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Analysis Of The Embryonic T Cell Antigens 11B5 And 9A5 In Mice.

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ANALYSIS OF THE EMBRYONIC T-CELL ANTIGENS 11B5 AND 9A5 IN MICE

by

JUDITH ALLISON CAIN

A DISSERTATION

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ABSTRACT OF DISSERTATION
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Title ANALYSIS OF THE EMBRYONIC T-CELL ANTIGENS 11B5 AND 9A5 IN MICE

The 11B5 and 9A5 monoclonal antibodies, raised against mouse fetal thymocytes, were used to detect differentiation antigens expressed during hemopoiesis. The 11B5 antibody identified a glycoprotein antigen of Mr 110,000 on the surface of most fetal thymocytes and a subpopulation (4%-10%) of adult thymocytes, while this antigen was not detectable on cells from other lymphoid and hemopoietic tissues.The 11B5 antibody, which like the previously-described M6 antibody may react with the thymocyte receptor for the *Dolichos bifluores* agglutinin, was used to examine the distribution and phenotypic characteristics of the subpopulation of thymocytes that express this marker. Cells with high levels of the 11B5 antigen express the cell surface molecules that typify immature thymocytes, namely IL2Rα, CD44 and heat stable antigen (HSA). As thymocytes begin to lose the 11B5 antigen they acquire CD3, CD4, and CD8. The $11B5^{hi}$ cells are scattered throughout the thymic cortex but are most concentrated in the outer cortex and are not present in the thymic medulla. We conclude that the 11B5 antigen is a useful marker for immature thymocytes throughout life.

The 9A5 antibody defines an antigen on thymocytes, a subpopulation of T cells, and a minor population of bone marrow cells.

The 9A5 antibody identifies a trypsin sensitive antigen on >80% of fetal thymocytes. The adult 9A5$^+$ thymocyte population includes both CD3$^-$ and CD3^{lo} cells, many of which express both CD4 and CD8. In spleen and lymph nodes, 9A5 is expressed by a subpopulation of CD4$^+$ T cells. In bone marrow, the antibody identifies approximately 10% of the non-adherent, mononuclear cells that are negative for B220, CD4, and μ chains. The 9A5$^+$ cells in bone marrow are also negative for the BP-2 granulocyte marker but 50% of them express the Mac-1 antigen. To test the differentiation potential of this bone marrow subpopulation, 9A5$^+$ cells injected into SCID mice indicated that 9A5$^+$ bone marrow cells can give rise to T lineage cells. The 9A5$^+$ subpopulation is enriched for CFU-S and, when cultured on S17 stromal cells, gives rise to B lineage cells. The 9A5 antigen therefore appears to be a hemopoietic progenitor cell marker, whose expression is retained by subpopulations of thymocytes and CD4$^+$ T cells.

Abstract Approved by: Committee Chairman Mary D. Cooper

Program Director Susan Jackson

Date 1/6/93 Dean of Graduate School W. A. Sibley

DEDICATION

I dedicate this dissertation to my mother, Dorothy A. Cain, for her support and understanding throughout the years. Her constant insistance that it is never too late to achieve a long-term goal has made it possible for me to complete this degree. I consider this research project a collaborative effort with her input being as important as mine.

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LIST OF ABBREVIATIONS

Av-PE	Avidin-phycoerythrin
CD	cluster designation
CFU-S	colony forming units-spleen
cμ	cytoplasmic IgM
CSF	colony stimulating factor
D	age in days after birth
E	embryonic age in days
DBA	*Dolichos bifluoros* agglutinin
FITC	fluorescein isothiocyanate
HSA	heat stable antigen
IL	interleukin
IL-2Rα	interleukin-2 receptor α chain
Ig	immunoglobulin
LTBMC	long-term bone marrow culture
mAb	monoclonal antibody
2-ME	2-mercaptoethanol
PNA	peanut agglutinin
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SCID	severe combined immunodeficient
sμ	cell surface IgM
TCR	T cell receptor

CHAPTER 1

LITERATURE REVIEW

The process of hemopoiesis generates mature blood cells which include eight distinct lineages; erythrocytes, granulocytes, monocytes, eosinophils, mast cells, megakaryocytes, T- and B-lymphocytes, as well as natural killer cells whose lineage is unknown. Early studies revealed that the ancestral cells of hemopoietic tissues in murine species originate in the yolk sac and migrate into the embryonic liver (Moore and Metcalf 1970) which is the main site of hemopoietic precursor cells in the developing fetus. Shortly after birth this role is transferred to hemopoietic tissues in the bone marrow and spleen. All lineages are derived from a common multipotent hemopoietic stem cell and maturation of most blood cells occurs in the adult bone marrow. The one exception are T-cells which develop and mature within the thymus.

Hemopoiesis: *In Vivo* Analyses

In the last thirty years research efforts have attemped to isolate and define the cells responsible for sustaining hemopoiesis. The development of clonal assay techniques established a hierarchy of cellular subsets within the hemopoietic cell compartment. Multipotential stem cells possess the capacity to self-generate and differentiate into lineage-committed progenitor (precursor) cells with a reduced ability for self-renewal. Progenitor cells in turn generate morphologically recognizable progeny which differentiate, lose their capacity for proliferation and become the

mature cells entering the blood (Metcalf 1988). The basic properties of hemopoietic stem cell were established following the development of the spleen colony technique which is based on the ability of individual cells to form colonies on the surface of the spleen (colony forming units-spleen, CFU-S) when injected into irradiated recipients (Till and McCulloch 1961). The true multipotent stem cell population in adult bone marrow is quiescent. When stimulated their basic properties include: the ability to self-generate as well as differentiate into all blood cell lineages, and the capacity to generate clonally expanding populations of maturing cells. These early *in vivo* studies assumed that when injecting a small number of nucleated bone marrow cells into irradiated recipients, each spleen colony was representative of progeny from a single stem cell (Till and McCulloch 1961). The observation that colonies were of erythroid, myeloid, or mixed composition and that mature cells were located in the outer regions of the colony led Till and McCulloch to the conclusion that stem cells were multipotent and generated clonally expanding cell populations. Their experiments also indicated the quiescent nature of early progenitors since bone marrow cells irradiated before injection retained the ability to form CFU-S. Later studies using bone marrow cells with radiation induced chromosomal abnormalities indicated that mixed splenic colonies originated from a single cell (Wu et al. 1967). However, one limitation of the CFU-S assay was that lymphoid cells were usually scattered throughout the spleen and did not form discrete colonies, therefore multipotent activity of stem cells along these lineages could not be determined. Yet, the observation of isolated splenic cells morphologically distinct from CFU-S which bore the same chromosomal

abnormalities as cells found in colonies suggested that they were produced by the same stem cell (Wu et al. 1968).

Although the generation of CFU-S in irradiated mice injected with bone marrow cells was considered a result of stem cell activity, the possibility that splenic colonies originated from a heterogeneous cell population composed of more mature progenitor cells and not from single stem cells could not be eliminated. The currently accepted interpretation is that spleen colonies observed 8-10 days after transplantation are the result of proliferation of more mature cells, while some colonies which appear later at 11-14 days after transfer are derived from more primitive cells (Magli, Iscove and Odartchenko 1982).

A number of other assays have been used to investigate the hemopoietic activity of bone marrow cells including the ability to rescue a recipient from radiation death (Visser 1984), and injection of primary colonies into a second irradiated recipient which tested for self renewal by stem cells (Siminovitch, McCulloch and Till 1963). However, the question of what population of cells is responsible, stem cell or precursor, was still unanswered. The next step in the quest for the stem cell was to separate populations of bone marrow cells and measure the efficiency of these subpopulations in the CFU-S or other assay systems. The first procedure used was separation by velocity sedimentation on a serum gradient (Worton, McCulloch and Till 1969). Populations of cells differing in size were tested first for generation of CFU-S, and then the primary spleen colonies were removed and transfered into a second irradiated recipient (Siminovitch, McCulloch and Till 1963). The smaller lower density cells were enriched for multipotent precursor (stem) cells having a greater capacity to form secondary CFU-S than larger cells

Separation of mulitipotent cell populations by cell surface antigens began with the development of the fluorescence activated cell sorter (FACS: Herzenberg and Sweet 1976). The flow cytometry technique allowed segregation of populations of cells based on their physical characteristics. Cells could be isolated by size and granularity (forward and side scatter configurations, respectively), and by expression of surface antigens recognized by reagents conjugated to fluorescent dyes. Further enrichment of CFU-S producing cell populations using flow cytometry was accomplished by cell surface binding of wheat germ agglutinin (WGA) and antibodies to MHC class I molecules (Visser and Bol 1981; Visser et al. 1984). Medium sized cells with high expression of MHC class I and WGA ligands were 135-fold enriched for CFU-S activity and 180-fold enriched in the ability to rescue lethally irradiated mice. Initial attempts to isolate murine stem cells by high surface expression of the Thy-1 antigen, as described in the rat (Williams 1976), were not successful due to very low expression of this antigen on mouse bone marrow cells. The first indication that Thy-1 was present on mouse hemopoietic precursor cells was reported by Basch (1982). Muller-Sieburg (1986) used a negative selection protocol to enrich for B-lineage precursor cell activity by staining bone marrow cells with antibodies against mature lineage markers of T cells (CD4, CD8), B cells (B220), macrophages (Mac-1), and granulocytes (GR-1), and isolated the population lacking expression of these differentiation antigens (lineage negative, Lin^-) by flow cytometry. When antibodies against the Thy-1 molecule were included in the negative selection procedure, B-cell progenitor activity was lost. When the precursor cell activity of $Thy\text{-}1^{lo}$ Lin^- cells was analyzed, a 100-fold increase in B-cell progenitors assayed

in culture, and a 200-fold enrichment for day ten CFU-S was achieved as compared to the efficiency of the WGA plus anti-class I isolation method (Muller-Sieburg, Whitlock and Weissman 1986).

Spangrude, Heimfeld and Weissman (1988) reported the isolation of a population of murine bone marrow cells that had the greatest stem cell activity so far described. In this study the Thy-1^{lo}Lin$^-$ bone marrow cells were subdivided based on the binding of a monoclonal antibody (mAB) (clone E13 161-7) which marked several hematolymphoid subsets including bone marrow thymocyte progenitors (Aihara et al. 1986). The antigen recognized by this antibody was designated stem cell antigen-1 (Sca-1) and was later described as a member of the Ly-6 antigen family (Spangrude, Heimfeld and Weissman 1988; van de Rijn et al. 1989). Sca-1 divides the Thy-1^{lo}Lin$^-$ cells into a minor population of Sca-1$^+$ cells and a major population of Sca-1$^-$ cells. Although both subsets had similar activity in CFU-S analyses, only the Sca-1$^+$ precursors developed into thymocytes when injected intrathymically and were able to rescue lethally irradiated mice. It was reported that forty Thy-1^{lo}Lin$^-$Sca-1$^+$ cells could repopulate blood T cell, B cell, and myeloid lineages when transferred into irradiated recipients (Spangrude, Heimfeld and Weissman 1988). The conclusion from this study was that Thy-1^{lo}Lin$^-$Sca-1$^+$ bone marrow cells are probably the only pluripotent hemopoietic stem cells in the mouse since this population of cells satisfied the major criteria necessary for classification as stem cells: most were not in cell cycle and therefore quiescent; they represented a small number of cells (0.05-0.1% of the total bone marrow population); they were able to differentiate into all blood lineages; Thy-1^{lo}Lin$^-$Sca-1$^+$ bone marrow cells could rescue a recipient from radiation death, and could develop into clonally expanding

populations of maturing cells. The one property not addressed was the long-term reconstitution ability of the Sca-1$^+$ subset.

Other cell surface markers have been utilized to enrich the stem cell population from mouse bone marrow. Thy-1^{lo} Lin$^-$ bone marrow cells were divided into two populations based on the expression of the Fall-3 mAb which detects an antigen distinct from Sca-1 (Muller-Sieburg 1991). This study reported that B cell precursor activity as measured by the Whitlock-Witte culture system was within the Thy-1^{lo}Lin$^-$Fall-3$^-$ bone marrow cells, whereas pluripotent stem cell activity was mostly restricted to Thy-1^{lo}Lin$^-$Fall-3$^+$ cells since this population rescued lethally irradiated mice. High expression of the Qa-M7 antigen was evident on cultured cells possessing high proliferative potential (noncommitted progenitors) but not on populations which have differentiated into myeloid precursors (Bertoncello et al. 1986). The antigen defined by the mAb AA4.1, although present on some mature lineages, detected an immature cell population in E14 fetal liver that reconstituted irradiated recipients with all blood cell lineages (Jordan, McKearn and Lemischka 1990). In addition the dye rhodamine-123 which labels mitochondrial membranes was used to separate metabolically active cell populations. Small resting cells that did not stain well with this dye had a greater reconstitution potential than highly labeled proliferating cells (Spangrude and Johnson 1990).

The conclusion derived from *in vivo* data was that the stem cell pool described to date is a heterogeneous population of cells composed of subsets that are negative for lineage markers but are in different stages of the cell cycle, have differing capacities for self-renewal, and which may already be committed to a specific lineage. The true stem cell pool is extremely small and remains in a resting state and only a few cells initiate

a cycle of replenishment under conditions such as radiation poisoning. It would appear that the bone marrow subpopulations described in these various assay systems are actually overlapping populations of cells (Spangrude 1991; Heimfeld and Weissman 1992). A cell surface antigen specific for hemopoietic stem cells has not been reported and mAb which recognize new antigens are necessary to unravel the complexity of the present descriptions of the stem cell phenotype. Nonetheless, the isolation of a self regenerating, multipotent stem cell awaits the development of *in vitro* culture systems able to support the expansion of single cells and test their developmental potential (Spangrude 1991).

