surface markers detected on null small lymphocytes in the present work were also expressed by some other cell populations in the bone marrow. The observed incidence of FcR on 14.8$^+$ small lymphocytes but not on Thy1$^+$ cells in the present work accords with previous data concerning FcR expression by sIgM$^+$ B lineage cells in the bone marrow (Yang et al, 1978; Yang & Osmond, 1979). The observed expression of NK1.1 by small lymphocytes having low densities of surface Thy1 antigen (Thy1^{lo} cells) suggests that the Thy1$^+$

cells in bone marrow, which, as already noted, are characterized by a wide range of Thy1 density, and may be functionally heterogeneous. Studies on population dynamics have shown that the subpopulations of $Thy1^{+}$ cells in the bone marrow are kinetically distinct. $Thy1^{hi}$ cells have a slow rate of renewal. This accords with the view that $Thy1^{hi}$ cells consist mainly of long-lived recirculating T lymphocytes from the blood (Ropke & Everett, 1973; Sprent & Basten, 1973; Press et al, 1977). $Thy1^{lo}$ cells, on the other hand, are rapidly-renewed. If so, $Thy1^{lo}$ expression would appear to be either a separate subset of NK cells or a feature of terminal maturation of NK cells. Approximately one half of the $NK1.1^{+}$ cells in the spleen have been shown to express Thy1 (Hurme & Sihvola, 1984; Hackett et al, 1986). The observed expression of NK1.1 by some $Thy1^{lo}$ small lymphocytes, however, suggests that these cells, in addition to the $14.8^{-}Thy1^{-}$ null small lymphocytes, form part of the rapidly-generated lineage of NK cells in the bone marrow. Alternatetively, not all $Thy1^{lo}$ cells in the bone marrow are necessarily NK cells. Possibly, these $Thy1^{lo}$ cells in the mouse bone marrow may include some newly-formed cells from the thymus (Rocha et al, 1983) or primitive hemopoietic progenitor cells (Muller-Sieberg et al, 1986; Sprangrude et al, 1988a).

Little is known about the progenitors and maturational stages of NK cells within the bone marrow. Previous studies

have shown the existence of transplantable NK cell precursors in the bone marrow, distinct from the pluripotential stem cell, capable of reconstituting NK activity in the mouse (Hackett et al, 1985a,b). Upon exposure to various lymphokines, immature precursors develop into functionally mature NK cells capable of lysing their target cells (Herberman, 1982). Previous work has given no information of the possible incidence and actual numbers of NK cells produced in the bone marrow. The present study now identifies null small lymphocytes in the bone marrow by direct cytological criteria at the single cell level, and determines the turnover and production rate of a population of cells which may represent differentiating NK cells.

Other potentials of null small lymphocytes in bone marrow

It is not excluded that some 14.8^{-}Thy1^{-} null small lymphocytes in the bone marrow could have potentials other than those of NK cells. Some resting hemopoietic precursors of low incidence in bone marrow are non-dividing cells of small-medium size (Trentin, 1973). The finding that a small fraction (6%) of the null small lymphocytes may be slowly-renewed cells, representing 0.1% of the total nucleated cells in the bone marrow, along with the early detection of null small lymphocytes during fetal development, could be consistent with them being such resting progenitor cells. A few of the null small lymphocytes in the bone marrow could

also represent early B cell progenitor cells before the expression of the B220 glycoprotein (Park & Osmond, 1987). A population of lymphoid cells in mouse bone marrow expresses the nuclear enzyme, terminal deoxynucleotidyl transferase (TdT) (Bollum, 1974; Gregoire et al, 1977) putatively representing early B progenitor cells at the stage of Ig heavy chain gene rearrangement (Desiderio et al, 1984; Campana et al, 1985; Park & Osmond, 1987). Some of these TdT^+ cells lack B220 and are of small to medium size. While the expression of TdT in null small lymphocytes as defined in the present work has yet to be verified directly, it may be calculated that such TdT^+ early progenitor cells at the most could account for only a small subset (<15%) of null small lymphocytes in bone marrow.