Hemopoiesis: *In Vitro* Analyses

The ideas generated by the *in vivo* studies were corroborated and often refined by the development of tissue culture systems which supported the growth of progenitor cells *in vitro* (Pluznik and Sachs 1965; Bradley and Metcalf 1966). These techniques provided a means of growing colonies *in vitro* from single cell suspensions of bone marrow. The growth of marrow cells in agar required feeder layers of neonatal mouse kidney or embryonic mouse cells. There was no growth observed with feeder layers of adult liver or peritoneal cavity cells, and limited colony formation was detected in cultures supplemented with splenocytes or thymocytes. Morphological examination of colonies identified cells of macrophage, granulocyte, and mast cell lineages (Bradley and Metcalf 1966). This data was comparable to the *in vivo* CFU-S results reported by Till and McCulloch (1961).

The observation that colony growth from bone marrow or spleen cells in culture was dependent on the presence of feeder layers suggested that soluble products secreted by these cells influenced the proliferation and

differentiation of progenitor cells. These growth stimulators were designated as colony stimulating factors (CSF). Four distinct CSF molecules which control the production of granulocytes and macrophages in the mouse have been purified and shown to regulate the survival, proliferation and differentiation commitment of progenitor cells (Metcalf 1985). In cultures of bone marrow cells, macrophage CSF (M-CSF) stimulated formation of predominately macrophage colonies (Metcalf and Burgess 1982). Granulocyte-CSF (G-CSF) was specific for granulocyte colony generation and granulocyte-macrophage CSF (GM-CSF) stimulated the generation of both cell lineages (Metcalf and Nicola 1983). Multi-CSF (IL-3) activity resulted in growth of granulocyte, macrophage, and eosinophil colonies (Metcalf et al. 1987).

In vitro colony formation in the presence of CSF was used to prove the bipotential nature of bone marrow precursor cells and the limited self-renewal of these stem cell progeny (Metcalf 1980; Suda, Suda and Ogawa 1984). The procedure involved initiation of bone marrow cultures in the presence of GM-CSF followed by separation of daughter colonies and transfer of individual cells to new cultures. The resultant progeny were analyzed after six days of culture in the presence of GM-CSF revealing the ability of paired cells to differentiate into both granulocytes and macrophages. (Metcalf 1982). These data clearly indicated that these two lineages share a common precursor cell that could differentiate along separate pathways when stimulated by CSF. To determine if this differentiation commitment was reversable, daughter cells were cultured in the presence of GM-CSF and then switched to a media containing M-CSF. Under these conditions precursor cells retained the ability to form granulocyte colonies. Alternatively cultures initiated with M-CSF and then

switched to GM-CSF remained committed to the macrophage lineage (Metcalf 1988). These data indicated that once committed, a cell cannot revert to a bipotential progenitor. Since CSF did not bind to lymphocytes (Nicola 1987) these assays, like the *in vivo* CFU-S analysis, cannot detect differentiation along the B- or T-lineage pathways.

In the intact organism, the process of hemopoiesis depends on the intimate contact of stem cells with adherent cells, generically termed stromal cells, present in the bone marrow and spleen (Spangrude 1991). In order to mimic a more natural microenvironment *in vitro*, two systems of long-term bone marrow cultures (LTBMC) were developed, one for myelopoiesis and another for lymphopoiesis. The Dexter culture conditions which support myeloid development included a complex adherent layer of bone marrow stromal cells and media containing a mixture of horse serum plus hydrocortisone and an incubation temperature of 33^{o}C (Dexter, Allan and Lajtha 1977). Dexter cultures did not support B or T cell maturation, although differentiative potential along the B- lineage was maintained (Dorshkind and Phillips 1982). Studies on the regulation of B lymphocyte production were facilitated by the development of the long-term B cell culture system (Whitlock and Witte 1982). The culture conditions were optimal for the growth of B lymphocytes and their progenitors while myeloid cells and their precursors were depleted within a few weeks (Whitlock, Robertson and Witte 1984). Immature progenitors under both culture conditions preferentially seeded within the adherent layer (Coulombel, Eaves and Eaves1983) and proliferated to form clusters of tightly packed cells called cobblestone areas (Ploemacher 1989). These assays were used to define subpopulations of cells in bone marrow previously demonstrated to have

high day fourteen CFU-S activity. The Thy-$1^{lo}Lin^-$ cells contained the greatest number of *in vitro* colony forming cells (Muller-Sieburg et al.1988), and the Thy-$1^{lo}Lin^-Sca\text{-}1^+$ subpopulation had the greatest differentiative activity in both Dexter and Whitlock-Witte culture conditions (Heimfeld and Weissman 1992).

Since the bone marrow microenvironment in LTBMC maintained hemopoiesis without the addition of exogenous growth factors, many stromal cell lines have been isolated in order to determine the identity and nature of the soluble mediators (cytokines) produced by the bone marrow microenvironment. Several generalizations have been determined from the study of stromal cell lines: these cells are generally nonphagocytic and lack cell surface markers characteristic of hemopoietic cells; stromal cell lines secrete soluble mediators which act on progenitor cells; however none have been identified that support the differentiation of a single hemopoietic lineage (Dorshkind 1990).

The regulation of hemopoietic stem cell development depends on stromal cell derived cytokines. Many of the factors have been purified and cloned and, when added to cultures separately, appeared to be primarily involved in the later stages of development (Spangrude 1991), whereas early progenitors required as many as three cytokines to induce colony formation *in vitro* (Bartelmez et al. 1989; Iscove and Yan 1990). Very large colonies derived from high proliferative potential (HPP) bone marrow cells were grown in semi-solid agar in the presence of Interleukin-1 (IL-1), Interleukin-3 (IL-3), or M-CSF either alone or in combination. No colony growth was observed in the presence of any single factor, while a limited number of colonies were generated when M-CSF plus IL-1 or IL-3 were added. A combination of all three factors induced a major population of

HPP-colony forming units in culture (HPP-CFC). Further additions of G-CSF or GM-CSF did not increase colony formation (Bartelmez et al. 1989). In another study, a combination of IL-6 and IL-3 was shown to stimulate hemopoietic progenitor cells from mouse spleen (Okada et al. 1991).

The requirement for interactions of stem cells with a supportive microenvironment was illustrated in studies with two mutant mouse strains with abnormalities at the white spotting (W) and Steel (Sl) loci located on chromosomes 5 and 10 respectively (Chabot et al. 1988; Geissler, Ryan and Housman 1988; Copeland et al. 1990; Zsebo et al. 1990). Mutations at either loci were responsible for defective development of neural crest-derived melanocytes, promordial germ cells and hemopoietic stem cells resulting in severe anemia, coat color abnormalities and sterility in homozygous animals (Russell 1979).

Abnormalities in W locus mutants resulted from defective stem cells while the defect in Sl locus mutants was due to a defective microenvironment. The product of the W locus was shown to be the cellular homolog (c-kit) of the viral oncogene v-kit, described as a transmembrane glycoprotein with a molecular weight of 150-165 Kd and a member of the tyrosine kinase family of receptors (Chabot et al. 1988; Geissler, Ryan and Housman 1988). A hemopoietic growth factor produced by stromal cells that maps to the Sl locus has been isolated and shown to bind to the c-kit product (Zsebo et al. 1990; Williams et al.1990). This factor has been called stem cell factor (SCF), c-kit ligand (KL) and mast cell growth factor (MGF). SCF was shown to be responsible for the Sl defect by reversal of anemia in heterozygous Sl/Sl^{d} mice by treatment with purified factor (Zsebo et al. 1990).

Lymphoid Lineages

The two types of immune responses, humoral (controlled by secreted antibodies) and cellular (responses resulting from cell to cell contact), are both primarily controlled by lymphocytes. Four decades ago, it was revealed that different lymphocyte lineages were responsible for these reactions. The identification of B-lineage cells and their function in humoral immunity and therefore antibody production was demonstrated using the chicken model. This was accomplished by immunization of normal chickens and those whose hind-gut organ, the *bursa of Fabricius*, had been removed (bursectomized) with a *Salmonella* derived antigen. An increased level of antibody was observed in serum from normal chickens, while only eight of seventy-five bursectomized birds produced antigen-specific antibody (Glick, Chang and Jaap 1956). The organ responsible for cellular immunity was determined in a study of allograft rejection in young thymectomized animals. Control groups with an intact thymus readily rejected skin grafts from an incompatable donor while grafts survived much longer in animals that had been thymectomized (Good et al. 1962; Miller 1961). This observation was corroborated in studies of human immunodeficiency diseases which determined that impairment of cellular immunity usually occurred with a defect in thymic development. Experiments that followed lymphocytes as they travelled throughout the body revealed that different cell types as well as different organs were involved in humoral versus cellular immunity. It was concluded that two pathways of lymphocyte development occurred: one where cells responsible for cellular immunity developed in the thymus (T cells), and another requiring residence in the bursa resulting in bursa-derived lymphocytes (B cells) (Moore and Owen 1967). The organs responsible

for production of B cells in mammals, which lack a bursa, was shown to be the fetal liver during embryonic development and the bone marrow after birth (Owen, Cooper and Raff 1974; Owen et al. 1977).

B and T lymphocytes are both derived from hemopoietic stem cells as are the other blood cell lineages. It has been proposed that a separation exists in the stem cell pool whereby multipotent stem cells can develop into lymphoid restricted and myeloid restricted stem cells (Phillips 1989). This concept was supported by a study where severe combined immunodeficient (SCID) (Bosma, Custer and Bosma 1983) mice injected with cells from long term bone marrow cultures were able to reconstitute T- and B-cell function in recipient animals while myeloid repopulating ability was markedly deficient (Phillips 1989). However, since limited myeloid differentiation was present in this study, the existance of lymphoid restricted stem cells was not proven and remains controversial.

B Cell Development

The stages of B cell development have been defined by the expression of cytoplasmic and surface immunoglobulin of the IgM isotype (cμ. sμ), surface IgD (sδ), nuclear deoxynucleotidyl transferase (TdT), and a number of surface antigens recognized by monoclonal antibodies. The earliest recognizable stage after the hemopoietic stem cell was designated pro-B (progenitor for B cells) and was defined as cells which have no detectable cμ or sμ but express nuclear TdT (Phillips 1989; Park and Osmond 1989). These differentiate into the $c\mu^+$ pre-B cell (Raff et al. 1976). Expression of sμ is characteristic of an immature B-cell (Owen et al. 1977; Burrows et al. 1978; Ryser and Vassalli 1974; Osmond and Nossal 1974; Landreth, Rosse and Clagett 1981) while mature B-cells can be recognized by expression of both sμ and sδ (Goding, Scott and Layton

1977; Maki, Roeder and Traunecker 1981; Moore, Rogers and Hunkapiller 1981). Loss of IgD, and in some instances, the occurance of isotype switching is followed by the final stage- the differentiation into antibody secreting plasma cells (Coffman 1983).

Immunoglobulin molecules are composed of two heavy (H) chain and two light (L) chain molecules covalently linked to form divalent (in the case of IgG) or multivalent (IgM) antibody recognition sites. The functional diversity required by the immunoglobulin antigen receptor is derived through rearrangement of germline DNA sequences which encode the various regions of these molecules.The H chain is composed of variable regions (V), diversity segments (D) and joining regions (J) which are first rearranged on the chromosome by deletion of intervening sequences in the order D to J and then V to DJ before transcription occurs (Alt et al. 1981). Rearrangement to the constant region (C) (VDJ to C) occurs by RNA splicing to produce mRNA molecules in which the V, D, J, and C sequences are contiguous. These are the mRNA molecules that are translated into H-chain polypeptides (Guise et al. 1990). Following rearrangement of the heavy chain locus, μ chains are produced in the cytoplasm, and the cell is recognized as a pre-B cell. There are no light chains present at this stage since rearrangement at this locus has not occurred. The formation of L chain molecules follows the pattern of κ chains before λ and involves rearrangement of two segments, V and J (Selsing et al. 1990). The translation of the light chain gene and the formation of IgM monomers marks the transition from pre-B cells to immature B cells (Cooper and Burrows 1990).

One member of the CD45 family of common leukocyte antigens was shown to be expressed on most B-lineage cells in the mouse from

the pro-B to the mature B-cell stages, but was lost upon differentiation into plasma cells (Kincade 1987). The molecule expressed on B cells (B220) is the largest member of this family having a molecular weight of 220,000 and is the antigen recognized by the 14.8 mAb (Kincade et al. 1989). The pro-B cell stage of development was divided into three levels based on the expression of TdT and B220. Since TdT expression was correlated with insertion of short nucleotide sequences at recombination junctions during immunoglobulin gene rearrangements (N region additions) (Desiderio et al. 1984), the presence of this enzyme was considered a characteristic of B cell commitment. The first recognizable B lineage cells were reported as $TdT^{+}14.8^{-}$ (Park and Osmond 1989). These cells acquired the B220 antigen before TdT activity was no longer detectable. The sequence of maturation described for pro-B cells was $TdT^{+}14.8^{-}$ cells matured to $TdT^{+}14.8^{+}$ and then to a $TdT^{-}14.8^{+}$ phenotype (Park, 1989). The BP1/6C3 antigen has a more restricted distribution (Cooper et al. 1986; Wu et al. 1989). This disulfide linked homodimer of Mr 140,000 is expressed on the surface of $B220^{+}$ pre-B cells and newly formed B cells in bone marrow but not on B cells in peripheral lymphoid tissues. Another molecule expressed on pre-B cells is the BP-3 antigen which has a core size of Mr 32,000 and variable sizes of surface forms (Mr 36,000-48,000) because of different degrees of glycosylation (McNagny, Cazenave and Cooper 1988). This glycoprotein, in contrast to the BP-1/6C3 antigen, is expressed on splenic B cells and is lost as cells increase surface IgD expression. The Bp-3 antigen is also found on myeloid cells and the level of expression increases as these cells mature. Lyb2 (CD72) is expressed from the $c\mu^{-}B220^{+}$ pre-B cell stage through the $s\mu^{+}$ B cell stage (Kincade 1987). Recently the CD72 molecule in human B

cell lines was shown to be the ligand for CD5, an antigen present on T cells and some B cells. It was suggested that this could be a mechanism of communication between B and T cells (Van de Velde et al. 1991).

Studies on the *in vitro* regulation of B-cell development in Whitlock-Witte LTBMC (1982) and in cultures of bone marrow derived stromal cell lines have described two classes of growth factors that stimulate B-cell progenitors: those that stimulate proliferation and others that control differentiation (Dorshkind 1990). Purification of factors from stromal cell conditioned media identified mediators with apparent molecular weights of 10 Kd (Lemoine et al. 1988), 30-40 Kd (Song et al. 1985), and the 14.9 Kd cloned IL-7 molecule (Namen et al. 1988; Namen et al. 1988) which were able to induce B-cell proliferation. The primary target for IL-7 among B-lineage cells was the $c\mu^+sIg^-B220^+$ large pre-B cell, although there was stimulatory activity on $B220^-$ progenitors. However, the differentiation of sIg^- pre-B cells to sIg^+ B-cells was not a function of IL-7 (Henny 1989; Lee et al. 1989). In addition, IL-7 was shown to stimulate thymocytes and T-cells thus demonstrating its proliferative activity on more than one cell lineage (Morrissey et al. 1989).

The existance of mediators that stimulate differentiation was first demonstrated by Landreth et al. (1985) who reported that expression of the B220 antigen and cμ by B-cell progenitors was induced by a factor present in the urine of cyclic neutropenia patients. A similar activity was reported for mediators isolated from the murine stromal cell line, S17, that produces factors with molecular weights of 10-Kd and 60-Kd (Collins and Dorshkind 1987; Landreth and Dorshkind 1988). The demonstration of synergistic effects of IL-7 and the S17-derived factors on B-cell progenitors revealed that differentiation to the $c\mu^+$ pre-B cell stage by S17

mediators occurs before the proliferative activity of IL-7 (Billips et al. 1992). Differentiation of the pre-B cells to sIg^+ B-cells is not induced by the S17 cell line (Collins and Dorshkind 1987), and the mature B-cell is not responsive to the proliferative effect of IL-7 (Henny 1989).

Another interleukin, IL-4, was shown to play a role in the terminal stages of lymphopoiesis by acting to induce the differentiation of pre-B cells to $c\mu^+$ B-cells (King, Wierda and Landreth 1988; Hofman et al. 1988). In contrast, IL-4 was shown to have an inhibitory effect on the differentiation of B-cell precursors to more mature stages (Rennick et al. 1987; Peschel, Green and Paul 1989). It was suggested that IL-4 inhibits growth and differentiation of immature precursors, while it synergizes with other factors to promote the growth of more mature progenitors (Rennick et al. 1987).

T Cell Development

Immunocompetent T-lymphocytes are derived from blood-borne stem cells by a series of developmental events that occur within the thymus (Moore and Owen 1967; Miller and Osoba 1967). The primary classification of developing thymocytes was based on their surface expression of molecules involved in mature T-cell function namely CD4 (Ceredig et al. 1983), CD8 (Ledbetter et al. 1980; Sarmiento, Glasebrook and Fitch 1980), and the T-cell receptor antigen (TCR) complex. A secondary classification of immature cells devoid of the CD4, CD8 and TCR antigens was based on several additional surface molecules which divided thymocytes into distinct subpopulations. The heat stable antigen (HSA) (Bruce et al. 1981; Crisp, Moore and Husman 1987), Thy-1, CD5, Pgp-1 (CD44) (Gause, Mountz and Steinberg 1988), the peanut agglutinin receptor (PNA) (Fowkes et al. 1985), IL-2R (Smith 1987), and MEL-14, an

antigen associated with lymphocyte homing (Gallatin, Weissman and Butcher 1983), have all been identified as antigens expressed on immature thymocytes. The functions of these molecules in early thymocyte development is unknown. In adult mice, Pgp-1, PNA and IL-2R expression increases upon activation of T-cells and these antigens may function similarly when expressed on early thymocytes (Scollay et al. 1988). The Pgp-1 antigen is the principal cell surface receptor for hyaluronate, and has a role in cell adhesion (Aruffo et al. 1990), and it has been suggested that the CD5 molecule is involved in communication between B- and T-cells (Van de Velde et al. 1991),

Thymocyte precursor cells, defined as having the capacity to reconstitute both thymic and peripheral T-cell populations, were first described by Kadish and Basch (1977) and were identified as being predominately $CD4^-CD8^-CD5^{lo}$ cells (Fowlkes et al. 1985). This identification proved to be too simple following the discovery that the $CD4^-CD8^-$ thymocyte subpopulation was heterogeneous and could be divided into at least eleven distinct subsets based on the surface expression of other antigens (Scollay and Shortman 1985; Wilson et al. 1988). A developmental pathway for thymocytes which lack surface expression of CD3, CD4 and CD8 was proposed (Pearse et al. 1989; Petrie et al. 1990), and was based on the expression of Pgp-1, HSA, and IL-2Rα surface markers. The most immature subset was described as $Pgp\text{-}1^+IL\text{-}2R\alpha^-HSA^+$ according to cell cycle analysis, reconstitution potential and the status of TCR gene rearrangements. Since the TCRβ and γ genes were not in germline configuration in this subset, a subsequent study reported that the earliest T-lineage precursor cell containing germline TCR genes expresses a low level of CD4 on the cell surface (Wu et al. 1991).

The next stage of thymocyte development is the acquisition of both CD4 and CD8 on the cell surface (double positive cells). Intermediate stages of $CD3^-CD4^+CD8^-$ and $CD3^-CD4^-CD8^+$ cells which develop into double positive thymocytes have been described, but the functional significance of these populations is unknown (MacDonald, Budd and Howe 1988; Hugo et al. 1990; Matsumoto et al. 1991). The $CD4^+CD8^+$ thymocyte subset, located predominately in the thymic cortex, represents approximately 85% of all thymocytes in the adult mouse, and around 50% of these express low levels of TCR. This subset is also characterized by variable expression of CD5 and MHC class I antigens which increase as the cells mature (reviewed in Scollay et al. 1988). The process of positive and negative selection, which is the mechanism that determines the TCR repertoire expressed on mature cells, occurs at the double positive stage of development and results in the death of the majority of thymocytes (Blackman, Kappler and Marrack 1990; von Boehmer 1991). Those that survive subsequently lose the expression of either CD4 or CD8 to become the mature single positive T-cells which enter the thymic medulla before migrating to peripheral lymphoid organs.

The T-cell receptor complex is composed of two covalently linked protein chains which form heterodimers that are noncovalently associated with the CD3 membrane complex composed of five proteins ($\gamma,\delta,\varepsilon,\zeta,\eta$) (Samelson, Harford and Klausner 1985). There are two types of TCR expressed on T-cells, those composed of either $\alpha\beta$ or $\gamma\delta$ heterodimers. The genes encoding these proteins have been sequenced and are similar to the immunoglobulin genes, containing non-contiguous variable (V), diversity (D; for β and δ) and joining segments (J) gene segments which assemble by gene rearrangements. (Hedrick et al. 1984; Yanagi et

al.1984; Saito et al. 1984; Chien et al. 1987). In the mouse the TCR α and δ genes are located on chromosome 14, the δ gene being located between the Vα and Jα segments, and the β and γ genes are present on chromosomes 6 and 13 respectively (Fowlkes 1989). During thymocyte development sequential process of rearrangement occurs where the β chain is rearranged before the α chain and similarly γ is rearranged before δ for the second type of TCR.

During fetal T cell development, precursor cells first enter the thymus between embryonic days 10-12 (E10-12) (Jotereau et al. 1987), and rapidly upregulate expression of the Thy-1 antigen. The majority of cells in the fetal thymus are $CD4^-CD8^-$ until E16, when $CD4^+CD8^+$ thymocytes first appear and this population is dominant (70%) by E17. One day later, mature $CD4^+$ and $CD8^+$ cells are present (Penit and Vasseur 1989). By birth, thymocytes display a CD4/CD8 phenotype similar to an adult animal. Intermediate phenotypes of immature $CD4^+CD8^-CD3^-$ and $CD4^-CD8^+CD3^-$ cells are evident on E15 (Penit and Vasseur 1989). Surface expression of CD3 occurs on E14-15 in association with the γδ TCR, followed two days later by a distinct subpopulation of cells expressing αβ TCR (Havran and Allison 1988). The T cell specific marker IL-2Rα is evident early in embryonic life on E13, peaks on E15, and declines sharply as cells acquire CD4 and CD8 (Penit and Vasseur 1989). The surface antigens CD44 (Pgp-1) and HSA, although not specific for T cell precursors, have been used to characterize stages in thymocyte development. Both proteins are expressed on most fetal thymocytes before E15 (Crispe and Bevan 1987). The sequence of surface antigen expression is similar in the adult (reviewed in Scollay et al. 1988) although there are several notable differences between fetal and adult T-cell

development. In the embryonic thymus developmental events occur more rapidly, major subpopulations are present which are very rare in the adult, and certain TCR V genes are used preferentially. For example, the Vγ3 gene is expressed first in the fetal thymus although its usage is rare or absent in the adult (Havran and Allison 1988). It was suggested that this occurs because of a difference between the stem cells that colonize the embryonic thymus and those that seed the adult thymus (Ikuta et al. 1990). In addition to Vγ3 expression, it was proposed that Vγ4 expressing thymocytes, CD5 B-cells and fetal erythrocytes were only derived from embryonic stem cells (Ogimoto et al. 1990; Ikuta et al. 1990). An earlier study demonstrated that thymocytes and T-cells present during the first week of life were derived from cells which first seed the thymus on E10-12. After postnatal day seven, these are rapidly replaced by the progeny of later arriving stem cells (Jotereau et al. 1987). There are several possible explanations for the differences in behavior between fetal, neonatal and adult thymocytes including postnatal appearance of regulatory factors, accessory cells and antigens, however, the possibility that early T-cells are derived from a stem cell that is distinct from the cell that produces T-cells in later life has not been eliminated.

Background And Experimental Strategy

Origins Of Experimental Mice

The mouse has provided an invaluable animal model in biomedical research due to its small size, ease of maintenance and short gestational time. Progress in many scientific disciplines has been enhanced by the development of inbred homozygous laboratory strains of *Mus musculus domesticus* (the domestic mouse) which reduce experimental variations based on heterogeneous genetic backgrounds. Although there are genetic

differences between laboratory strains, outbred (wild) mice have a greater polymorphism than that seen between domestic mice. The wild mouse species have been invaluable in studies of evolutionary divergence based on geographical location, external morphology, biochemical characteristics and descriptions of conserved genetic loci among members of the genus *Mus* (Thaler 1986). Domestic mice are descendents of the west European house mouse whose origins are not fully understood. Feral (wild) mice, although preferring environments devoid of humans, nonetheless have also populated Europe. One species, *Mus spretus*, is thought to have migrated from North Africa via sailing ships and it has been estimated that this species has been genetically isolated from domestic strains for one to three million years (Thaler 1986). The study of gene products within the *Mus* genus has led to a classification based on biochemically divergent groups. Domestic mice are classified as Mus biochemical group 1 (Mus 1) while the wild mouse species, *Mus spretus*, is a member of biochemical group 3 (Mus 3) (Bonhome et al. 1984).

Experimental Strategy

The original goal of this dissertation was to produce a panel of monoclonal antibodies (mAb) with specificity for novel T cell differentiation antigens, to characterize the antigens recognized by these antibodies, and to identify their expression during ontogeny. Monoclonal antibodies with specificity for important differentiation antigens such as CD4 and CD8 have been produced by hyperimmunizing rats, however, markers for detecting developmental stages preceeding the appearance of these two antigens are scarce. Other cell surface molecules present on immature thymocytes such as CD5, HSA, Pgp-1 and PNA have been identified, yet their usage in

defining stage specific cell populations is restricted because of their presence on other cell types.

This study has taken advantage of the genetic diversity between Mus 1 and Mus 3 mice to detect differentiation antigens present on immature thymocytes. A panel of monoclonal antibodies was produced by hyperimmunizing *Mus spretus* (wild mice, Mus 3) with fetal thymocytes from inbred strains (Mus 1). By using such an immunization protocol, highly conserved molecules such as Thy-1 and Ly-2 which have a limited polymorphism of only two and three alleles, respectively, among members of the *Mus* genus (Figueroa 1986) will not be antigenic. In contrast, epitopes expressed on cells present in domestic species (Mus 1) but not in wild mice (Mus 3) because of genetically diverse ancestry or evolutionary mutational alterations can be revealed.