Other possible candidates for the null small lymphocytes produced in the bone marrow includes immunoregulatory cells. These cells include natural suppressor (NS) cells which occur in adult bone marrow and neonatal spleen, and can suppress primary IgM antibody responses (Duwe & Singhal, 1979; Bains et al, 1982; Mortari et al, 1986), mitogen responses (Maier et al, 1985) mixed lymphocyte reactions (Dorshkind et al, 1980; Strober, 1984; Oseroff et al, 1984) and self-reactive cytotoxic T cell clones (Muraoko & Miller, 1980). Phenotypically, these suppressor cells express FcR but lack markers or characteristics of normal mature T cells and B cells ie. sIg^- $Thy1^-$ Ia^- Lyt^-, or macrophages ie. non-adherent

and indomethacin insensitive (Duwe & Singhal, 1979; McGarry & Singhal, 1982; Oseroff et al, 1984; Strober, 1984; Maier et al, 1986). Morphologically, NS cells appear to be lymphoid (Oseroff et al, 1984) and their suppressive activity is mediated by the release of a soluble glycolipid mediator (Duwe & Singhal, 1978; Saffran et al, 1986; Mortari & Singhal, 1988).

Collectively, however, the available data are consistent with the hypothesis that the 14.8$^-$Thy1$^-$ null small lymphocytes which are produced in large numbers and express NK cell-associated surface markers in mouse bone marrow mainly represent immature cells of the NK lineage. If so, the present work provides a basis on which to examine the central genesis of NK cells during tumor bearing and other perturbations in vivo.

In examining the potential significance of lymphocyte production in the bone marrow, the present study has demonstrated that the growth of transplanted tumor cells in mice results in a marked selective increase in the number of null small lymphocytes in the bone marrow and spleen, and is enhanced by administering the prostaglandin inhibitor, indomethacin. The effect in the bone marrow is accompanied by expansion of small lymphocyte populations expressing NK1.1 and Thy1^{lo}. These findings are in accord with the hypothesis that the continuous production of null small lymphocytes in the

bone marrow represents the neogenesis of cells of the NK cell lineage, potentially responsive to the stimulus of tumor growth.

Lineage identity of null lymphocytes during tumor-bearing

The proposed NK lineage identity and functional role of null cells generated in the bone marrow receive support from the present work. The similarity in the responses to the two separate tumors, differing widely in phenotype, growth characteristics and metastatic properties (Lala, 1972; Claësson & Olsson, 1977; Lala et al, 1985; Brodt, 1986; Balducci & Hardy, 1988), and using two different mouse strains suggest that the expansion of null cell populations is a general response to the growth of tumor cells in mice. The response is , moreover, shown selectively by null cells, as compared with mature B and T cells. The results accord with reported increases in the number of splenic lymphocytes lacking surface IgM and Thy1 in EA tumor-bearing mice (Lala et al, 1985), peaking two days later than the $14.8^{-}Thy1^{-}$ null lymphocytes in the bone marrow. Since lymphocytes migrate from the bone marrow to peripheral lymphoid organs (Brahim and Osmond, 1970, 1976) it is highly probable that this phenomenon represents a precursor-product relationship in which bone marrow null cells migrate to the spleen. The present finding that the population of null cells in the bone marrow displaying NK1.1 also increases during the early stages of

tumor growth strongly suggests that the B220$^-$Thy1$^-$ bone marrow lymphocytes are related to the NK cell lineage. A similarly transient elevation of NK functional activity has been observed in the spleen EA tumor-bearing mice (Lala et al, 1985).