From a panel of eighteen hybridoma clones, the antigens recognized by the 11B5 and 9A5 antibodies have been characterized in this dissertation. Chapter 2 describes the biochemical characteristics of the 11B5 antigen and its expression during fetal and adult thymopoiesis. In addition, the subpopulation of immature thymocytes that express this antigen identifies a previously unrecognized intermediate in the T-cell differentiation pathway.

The characteristics of the 9A5 antigen are described in chapter 3. This antigen is expressed during early fetal thymocyte development and is retained by a subpopulation of $CD4^+$ T-cells. In addition, a minor subpopulation of bone marrow cells which expresses this antigen have the capacity to differentiate into myeloid, B- and T-lymphoid cells.

CHAPTER 2

EXPRESSION OF THE 11B5 ANTIGEN DURING T-CELL DEVELOPMENT

Introduction

Cells which populate the thymus are derived from the same hemopoietic stem cells which generate all blood cell lineages. Although there is evidence for extrathymic maturation (Speiser, Stubi and Zinkernagel 1992; Lefrancois et al.1990), the major T-cell developmental pathway requires a thymic microenvironment where the mechanisms of positive and negative selection determine the repertoire of T-cell receptors (TCR) that will be expressed on the minority of cells that survive and migrate to peripheral lymphoid organs (Blackman, Kappler and Marrack 1990; von Boehmer, 1991).

While the initial stages of thymocyte development have been extensively studied (reviewed in Scollay 1991; Fowlkes et al. 1985), the pathway from entry into the thymus to development of surface $CD4^+CD8^+$ thymocytes is not fully understood. A maturation scheme for the murine thymocyte development has been outlined using antibodies specific for the heat shock antigen (HSA), CD44 (Pgp-1), and IL-2Rα molecules to distinguish maturation levels within the $CD4^-CD8^-CD3^-$ thymocytes, however, none of these antigens are restricted to T-lineage cells (Pearse et al. 1989).

The present study has taken advantage of the genetic diversity between domestic and wild mice to produce a panel of monoclonal

antibodies (mAb) which recognize differentiation antigens expressed during murine T-cell development. A classification within the genus *Mus* divides the different species into four biochemical groups (Bonhomme et al. 1984). Domestic species (Mus group 1) include most of the laboratory strains of mice while the wild mouse species used in this study (*Mus spretus*) is a member of Mus group 3. The immunization strategy used can therefore generate antibodies that detect differences in allelic forms of conserved molecules as well as antigens not expressed in wild mice due to genetic isolation or mutation.

In the present study, we have immunized wild mice with fetal thymocytes from inbred mice to create a panel of monoclonal antibodies which recognize T-cell differentiation antigens. One of these mAb, 11B5, is specific for an antigen of Mr 110,000 which is expressed on the surface of immature thymocytes during embryonic and adult life. Our studies define the 11B5 cell surface glycoprotein as a differentiation stage specific marker of thymocytes.

Materials and Methods

Mice

Mus spretus(Spain), DBA/2J, C3H/HeJ, and C3D2F1 mice were purchased from Jackson Labs (Bar Harbor, MA). DBA/2, C57Bl/6, and Balb/c strains were purchased from Charles River (Raleigh, NC). C3D2F1XDBA/2J mice were bred in the animal facility at the University of Alabama at Birmingham. The date that a vaginal plug was observed was designated as day 0 of gestation.

Cell Lines

The mouse tumor cell lines A20, EL-4, BW5147, and Yac-1 were purchased from the American Type Culture Collection (ATCC, Rockville,

MD). S49.1 and L6911 cell lines were a gift from Dr. Elizabeth Evans (Becton Dickinson, Mountain View, CA).

Antibodies and Immunofluorescent Reagents

Anti-CD3-FITC (clone 145-2C11), anti-CD4-FITC (clone GK1.5), anti-IL-2R-FITC (clone 7D4), anti-Thy-1-FITC (clone 30-H12), and anti-Pgp-1-FITC (clone IM7.8.1) were gifts from Dr. John H. Eldridge (University of Alabama at Birmingham). Goat anti-mouse IgM-FITC and goat anti-rat Ig were purchased from Southern Biotechnology Associates (Birmingham, AL). Anti-HSA (J11d) was a gift from Dr. Jonathan Sprent (Scripps Institute, LaJolla, CA). Anti-CD8-FITC was purchased from Becton Dickinson (Mountain View, CA). The 11B5 antibody was purified from the 11B5 hybridoma culture supernatant by affinity chromatography using a column of goat antibodies to mouse IgM (Southern Biotechnology Associates, Birmingham, AL) coupled to sepharose 4B (Pharmacia, Piscataway, NJ). The purified 11B5 antibody was biotinylated with NHS-LC-biotin (Pierce, Rockford, IL) by a published method (Kronick, 1986). *Dolichos bifluores* agglutinin conjugated to FITC (DBA-FITC) was purchased from Vector Laboratories (Burlingame, CA).

Hybridoma Production

Mus spretus (Mus biochemical group 3) were immunized with E15-17 fetal thymocytes from inbred C3D2F1XDBA/2J mice (biochemical group 1) were injected four times over a two week period and regional lymph node cells were fused one day after the last immunization with the AG8.653 myeloma variant (Kearney et al. 1979). Hybridoma supernatants were screened by indirect immunofluorescence staining of E15-17 thymocytes using goat anti-mouse-FITC (Southern Biotechnology Assoc., Birmingham Al) as the indicator antibody. Positive clones were subcloned

twice and the antibody isotypes determined by reactivity with isotype-specific goat anti-mouse antibodies (Southern Biotechnology Assoc., Birmingham, AL).

Immunofluorescence

Cells ($0.1\text{-}1x10^6$) from adult and fetal mouse tissues were incubated on ice with a saturating amount of antibody for fifteen min and washed before addition of the fluorescein conjugated indicator antibody or in the case of biotinylated antibodies, phycoerythrin conjugated streptavidin (Av-PE; Southern Biotechnology Assoc., Birmingham, AL). Isotype matched antibodies with irrelevant specificity were used as controls. Samples were analyzed by flow cytometry on a FACSCAN (Becton Dickinson, Mountain View, CA).

Immunofluorescence Cell Sorting

Thymocytes stained with rat anti-CD8-FITC were removed by panning on petri dishes coated with goat anti-rat Ig. Nonadherent cells were stained with CD3-FITC and CD4-FITC and in some experiments with 11B5-biotin and Av-PE, followed by sorting on a FACSTAR IV PLUS (Becton Dickinson). Negative and positive populations were collected in fetal calf serum.

Immunoprecipitation of Cell Surface Molecules

Fetal thymocytes ($5\text{-}15x10^7$, E15-17) were surface labeled with Na^{125}Iodine (1.0 mCi/$5.0x10^7$ cells; Amersham Corp., Arlington Heights, IL) by the lactoperoxidase method (Goding, 1980) and lysed with 0.5% NP-40 in 0.05M TRIS-HCL (pH 7.5) containing 0.02% sodium azide, 50.0mM iodoacetamide, 1.0mM PMSF, 20.0mM ε-amino N-caproic acid, and 0.01% soy bean trypsin inhibitor (SBTI). Immunoprecipitation was accomplished by a solid phase immunoprecipitation technique (Cooper et

al. 1986) in which concentrated cell lysates were precleared in control antibody wells before incubation in microtiter plates precoated with goat anti-mouse Ig followed by either irrelevant antibody or the 11B5 antibody. After incubation (24 hrs at 4°C) and extensive washing with 0.05M Tris-HCl (pH 8.0) containing 0.4M NaCl, 0.5% NP-40 and 0.5% deoxycholate, bound molecules were dissociated by addition of Laemmli's sample buffer (Laemmli 1970), and analysed under reducing and nonreducing conditions on a 7.5% or 10.0% polyacrylamide gel (SDS-PAGE). Labeled proteins were detected by autoradiography on XAR-5 film (Eastman Kodak, Rochester, NY) with Cronex Lightening Plus intensifying screens (Dupont, Wilmington, DE).

Enzyme Analysis

Immunoprecipitated antigen was subjected to N-glycanase™ (Genzyme, Boston, MA), neuraminidase (TypeX, Sigma Chemical Co., St. Louis, MO) and O-glycanase™ (Genzyme, Boston, MA) treatments according to the manufacturers instructions. For glycophosphatidylinositol-anchor analysis of the 11B5 antigen, SCID thymoma cells ($1.0x10^6$) were treated for 45 min at 37°C with phosphatidylinositol-specific phospholipase C (PI-PLC; Sigma Chemical Co., St. Louis, MO) in Hanks balanced salt solution (HBSS, pH 7.4), 10mM Hepes and 1.0% BSA. Treated and control cells (incubated in buffer only) were analysed for antigen expression by immunofluorescence. Trypsin (Sigma Chemical Co., St. Louis, MO) and Pronase (Calbiochem, San Diego, CA) treatments of SCID thymoma cells were accomplished by incubation with concentrations up to 200 µg/ml PBS (pH 7.4) for 30 min at 37°C. Control and test samples, washed with soybean trypsin inhibitor (50 µg per ml PBS), were analyzed by immunofluorescence for the 11B5 antigen.

Cell Cycle Analysis

Cells ($0.6\text{-}1.0 \times 10^5$) were washed in PBS (pH 7.3) and fixed in 50% cold ethanol before treatment with Ribonuclease A (1.0mg/ml PBS, Sigma Chemical Co., St. Louis, MO) for 20 min at 37°C and propidium iodide (20mg/ml). The cells were analysed by flow cytometry on a FACSCAN instrument (Becton Dickinson, Mountain View, CA).

Immunohistology

Tissues embedded in O.C.T. (Miles, Eikhart, IN) and frozen in liquid nitrogen were sectioned, fixed with acetone and stained by the immunoperoxidase technique (Bucy, Chen and Cooper 1989) before microscopic evaluation.

Lectin Binding Assays

Yac-1 thymoma cells (1.0×10^5) were incubated for 30 min. at 22°C with 300μg of the DBA inhibitor methyl-2-acetamido-2-deoxy-β-D galactopyranoside (10mg/ml PBS) (Calbiochem, San Diego, CA), before incubation with DBA-FITC or the 11B5 antibody followed by goat-anti-mouse-IgM-FITC and immunofluorescence analysis.

Results

Production of the 11B5 Antibody

Lymph node cells from wild mice, *Mus spretus*, hyperimmunized with embryonic thymocytes (E15-17) from inbred mice, C3D2F1xDBA/2J, were fused with a myeloma variant, and the resultant hybridomas were screened for production of antibodies reactive with surface antigens of donor fetal thymocytes (E15-17). Out of a panel of eighteen hybridoma clones selected by this antibody screening strategy, the 11B5 clone was selected. The 11B5 antibody typed as IgMκ was found to react with thymocytes from all of the inbred mouse strains tested including Balb/c,

C3H/HeJ, DBA/2 and C57Bl/6 and with thymocytes from *Mus spretus*. The antigen recognized by this mouse autoantibody was characterized in the following studies.

Tissue Distribution and Tumor Cell Expression of the 11B5 Antigen

Cells in the bone marrow, lymph nodes, spleen, and thymus from adult DBA/2J mice (6-8 weeks), fetal liver (E14 - E17), neonatal spleen (D1 - D5) and thymus from fetal to adult ages were examined by cell surface immunofluorescence. The thymus was the only one of these tissues found to contain cells that express the 11B5 antigen, and the frequency of $11B5^+$ thymocytes declined as a function of donor age (see below). Because of this pattern of thymic expression, a panel of cell lines was tested for reactivity with the 11B5 mAB (Table 1). The antigen was not expressed on the B-lineage cell lines. Two of the six T-lineage cell lines, the Yac-1 thymoma and a SCID thymoma, expressed the 11B5 antigen. Notably, both of these $11B5^+$ cell lines displayed an immature thymocyte phenotype while the $11B5^-$ T-cell lines were characterized by more mature phenotypes.

TABLE 1: Tumor cell expression of the 11B5 antigen

Cell line	Phenotype	11B5 expression
18.81	Pre-B	-
A20	Mature B	-
Yac-1	Immature T	+
SCID	Immature T	+
EL-4	Mature T	-
BW5147	Mature T	-
S49.1	Mature T	-
L6911	Mature T	-

Expression of the 11B5 Antigen as a Function of Age

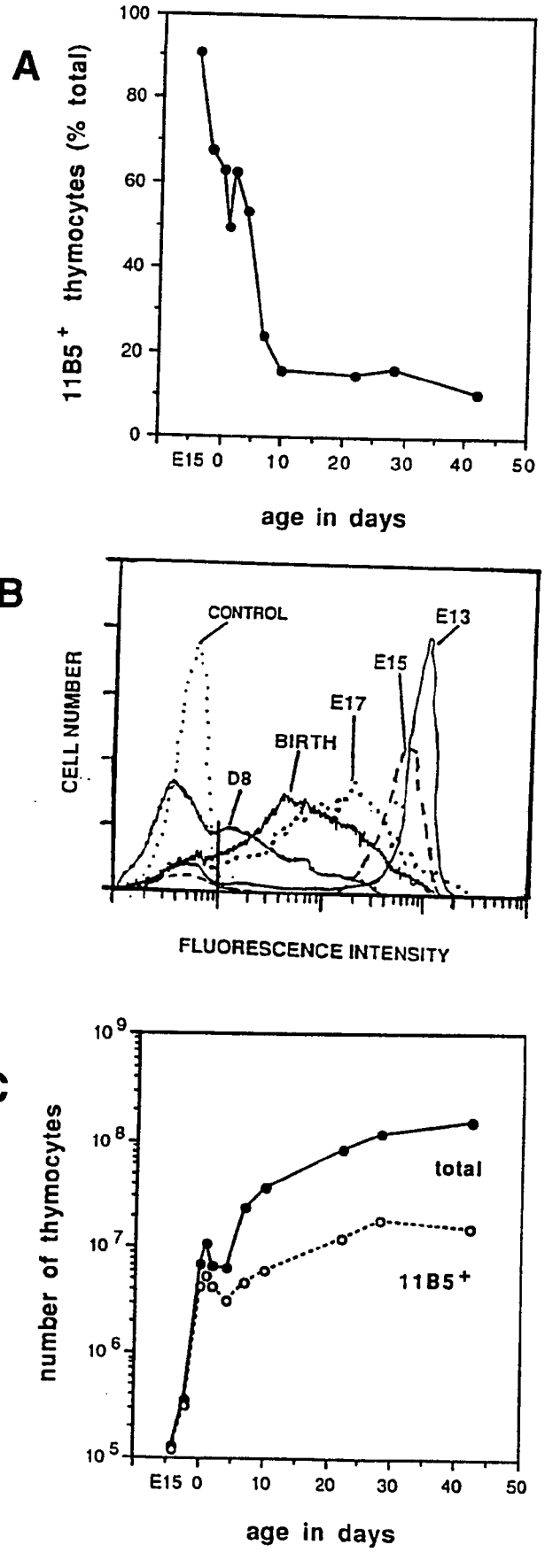
The immunofluorescence profiles for expression of 11B5 in the thymus revealed a sharp decline in the percentage of 11B5$^+$ cells over the last few days of embryonic life and after birth (Fig. 1A). From peak levels of 90% 11B5$^+$ thymocytes on E15, the frequency of positive cells dropped to around 15% or less by the second week after birth. In addition, the levels of the 11B5 antigen on the subpopulation of thymocytes expressing this molecule decreased as a function of age. Early in development (E13) when >85% of thymocytes were 11B5$^+$, the peak fluorescence intensity was 200 times the control value. The intensity decreased to 125 times at E15 and 12 times the background level at birth. While a bright homogeneous staining pattern was observed at E13, broad peaks including both bright to dim subpopulations were found at E17 and all ages thereafter (Fig. 1B). Despite the rapid developmental decline in the frequency and intensity of thymocyte expression of the 11B5 antigen the absolute numbers of 11B5$^+$ cells increases because of the enormous growth of the total thymocyte population (Fig. 1C).

Phenotype of 11B5$^+$ Thymocytes as a Function of Age

Embryos

Since E13 thymocytes showed a bright homogeneous staining pattern for the 11B5 antigen, this molecule appears to be one of the earliest markers expressed during thymic ontogeny. In the E13 thymus, 87% of the cells expressed 11B5 while only 70% expressed Thy-1. On E17 (Fig. 2), approximately 50% of thymocytes expressed the 11B5 antigen at levels varying from very low to very high (panel A). Essentially all 11B5$^+$ cells express Thy-1 at relatively high levels (panel B). Only the bright 11B5 subpopulation express IL-2Rα (panel C), and these cells

Figure 1. Thymic expression of the 11B5 antigen as a function of age. Viable cells were incubated with biotinylated 11B5 antibody or control antibody and bound molecules were detected by phycoerythrin conjugated streptavidin. Analysis was by automated flow cytometry. (A) Thymus from DBA/2 mice at ages E15-D42 plotted as the % of thymocytes bearing the 11B5 antigen versus the age in days. (B). Immunofluorescence analysis of 11B5-reactive thymocytes from DBA/2 mice at various ages. (C). The total number of thymocytes (——) compared with the absolute number of $11B5^+$ thymocytes (- - - -) versus age in days.


A
100
80
60
40
20
0
11B5+ thymocytes (% total)
E15 0
10
20
30
40
50
age in days
B
CONTROL
E13
E15
E17
BIRTH
D8
CELL NUMBER
FLUORESCENCE INTENSITY
C
10 9
10 8
10 7
10 6
10 5
number of thymocytes
total
11B5+
E15 0
10
20
30
40
50
age in days

lacked both CD4 and CD8 (panels D and E). In contrast, thymocytes expressing low levels of the 11B5 antigen lacked IL-2Rα but express CD4 and CD8. This data suggests that expression of the 11B5 antigen declines with the onset of CD4 and CD8 expression.

Neonates

While very few thymocytes in newborn mice express high levels of the 11B5 antigen (<1%), approximately 9% express relatively low levels. Most of the $11B5^{lo}$ cells express CD4 and CD8 (Fig. 3A and Fig. 3B). Of these $11B5^{lo}$ cells, only 3% express low levels of CD3. The CD3 bright population of mature thymocytes lack the 11B5 antigen (Fig. 3C). These data further suggest loss of 11B5 expression with thymocyte maturation.

Adults

Although the frequency of $11B5^{+}$ thymocytes in mature mice is relatively low, and most are $11B5^{lo}$, a small subpopulation of $11B5^{hi}$ thymocytes persists into adulthood (Fig. 4). These $11B5^{hi}$ cells express IL-2Rα but not CD4 or CD8, whereas the $11B5^{lo}$ cells are IL-2Rα^{-} $CD4^{+}CD8^{+}$. The Mature $CD4^{+}CD8^{-}$ cells, when isolated by cell sorting, do not express the 11B5 antigen (Fig. 5A), suggesting that $CD4^{+}CD8^{+}11B5^{lo}$ cells are within the double positive thymocyte subset. Further analysis of thymic subpopulations revealed that $CD4^{-}CD8^{-}$ cells contained 30-50% $11B5^{+}$ cells varying in intensity from high to low levels of expression (Fig. 5B). These $CD4^{-}CD8^{-}11B5^{+}$ thymocytes represent 0.5-1.0% of the adult thymocyte population. Approximately 9.0% of $11B5^{lo}$ cells expressed CD3, and these may represent γδ TCR bearing cells.

To examine further the phenotype of immature adult thymocytes that express the 11B5 antigen, the $CD3^{-}CD4^{-}CD8^{-}$ population was isolated

Figure 2. Two-color immunofluorescence analysis of the 11B5 antigen and T-cell antigens expressed on fetal (E17) thymocytes. Viable cells were incubated with biotinylated 11B5 and with FITC-conjugated control antibody (A) or antibodies toThy-1 (B), IL-2Rα (C), CD4 (D) or CD8 (E).

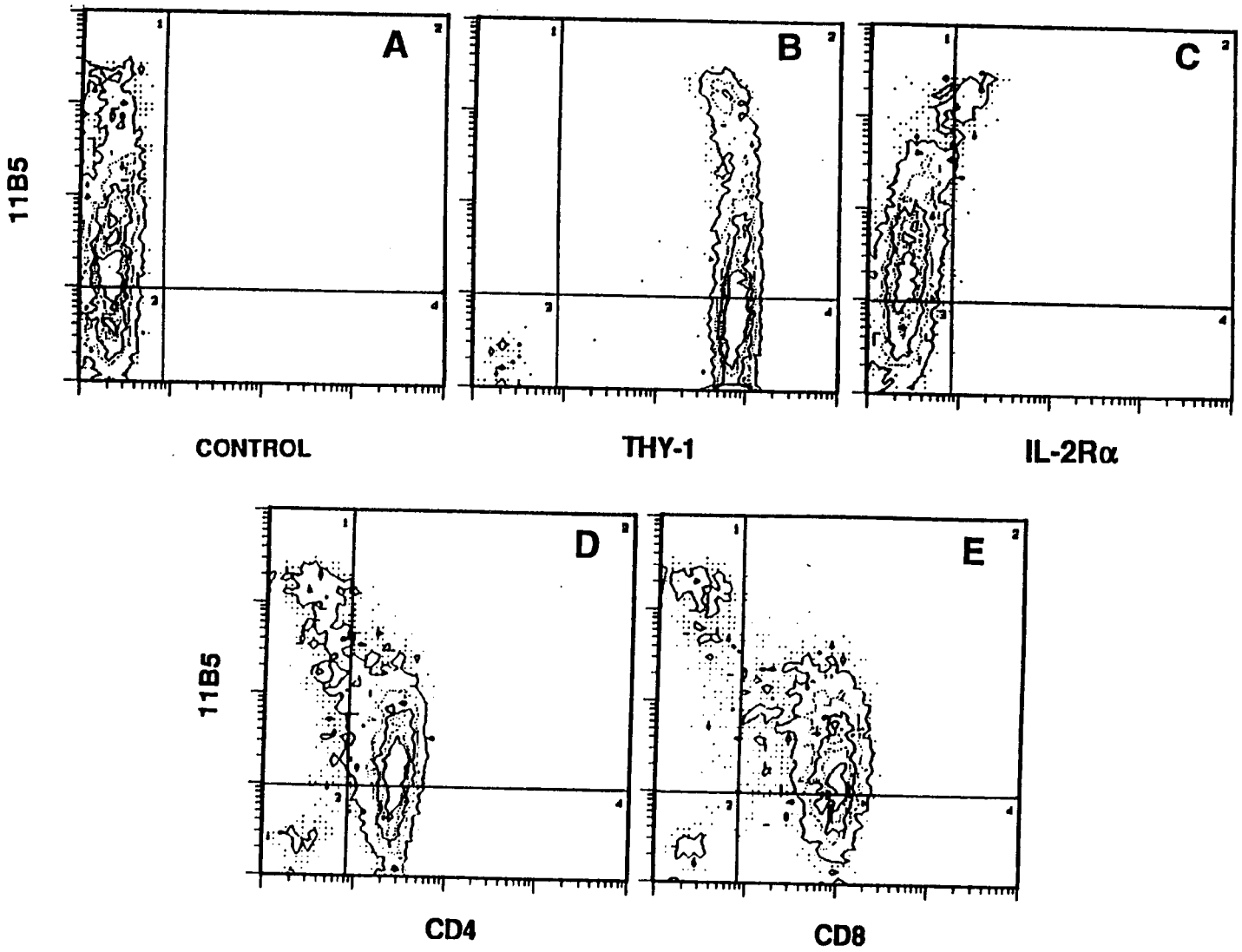
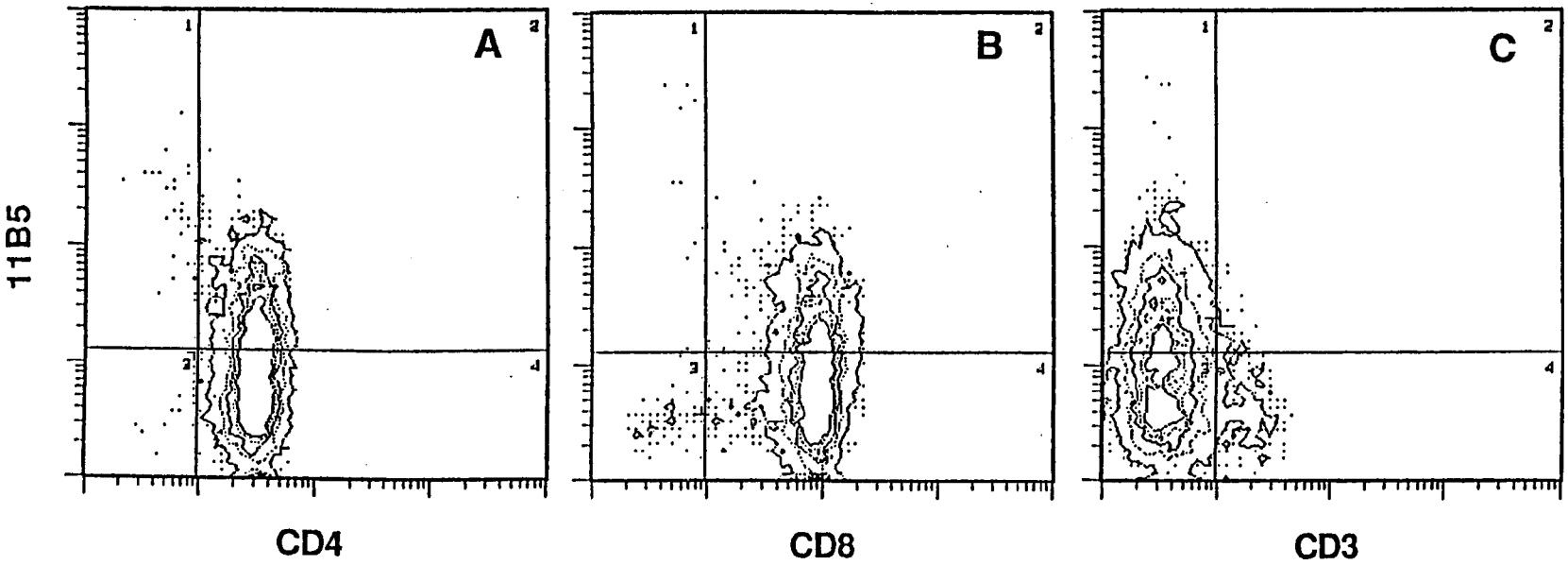

A
B
C
11B5
CONTROL
THY-1
IL-2Rα
D
E
11B5
CD4
CD8

Figure 3. Two-color immunofluorescence analysis of the 11B5 antigen and T-cell antigens on neonatal (D1) DBA/2 thymocytes. Viable cells were incubated with biotinylated 11B5 antibody and FITC-conjugated antibodies to CD4 (A), CD8 (B), or CD3 (C). Cell-bound 11B5 was detected by Av-PE.