Properties of Thy1^{lo} cells during tumor-bearing and scheme for NK cell maturation

Thy1$^+$ cells in the bone marrow fall into two distinct subgroups, based on radioautographic quantitation of Thy1 density. Thy1^{lo} cells have a remarkably rapid renewal rate and are now shown to be increased in numbers during early stages of tumor growth. As described previously, some Thy1^{lo} cells also express NK1.1, representing a subset of the NK1.1$^+$ small lymphocyte population in the bone marrow. Collectively, the findings in the tumor-bearing state support the hypothesis that some rapidly-renewing Thy1^{lo} small lymphocytes are part of the lineage of newly-generated NK cells which, in a sequence of terminal post-mitotic maturation, would pass from a null phenotype (NK1.1$^-$Thy1$^-$B220$^-$) to express NK1.1 (NK1.1$^+$Thy1$^-$B220$^-$) followed by low densities of Thy1 (NK1.1$^+$Thy1^{lo}B220$^-$), a phenotype which characterizes cells capable of NK cell function (Herberman et al, 1978; Hurme & Sihvola, 1984; Hackett et al, 1986) (fig. 37 and 38).

Mechanism of response

Three phases can be distinguished in the response of bone marrow null lymphocytes to tumor cell growth, (1) an initial rapid increase in numbers of null cells, NK1.1^{+} cells and Thy1^{lo} cells, (2) an abrupt truncation of this increase which is reversible, in the case of null cells, by indomethacin treatment and (3) a decline of null, NK1.1^{+} and Thy1^{lo} cells toward normal values. Neither the first nor the third phase are susceptible to indomethacin treatment. The precise mechanisms responsible for these changes remain speculative, though certain long-range soluble factors are candidates, accounting for the generalized effects of some localized tumors. Early tumor growth may result in the secretion of a variety of positive mediators either by the tumor cells themselves or from host cells (Kovacs et al, 1985), notably macrophages. A local and systemic surge of interferon (IFN) production has been correlated with elevated levels of NK cell functional activity in the spleen of tumor-bearing mice (Herberman et al, 1977; Djeu et al, 1979, 1980; Gaudernack & Olstad, 1983). A variety of tumors, including LLc, secrete colony stimulating factor (CSF) (Hardy & Balducci, 1986; Devlin et al, 1987; Young et al, 1989) which may promote a variety of stimulatory hemopoietic effects. Other conditions of macrophage activation produced by administration of various foreign agents can also produce an increase in null lymphocytes in the bone marrow together with a substantial

increase in B cell genesis (Fulop & Osmond, 1983; Pietrangeli & Osmond, 1985, 1987).

The effect of indomethacin treatment suggests that the increase in null cells in the spleen and bone marrow may be limited by the production of prostaglandins (PGE) during tumor growth. PGE can be produced by the monocyte-macrophage lineage of cells of tumor-bearing mice (Kurland & Bochman, 1978; Lala et al, 1985; Parhar & Lala, 1985, 1987, 1988), human T suppressor/cytotoxic cells (Garcia-Penarrabia et al, 1989), or, in some cases, by the tumor cells themselves (Owens et al, 1980; Rolland et al, 1980; Fulton & Levy, 1980; Fulton, 1984, 1987; Furuta et al, 1988). PGE can suppress immune responses in vitro (Goodwin & Ceuppens, 1983) and in vivo (Tilden & Balch, 1981; Parhar & Lala, 1985; Young & Hoover, 1986; Lala et al, 1988), including NK cell activity against a variety of tumor cells (Droller et al, 1978; Brunda et al, 1980; Lala et al, 1985; Parhar & Lala, 1985, 1987) by inhibiting the production of both interleukin 2 (IL-2) and IL-2 receptors (IL-2R) (Rappaport & Dodge, 1982; Hersey et al, 1983; Lala et al, 1988), essential for the development of functional NK cells (Henney et al, 1981; Suzuki et al, 1983). The 3-fold increase in null small lymphocytes in the spleen during the later time points of indomethacin-treated mice bearing tumors in the present study correlates with the doubling of NK cell functional activity previously observed under similar conditions (Brunda et al, 1980; Lala et al,

1986; Parhar & Lala, 1987).