A
B
C
11B5
CD4
CD8
CD3

from adult mice by cell sorting. This population was enriched in 11B5^{hi} cells which were found to be Thy-1^{+}IL-2Rα^{+}HSA^{+} and CD44$^{+/-}$ (Fig. 6).

Cell cycle analysis of CD3^{-}CD4^{-}CD8^{-}11B5^{+} thymocytes revealed that 19% of these cells were in the S phase and 2% were in the G2M phase versus 28% and 1%, respectively, for the CD3^{-}CD4^{-}CD8^{-}11B5^{-} population.

Intrathymic Distribution Pattern of 11B5^{+} Cells

Thymus sections were examined by immunohistochemistry in order to determine the location of 11B5^{+} cells (Fig. 7). On E17, the 11B5^{hi} thymocytes were scattered throughout the cortex but were more concentrated in the outer cortex (panel A). While the proportion of 11B5^{hi} thymocytes decreased as a function of developmental age, positive cells were found scattered throughout the cortex at all ages but were excluded from the medulla.

Biochemical Analysis of the 11B5 Antigen

In immunofluorescence experiments the antigen was found to be insensitive to trypsin and was unaffected by treatment with PI-PLC but was sensitive to Pronase.

When mouse fetal thymocytes (E15 -17) were surface labeled with ^{125}Iodine, and cell lysates were immunoprecipitated with the 11B5 antibody, the apparent molecular weight of the antigen was found to be 110,000 under both reducing and nonreducing conditions (not shown). For determination of the core size of the molecule, immunoprecipitated protein was subjected to N-glycanase treatment to determine the reduction in molecular weight after N-linked carbohydrates are removed, or were treated with neuraminidase and O-glycanase for molecular weight changes due to O-linked carbohydrates. The apparent molecular weight of the 11B5

Figure 4. Two-color immunofluorescence analysis of adult 11B5 reactive thymocytes. Thymocyte suspensions from DBA/2 mice were incubated with biotinylated 11B5 mAb and FITC-conjugated antibodies to CD4 (A), CD8 (B), or IL-2Rα (C). Cell bound 11B5 was detected by Av-PE.

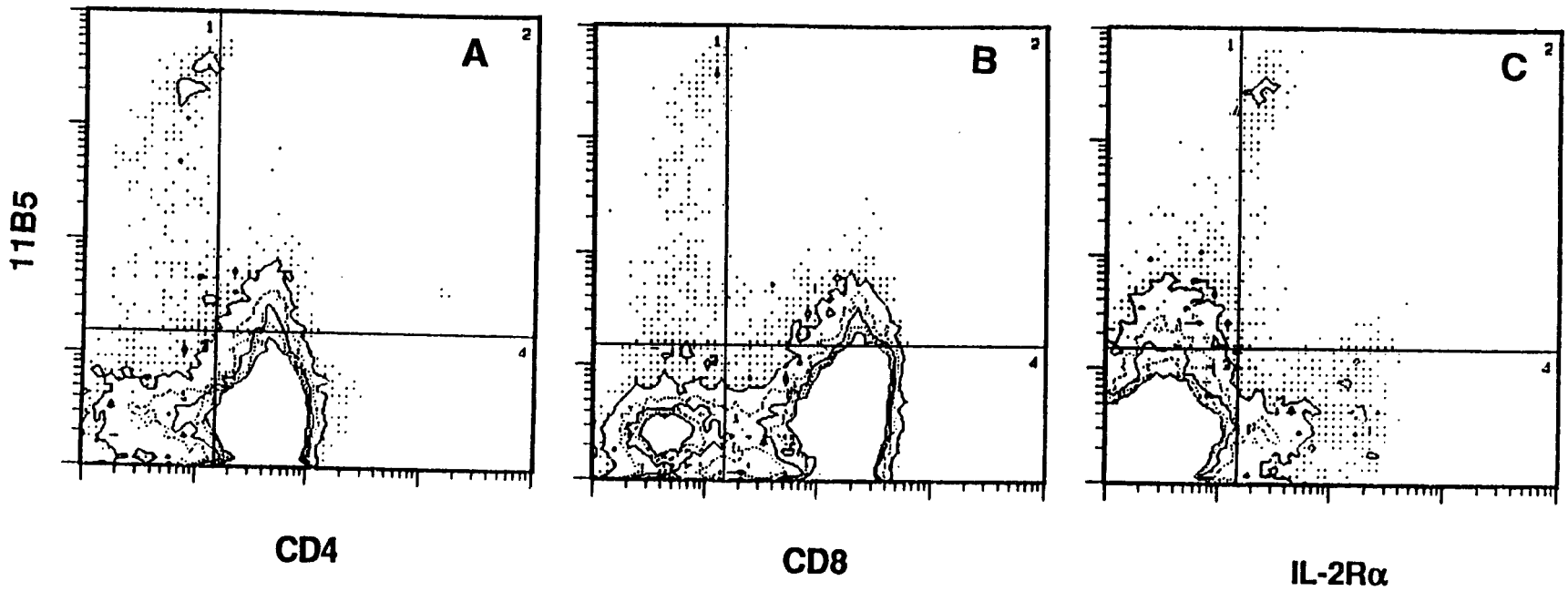

11B5
A
B
C
CD4
CD8
IL-2Rα

Figure 5. Adult 11B5$^+$ thymocytes are relatively immature. CD4$^+$CD8$^-$ (A) and CD4$^-$ CD8$^-$ (B) thymocyte subsets were isolated by cell sorting and incubated with biotinylated 11B5 detected with Av-PE and in (B) with FITC-conjugated antibody to CD3.

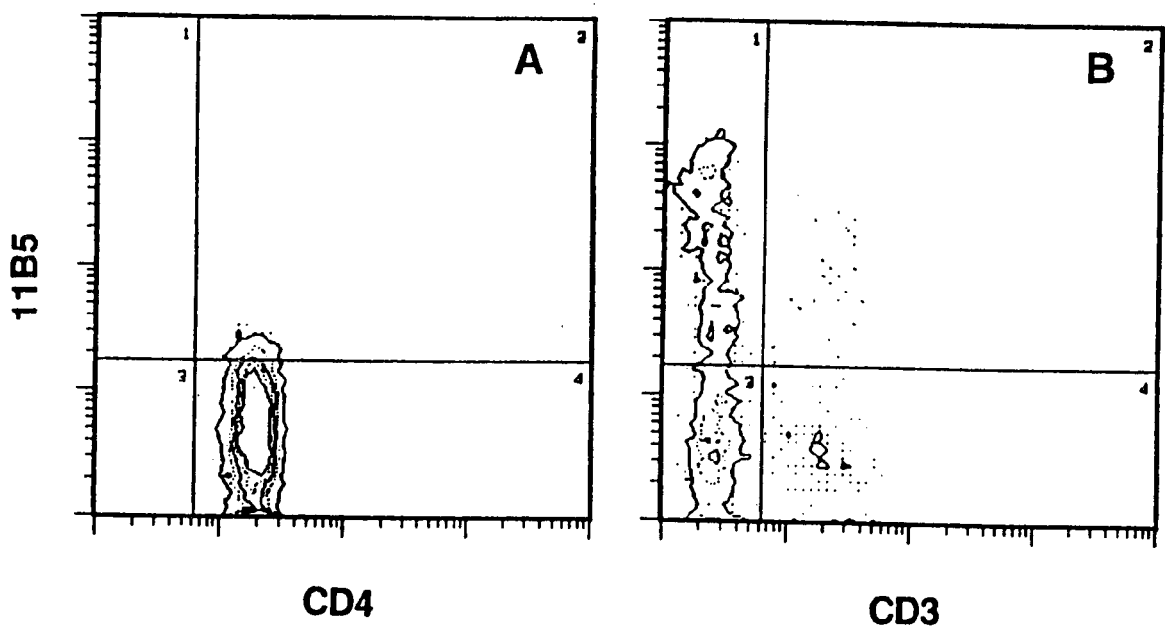

A
B
11B5
CD4
CD3

Figure 6. Phenotypic analysis of adult 11B5$^+$ thymocytes. The CD3$^-$CD4$^-$CD8$^-$ subset isolated by cell sorting was incubated with biotinylated 11B5 antibody detected by Av-PE and FITC-conjugated antibodies to irrelevant antigen (A),Thy-1 (B), IL-2Rα(C), or HSA (J11D)(D), or incubated with biotinylated Pgp-1 and 11B5 mAb detected by Av-PE and goat-anti-mouse IgM-FITC, respectively (E).

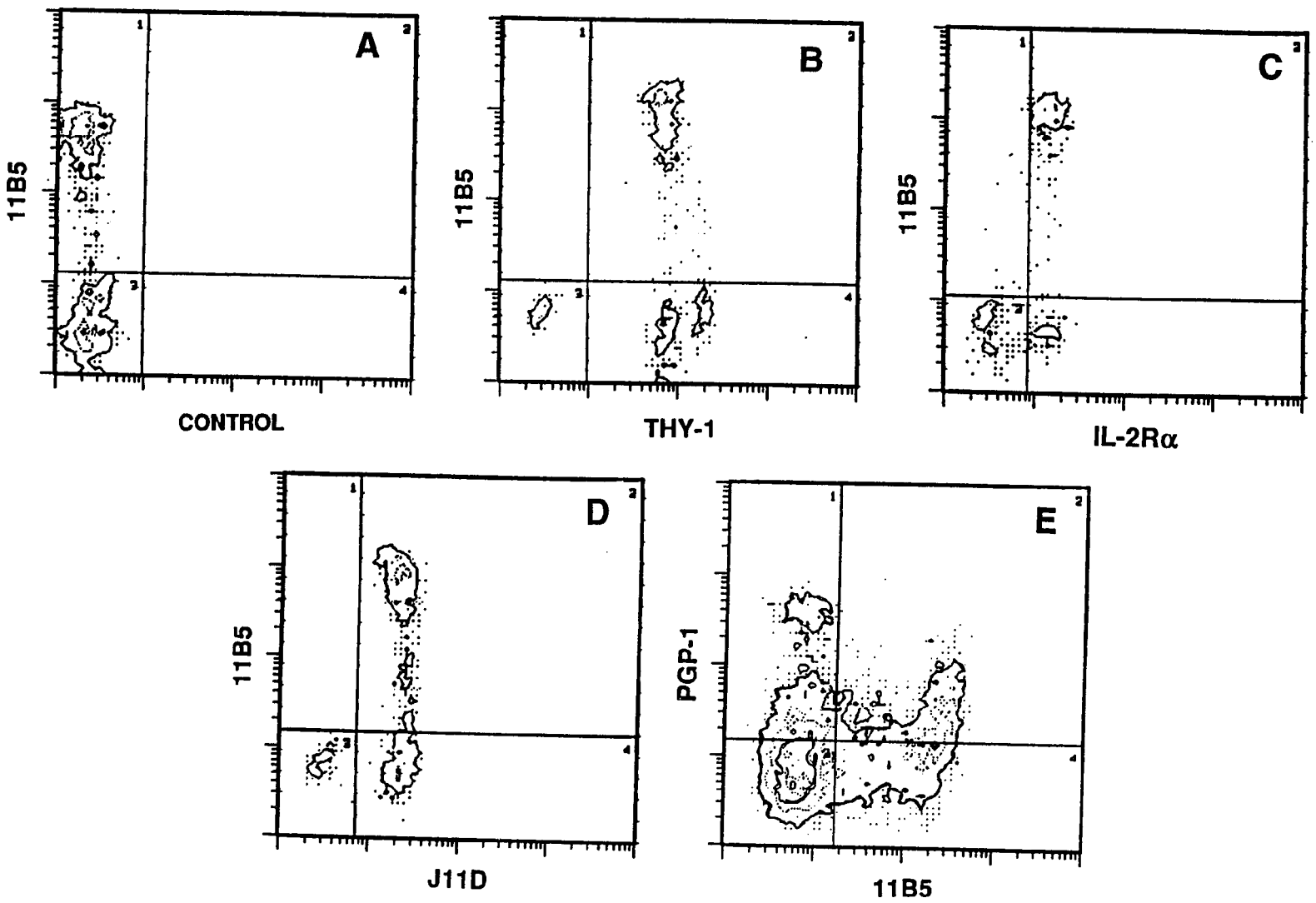
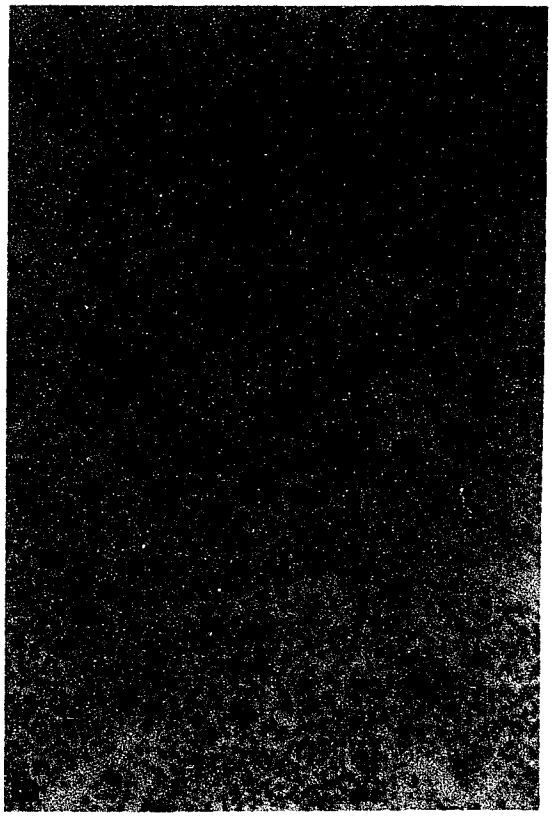
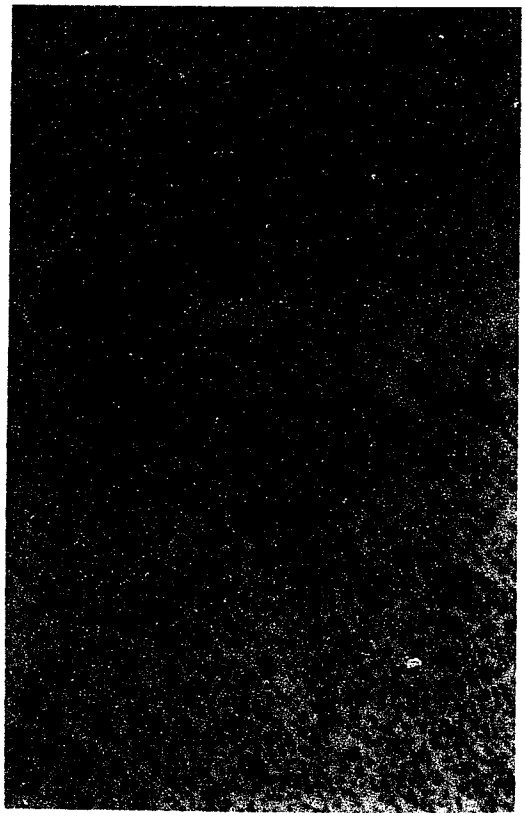
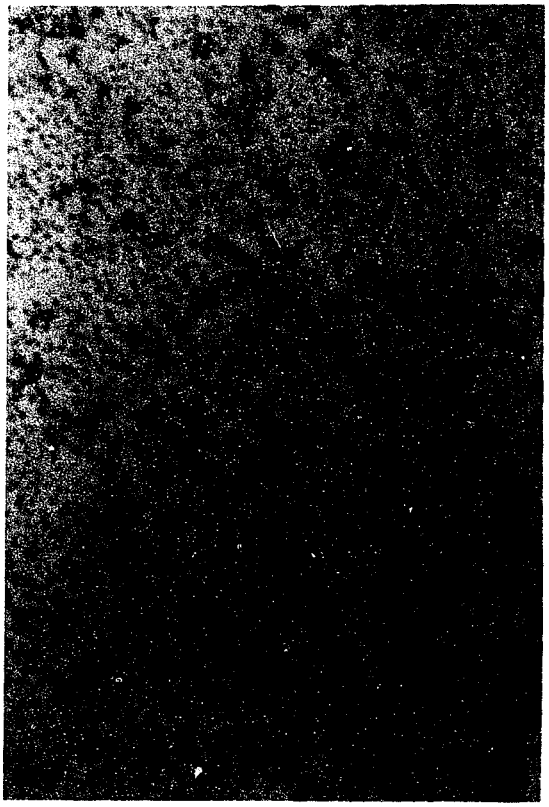
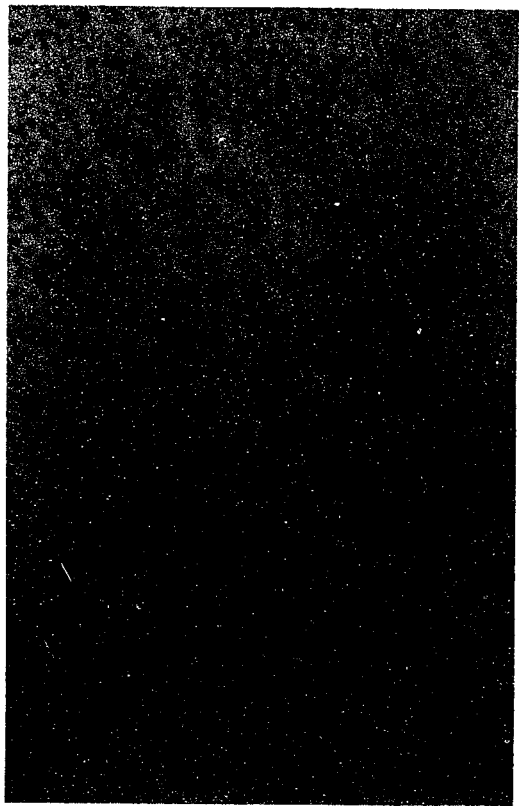

A
11B5
CONTROL
B
11B5
THY-1
C
11B5
IL-2Rα
D
11B5
J11D
E
PGP-1
11B5

Figure 7. Immunohistologic analysis of the intrathymic distribution of 11B5$^+$ cells. Sections of thymus from E17 (A), D10 (B), D21 (C), D42 (D) were stained with biotin-conjugated 11B5 followed by avidin-biotin peroxidase and the diaminobenzidine developing reagent. (m = medulla, c = cortex, sc = subcapsular)

antigen was reduced by 20,000 after N-glycanase treatment but was unaltered by treatment with neuraminidase alone or with neuraminidase plus O-glycanase (Fig.8).

Comparison of 11B5 and Lectin Binding

The 11B5 antigen and the FT-1 antigen, recognized by the M6 mAb (Kasai et al. 1983), have many similar characteristics. The specificity of the M6 antibody was shown to be the molecule recognized by *Dolichos bifluores* agglutinin (DBA). When we compared the staining profiles of the 11B5 mAB and DBA on neonatal Balb/c mice at D1, D3, and D9 (Fig. 9), very similar patterns of expression were observed at each time point. In addition, the two 11B5$^+$ cell lines (Yac-1 and Scid thymoma) also bind *Dolichos bifluores* agglutinin.

To determine if the antibody recognizes the same site as the lectin, competition assays for binding of both reagents with the DBA inhibitor methyl-2-acetamido-2-deoxy-β-D galactopyranoside were performed. Cells stained in the presence of the DBA inhibitor showed a 92% inhibition of DBA-FITC binding but <1% inhibition of 11B5 fluorescence intensity. When cells were stained first, added inhibitor reduced DBA-FITC binding by 90% and 11B5 binding by <1%. Even when increasing amounts of inhibitor were added to cells stained with below saturating levels of the 11B5 mAb, there was no reduction in fluorescence intensity. These data suggest that the 11B5 antibody and the DBA lectin do not bind to the same sites.

Discussion

The 11B5 antigen is a single chain, transmembrane glycoprotein with an Mr of 110,000 and a core size of 90,000 Mr after removal of N-linked oligosaccharides. The molecule is expressed on the surface of murine thymocytes during fetal and adult development and appears to be

Figure 8. Immunoprecipitation analysis of the apparent molecular weight and core size of the 11B5 antigen. Cell surface proteins labeled with $Na^{125}I$ were immunoadsorbed to the 11B5 antibody coated onto plastic wells and the precipitated molecules resolved by SDS-PAGE analysis on a 7.5% gel after treatment with carbohydrate cleaving enzymes. Lane 1- N-glycanase treated, Lane 2- nontreated control, Lane 3- buffer control, Lane 4- neuraminidase, Lane 5-neuraminidase followed by O-glycanase.

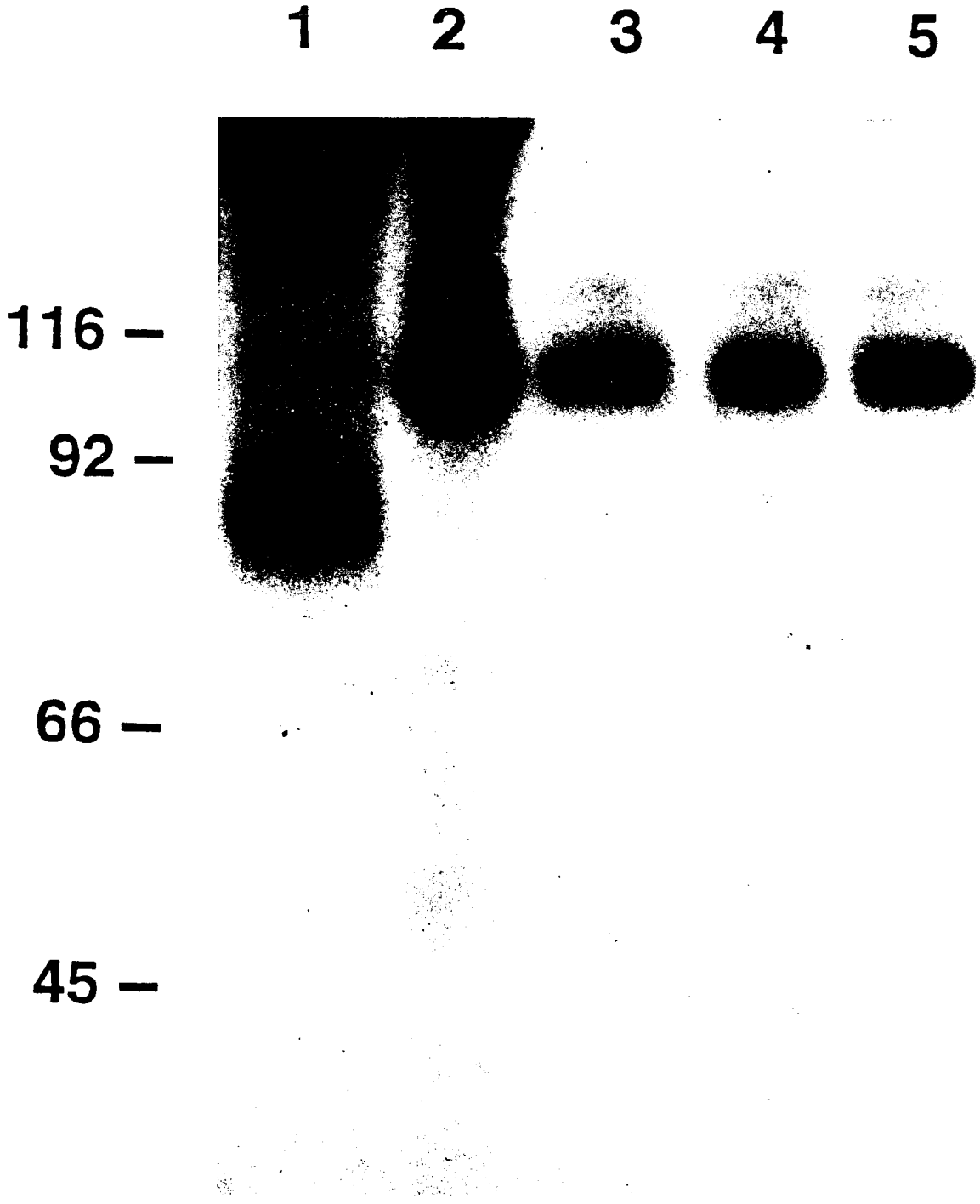
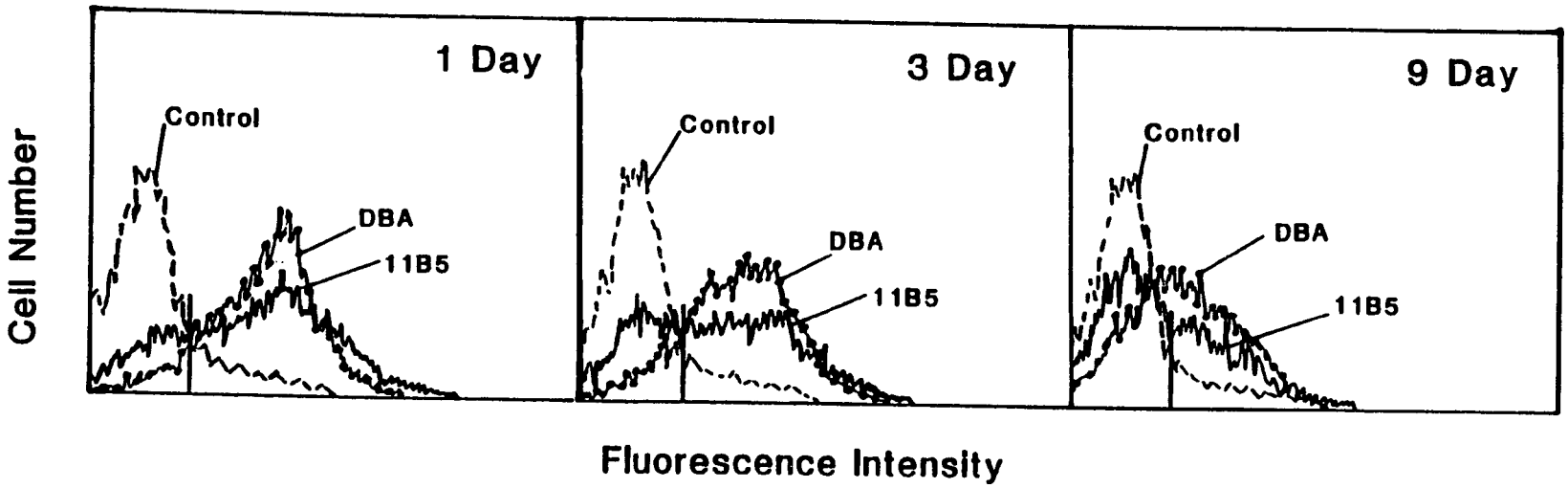

1
2
3
4
5
116 –
92 –
66 –
45 –

Figure 9. Comparison of the 11B5 mAb and *Dolichos bifluorous* agglutinin (DBA) binding to neonatal thymocytes. Viable cells from Balb/c mice on D1, D3, or D9 were incubated with 11B5- or DBA-biotin and bound molecules were detected with Av-PE.