PGE may be only one of many mechanisms in tumor-bearing hosts which depress immune cell functions and cause failure to defend against malignancy. Many tumor cells produce soluble immunosuppressive factors in a wide range of molecular weights (<1000 to >200,000) (Warabi et al, 1984; Cooperband et al, 1976; Cornelius & Norman, 1988) which can inhibit the production of interleukin 1 (IL-1) by mouse macrophages, and IL-2 by mouse spleen lymphocytes and human blood lymphocytes (Farram et al 1982; Hersey et al, 1983; Nelson & Nelson, 1988). Host natural suppressor (NS) cells can abrogate mitogen responsiveness of T and B cells in EA tumor-bearing mice (Subiza et al, 1989).

Suppressor T cells may play an important role in tumor survival (Lala & McKenzie, 1982; Brodt & Lala, 1983; Schatten et al, 1984; North, 1985) by suppressing immune functions, including NK cell activity (Garcia-Penarrabia et al, 1989). Such mechanisms could contribute to the ultimate indomethacin-insensitive decline of null lymphocytes now observed in the bone marrow and spleen of tumor-bearing mice.

Response of other lymphocyte cell types

$B220^{+}$ and $Thy1^{hi}$ cell populations also fluctuate in tumor-bearing mice. However, the B lineage cells in the bone marrow showed no consistent changes in the two tumor-bearing systems examined. It remains to be determined whether the increase in

14.8^+ cells observed in the spleen of LLc tumor-bearing mice represents an accumulation of B cells, possibly discharged from the bone marrow (Brahim & Osmond, 1976; Yang et al, 1978), a small subset of T cells which binds mAb 14.8 (Kincade et al, 1981), or a local splenic production of 14.8^+ B cells (Shortman et al, 1981). The sustained increase in the population of $Thy1^{hi}$ cells in the bone marrow of tumor-bearing mice suggests an increase in the entry of slowly-renewing T cells from the recirculating pool (Ropke & Everett, 1973; Rosse, 1976). In the spleen, an observed increase in $Thy1^+$ cells could reflect the response of cytotoxic T cells to the tumor cells (Brodt & Lala, 1983; Fuyama et al,1986), altered kinetics of T cell recirculation through the spleen, or the accumulation of NK cells expressing Thy1 (Hurme & Sihvola 1984; Hackett et al, 1986).

Conclusion

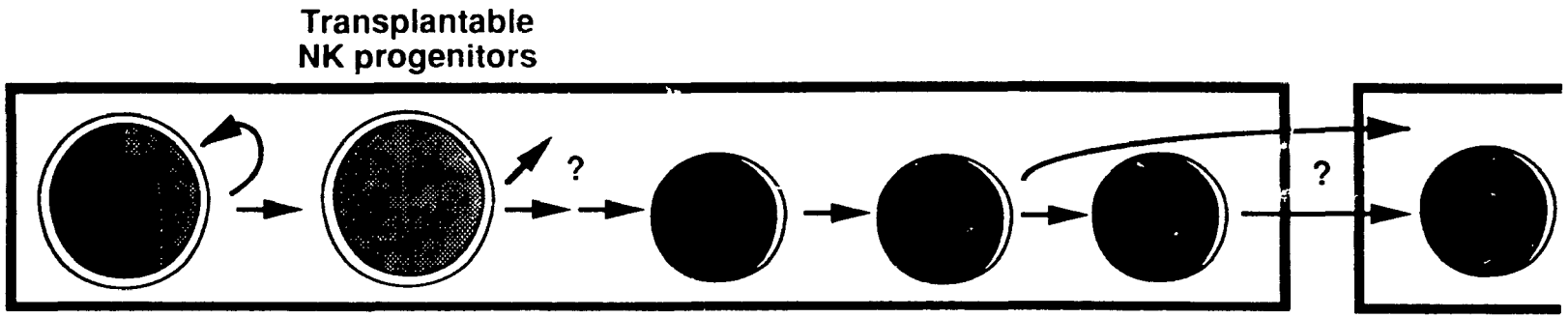
The present findings indicate that null, $NK1.1^+$ and $Thy1^{lo}$ small lymphocyte populations produced in the bone marrow are responsive to tumor growth in vivo and support the hypothesis that these cells are related to the NK cell lineage. The results also emphasise, however, that the response of these cells does not prevent progressive growth of transplanted tumor cells and that therapeutic attempts to improve the host response of null and NK cells by indomethacin administration may not be effective in late stages of malignancy. On the

other hand, the present work uses doses of tumor cells (10^6) known to be readily transplantable and to produce rapid tumor growth, reaching 10^9 cells by 14 days in the case of the EA tumor (Lala, 1972; Claesson & Olson, 1977). The secretion or induction of soluble factors by such a large initial number of rapidly-proliferating tumor cells could well outstrip the capacity of the null-NK cell lineage to respond adequately. It remains possible, nevertheless, that these bone marrow derived cells may normally provide a successful response against many naturally occuring clonal malignancies at the time of their initiation.

Fig. 38. A proposed scheme of NK cell differentiation in the bone marrow, as derived from the present study and others (Hurme & Sihvola, 1984; Hackett et al, 1985a,b, ; Pollack & Rosse, 1987)

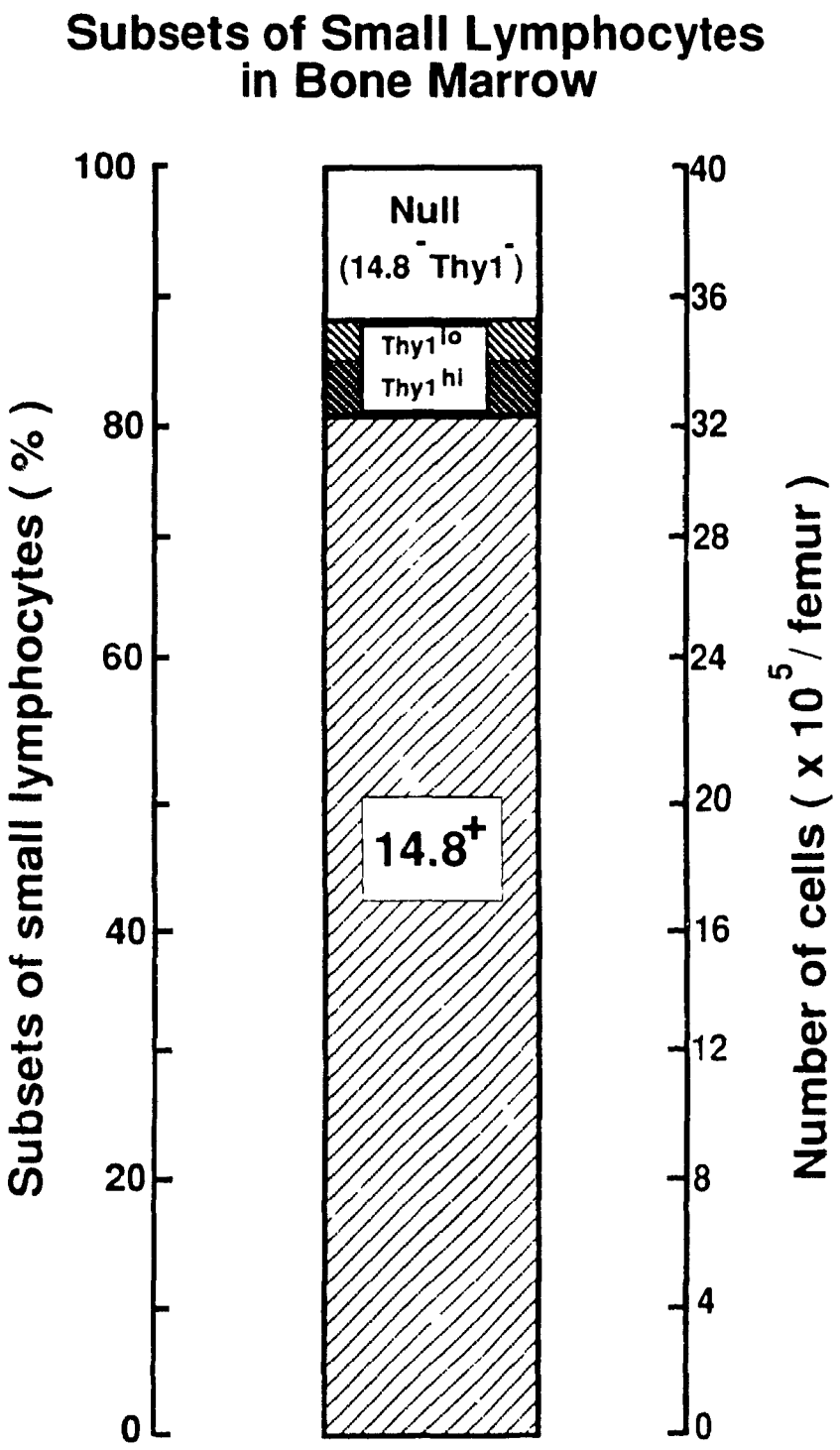
NK Cell Differentiation in the Bone Marrow

Stem Cell → Proliferating precursors → Non-dividing small lymphocytes (post-mitotic maturation) → Periphery



14.8	—	—	—	—	—
Thy 1	—	—	—	$+^{lo}$	$+^{hi}$
NK 1.1	—	—	+	+	+

Fig. 37. Histogram representing the proportions of the various lymphocyte subsets present in the mouse bone marrow.


Subsets of Small Lymphocytes
in Bone Marrow
Subsets of small lymphocytes (%)
100
80
60
40
20
0
Null
(14.8⁻ Thy1⁻)
Thy1lo
Thy1hi
14.8+
40
36
32
28
24
20
16
12
8
4
0
Number of cells (x 10⁵ / femur)

SUMMARY AND CONCLUSION

The objective of the thesis was to determine whether the small lymphocytes produced in large numbers in mouse bone marrow include cells other than virgin B lymphocytes, and if so, to characterize their kinetic and lineage properties.

To resolve those questions, a specific radioautographic technique was first developed to identify morphologically lymphoid cells containing the B lineage specific molecule, μ heavy chain of IgM in both surface μ^+ ($s\mu$) B cells and cytoplasmic μ^+ ($c\mu$) pre-B cells. Eighty percent of the small lymphocytes in the mouse bone marrow expressed μ chains, while 8% were $Thy1^+$ cells. The results demonstrated the presence of a population of null small lymphocytes which are not demonstrably of the B or T lineage, ie. μ^- $Thy1^-$, and thus may be part of a distinct cell lineage within the bone marrow.

Null small lymphocytes were subsequently revealed by an alternate radioautographic immunolabeling technique using mAb 14.8, which detects the B lineage-associated B220 glycoprotein on the surface of both B and pre-B cells, together with mAb anti-Thy1. The incidence of null small lymphocytes by these criteria (ie, 14.8^-Thy1^-) was consistent with the previous radioautographic techniques (μ^-Thy1^-). Null small lymphocytes were found to develop early during fetal life as assessed in the fetal liver at 11 days of gestation, suggesting that these cells may play an important role in the establishment of the

immune system. Cell kinetic studies using the deletion by hydroxyurea (HU) of cells in cell cycle, showed that null small lymphocytes in adult bone marrow are themselves non dividing, but have remarkably rapid renewal kinetics ($t_{\frac{1}{2}}$, 7.5 hr), distinct from B or T lineage cells, and a high total rate of production, by the entire bone marrow organ in the mouse, up to 1/3 the level of B cell production. Splenic null small lymphocytes turned-over rapidly, suggesting that these cells have only a short life span in the periphery. The data are consistent with the hypothesis that null small lymphocytes are continuously produced in large numbers in the bone marrow and then migrate to the spleen, constituting an independent cell lineage.

To determine the nature of the null lymphocytes within the bone marrow, double labeling techniques were used to detect NK cell related surface markers. Some 14.8^{-}Thy1^{-} null cells displayed the NK1.1 marker while larger proportions were also able to bind spontaneously to tumor cells and expressed Fc receptors. Kinetic experiments by HU deletion showed that NK1.1 is a late differentiation marker, suggesting that null small lymphocytes undergo an NK1.1^{-} to NK1.1^{+} transformation during their post-mitotic maturation period in the bone marrow. The Thy1^{+} cells in bone marrow have a wide range of Thy1 density and are kinetically heterogeneous. Thy1^{hi} cells have a slow rate of renewal, probably representing long-lived recirculating T cells from the blood stream. Thy1^{lo} cells, on

the other hand, are rapidly renewed. The expression of NK1.1 by Thy1^{lo} small lymphocytes suggest that these cells, in addition to the 14.8^{-}Thy1^{-} null small lymphocytes, form part of the rapidly-generated lineage of NK cells in the bone marrow. If so, Thy1^{lo} expression may represent either the terminal differentiation of NK cells or a separate subset of NK cells. These findings suggest that the production of null small lymphocytes in the bone marrow includes the neogenesis of NK cells, known from functional assays to be bone marrow derived and rapidly-renewed.

During the growth of a transplantable tumor, null small lymphocyte numbers increased in bone marrow and spleen but then decreased back towards normal values. Thy1^{lo} cells in the bone marrow also increased transiently in numbers during early stages of tumor growth. After administering indomethacin, a PGE inhibitor, to mice bearing Lewis lung carcinoma, the increase in null lymphocytes was enhanced, notably in the spleen. The proportion of bone marrow null small lymphocytes expressing NK1.1 increased considerably during tumor growth, supporting the hypothesis that the production of null cells in that organ includes the development of NK cells. Possibly, bone marrow-derived null cells may play an important role against naturally occuring malignancies within the host.

SIGNIFICANCE AND ORIGINALITY

Technical contributions:

A. Radioautographic immunolabeling techniques have been developed to reveal specific molecular markers on various bone marrow lymphocyte populations. These techniques detect:

1) pre-B cells containing cytoplasmic μ ($c\mu$)
2) mature B cells, early B progenitors, and pre-B cells, all of which express B220 glycoprotein, detected by mAb 14.8
3) T lineage cells bearing Thy1 and other allotypic markers defining various functional subclasses, (Lyt1, Lyt2, L3T4)
4) natural killer cells bearing the NK1.1 and asialo GM1 surface markers

Double labeling procedures were developed to detect null small lymphocytes radioautographically in conjunction with the labeling of NK cell-associated markers including:

1) the NK1.1 marker by avidin-biotin immunoperoxidase complex
2) tumor cell binding properties using the YAC.1 lymphoma
3) Fc receptors by erythrocyte rosetting techniques

Results:

B. These present observations are entirely original with

respect to:

1) the characterization of null small lymphocytes in the bone marrow, completly lacking both B and T lineage cell surface determinants ($B220^-, \mu^-, Thy1^-$)
2) the quantitation of the population dynamics of null small lymphocytes in bone marrow and spleen, and the demonstration of the magnitude of their production
3) the detection, and quatitation of $Thy1^{lo}$ small lymphocytes and the demonstration of their rapid renewal kinetics (in contrast with the slow renewal kinetics of $Thy1^{hi}$ cells)
4) the ontogenic development of null lymphocytes
5) the expression of NK1.1 by individual bone marrow small lymphocytes, including null cells
6) the response of null lymphocytes, $NK1.1^+$ and $Thy1^{lo}$ cells in the bone marrow to the growth of tumor cells, and the enhancing effect by indomethacin treatment

The findings are significant in revealing a large-scale production of null cells in the bone marrow which includes the neogenesis of NK lineage cells involved in tumor cell immunosurveillance. The work provides a direct cytological demonstration of the production of NK lineage cell maturation and renewal.

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