1 Day
3 Day
9 Day
Control
DBA
11B5
Cell Number
Fluorescence Intensity

lost as the cells mature from $CD4^-CD8^-$ to the mature $CD4^+$ or $CD8^+$ phenotype. We believe that the 11B5 mAb recognizes the same molecule as the previously described M6 antibody (Kasai et al. 1983). This study reported that the *Dolichos bifluores* agglutinin (DBA) receptor was the antigen recognized and described its molecular weight, tissue distribution and fetal thymus ontogeny. The 11B5 and M6 antigens have comparable molecular weights of 110,000 and 130,000, respectively, and this correlates with the antigen immunoprecipitated by DBA in the M6 report and in the present study (data not shown). The data are similar in fetal thymus studies where both antibodies show a decrease in the frequency of positive cells as the animal matures, as well as reacting with thymocytes before Thy-1 expression. The thymus is the only tissue reported to contain $11B5^+$ and $M6^+$ cells. In the present study, $11B5^+$ thymocytes were found in the adult as well as fetal thymus. Using a more sensitive reagent, Scofield, Yan and Farr (1989) detected DBA bearing thymocytes in adult animals. Although we lack definitive proof indicating that the 11B5 and M6 antigens are identical, the similarities in the data strongly suggest both antibodies recognize the same molecule as DBA. In the present study we have used the 11B5 mAB to describe a specific population of murine thymocytes and the transient expression of the 11B5 antigen during murine thymic development.

In the fetal thymus, cells that bear the 11B5 antigen appear as a bright homogeneous population during early development when all thymocytes are $CD4^-CD8^-$ and is expressed on >85% of cells. As the animal matures, thymocytes display a heterogeneous expression of the 11B5 antigen represented by both dim and bright populations and this corresponds with a reduction in the frequency of $11B5^+$ cells. This data indicates that bright

expression of the 11B5 antigen is restricted to an early developmental stage in thymocyte ontogeny. Evidence for the loss of the 11B5 antigen as a function of maturation is shown by the immunofluorescence data on E17 thymocytes where $11B5^{hi}$ cells do not express CD4 or CD8 but do express IL-2Rα (Fig. 2). In contrast, $11B5^{lo}$ bearing cells may express CD4 and CD8 at E17 and D1 (Fig. 3). In addition, in the D1 thymus when most thymocytes show low expression of the 11B5 antigen, some of these cells are also $CD3^{lo}$ while the mature $CD3^{hi}$ thymocytes are $11B5^{-}$.

In the adult, expression of the 11B5 antigen mimics that observed in fetal life in that the $11B5^{hi}$ thymocytes are IL-2Rα^{+}CD4^{-}CD8^{-} and low expression of the 11B5 antigen corresponds with an increased frequency of CD4 and CD8 bearing cells. Another similarity is the lack of $11B5^{+}$ thymocytes within the mature CD4 or CD8 single positive subsets shown by expression of the antigen on $CD3^{lo}$ but not $CD3^{hi}$ cells, and by the absence of $11B5^{+}$ cells in the medulla. Since early fetal thymocytes (E13, E15) show an intense, homogeneous expression of the 11B5 antigen, which decreases in the frequency of positive cells and their intensity as a function of maturation, we believe that the 11B5 antibody marks a population of cells which represent an early developmental stage in thymocyte ontogeny.

A maturation scheme for murine thymocyte development using the HSA, CD44 and IL-2Rα markers has been reported by Pearse et al (1989). Based on reconstitution potential, TCR gene rearrangements and cell cycle analysis of sorted populations, these researchers proposed a sequence whereby (1) the CD44^{+}IL-2Rα^{-} phenotype develops into (2) CD44^{-}IL-2Rα^{+} cells which mature to a (3) CD44^{-}IL-2Rα^{-} phenotype. All three subsets are HSA^{+}. The more immature subsets have the fewest

percent of cells cycling and have a greater thymic reconstitution potential. Most $CD3^-CD4^-CD8^-$ thymocytes which express the 11B5 antigen also bear HSA and IL-2Rα and the $11B5^{hi}$ cells express CD44. Therefore bright $11B5^+$ thymocytes are $CD44^+HSA^+IL\text{-}2R\alpha^+$. Based on the maturation sequence described above, this phenotype places $11B5^+$ thymocytes at a stage of development which falls between steps 1 and 2. CD44 is not detectable on the $11B5^{lo}$ thymocyte population. These data further indicate that loss of the 11B5 antigen occurs as a function of maturation.

Pearse et al. (1989) also analyzed the status of the TCR genes in cell subpopulations representing the three stages of maturation, revealing that all populations were at least 50% rearranged at the TCR β loci, whereas TCR α mRNA was not detectable until the third stage, after the loss of IL-2Rα expression, when a large proportion of cells is cycling. The $IL\text{-}2R\alpha^-CD44^+$ (stage 1), the $IL\text{-}2R\alpha^+$ (stage 2) and mature $IL\text{-}2R\alpha^-$ thymocytes (stage 3) contain 18%, 19%, and 59% in S+G2+M phases of the cell cycle, respectively. In the present study, the $11B5^+CD3^-CD4^-CD8^-$ population contains 21% of cells in S+G2+M phases, further supporting the immature status of $11B5^+$ thymocytes. While the status of the TCR genes for the 11B5 bearing adult thymocytes is not known, one would expect, based on the phenotype and the cell cycle analysis, rearrangement at the TCR β locus but little or no rearrangement at the TCR α locus.

Since at least 50% of the most immature $CD44^+IL\text{-}2R\alpha^-$ thymocytes in the study by Pearse et al. (1989) have rearrangement of the TCR β locus, Wu et al. (1991) searched for a population of adult thymocytes in which no TCR rearrangement had occurred. They reported a $CD4^{lo}$

precursor population with the TCR genes in germline configuration, containing fewer cycling cells, and a better reconstitution potential than the populations described in the earlier study. Since the $CD4^+$ population was eliminated from the present study, it is not known if this early precursor expresses the 11B5 antigen.

CHAPTER 3

EXPRESSION OF THE 9A5 ANTIGEN DURING HEMOPOIESIS AND T-CELL DIFFERENTIATION

Introduction

It has been estimated that domestic and wild species of mice have been genetically isolated from each other for one to three million years (Thaler 1986). A classification of species within the genus *Mus* which divides the different species into four biochemical groups has been proposed, based on differences in electrophoretic mobilities of serum proteins (Bonhomme et al. 1984). This study has taken advantage of the genetic diversity between domestic species (Mus biochemical group 1) and a wild mouse species (*Mus spretus*; Mus group 3) to generate a panel of monoclonal alloantibodies which recognize early differentiation markers. The immunization strategy used selects against the generation of antibodies specific for antigens conserved throughout the genus whereas molecules which have been altered by mutation or are lacking in some species may be antigenic.

The initial goal was to isolate monoclonal antibodies (mAb) which identify differentiation antigens expressed during early T-cell development. The antigen recognized by the 9A5 mAb was selected for analysis based on its expression on embryonic thymocytes. The analysis of the tissue distribution of the 9A5 antigen revealed that a minor subpopulation of immature bone marrow cells also bears this antigen. The expression of the 9A5 antigen during murine fetal and adult thymopoiesis and in peripheral

lymphoid organs is described. In addition, the subpopulation of bone marrow cells bearing the 9A5 antigen was analysed for its capacity to differentiate into myeloid, erythroid, T- and B-cell lineages by *in vivo* reconstitution assays, CFU-S analysis, and *in vitro* stroma induced B-cell differentiation.

Materials and Methods

Mice

Mus spretus (Spain), DBA/2J, C3H/HeJ, Balb/c, C3D2F1 and C57Bl/6 (Thy-1.1 congenic strain) mice were purchased from Jackson Labs (Bar Harbor, ME). DBA/2, C57Bl/6, BDF1 and AKR strains were purchased from Charles River (Raleigh, NC). The wild mouse strains MYL, Spret-1, SEI, XBS and Caroli were tested at the Pasteur Institute, Paris, France. C3D2F1XDBA/2J, DBA/2J and severe combined immunodeficient mice (SCID) (Bosma et al. 1983) were bred in the animal facility at the University of Alabama at Birmingham. The date that a vaginal plug was observed was designated as day 0 of gestation.

Cell Lines

The mouse T cell lines EL-4, BW5147 and Yac-1, and the A20 B cell line were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The S49.1 and L6911 T cell lines were a gift from Dr. Elizabeth Evans (Becton Dickinson, Mountain View, CA). Other tumor cells used were the pre-B cell lines 18.81 (Siden et al. 1979), 38B9 and L1-2 (Dr. Naomi E. Rosenberg, Boston, MA), and a SCID thymoma isolated in our laboratory at the University of Alabama at Birmingham.

Hybridoma Production

Wild mice (*Mus spretus*; biochemical group 3) were immunized with E15-17 fetal thymocytes from inbred C3D2F1XDBA/2J mice (biochemical

group 1). Animals were injected four times over a two week period and axillary, linguinal and mesenteric lymph node cells were fused one day after the last immunization with the AG8.653 myeloma variant (Kearney et al. 1979). Tissue culture supernatants from resulting hybridomas were screened by indirect immunofluorescence staining of E15-17 thymocytes using goat anti-mouse-FITC (Southern Biotechnology Assoc., Birmingham AL) as the indicator antibody. Positive clones were subcloned twice and the antibody isotypes determined by reactivity with isotype-specific goat anti-mouse antibodies (Southern Biotechnology, Birmingham, AL)

Antibodies and Immunofluorescent Reagents

Anti-CD3-FITC (clone 145-2C11), anti-CD4-FITC (clone GK1.5), anti-Thy-1-FITC (clone 30-H12), anti-Mac-1-FITC (clone M1/70) and anti-Thy-1.1-biotin (clone T11D7e2) were gifts from Dr. John H. Eldridge (University of Alabama at Birmingham). Goat anti-mouse IgM conjugated with FITC or RITC, goat anti-mouse IgG2a-FITC and streptavidin-phycoerythrin (Av-PE) were purchased from Southern Biotechnology Assoc. (Birmingham, AL). Anti-CD8-FITC (Becton Dickinson Mountain View, CA), αβTCR antibody (clone H57-597, gift from Dr. Ralph Kubo, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO), anti-H-2^k (clone H100-27/55, Accurate Chemical and Scientific Corp., Westbury, NY), and the anti-granulocyte antibody (clone BP-2, IgG3,κ) produced in our laboratory were also used. The anti-B220 (clone 14.8; Kincade et al. 1981) and anti-H-2^b (clone K10.56.4, gift from Dr. S. Kimura) antibodies were purified by passing tissue culture supernatants over a protein G column (Pharmacia, Piscataway, NJ). The 9A5 antibody was purified by passing the 9A5 hybridoma culture

supernatant over a column of goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL) coupled to sepharose 4B (Pharmacia, Piscataway, NJ). Bound antibody was eluted from the column with 0.2 M glycine HCL (pH 2.8), the pH was adjusted to 7.2, followed by concentration using an Amicon ultrafiltration unit (Amicon Corp. Danvers, MA) and dialysis against PBS (pH 7.4) containing 0.1% sodium azide. Antibodies to BP-1/6C3 (clone BP-1) (Cooper et al. 1986), CD4 (clone GK 1.5), and 9A5 were purified from ascites by the caprylic acid precipitation procedure (McKinney and Parkinson 1987). Purified CD4, B220 (clone14.8), H-2^b, BP-1, and 9A5 mAb were biotinylated with NHS-LC-biotin (Pierce, Rockford, IL) by a published method (Kendall, Ionescu-Matiu and Dreesman 1983).

Immunofluorescence

Cells ($0.1\text{-}1x10^6$) from adult and fetal mouse tissues were incubated on ice with a saturating amount of antibody for 15 min and washed twice before adding the fluorescein conjugated indicator antibody or in the case of biotinylated antibodies, phycoerythrin conjugated streptavidin (Av-PE). Isotype matched irrelevant mAb were used as negative controls. Samples were analyzed by flow cytometry on a FACSCAN (Becton Dickinson, Mountain View, CA)

Immunofluorescence Cell Sorting

Bone marrow cells from adult mice ($1.5x10^8$ cells) resuspended in RPMI 1640 media supplemented with 2mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY) , 5×10^{-5}M 2-ME, and 15% fetal bovine serum (FBS: Hyclone, Logan, UT) were adhered onto nylon wool columns for 1 hr at 37°C. Nonadherent cells were eluted with media, layered onto lympholyte M (Accurate Chemical and Scientific

Corp., Westbury, NY) and centrifuged at 1800 rpm 20 min. The mononuclear cell layer was washed with PBS containing 3% BSA (pH 7.4), incubated with the 9A5-biotinylated mAb and Av-PE and in some experiments with anti-Mac-1-FITC. Separation of populations was by sorting on a FACSTAR IV PLUS instrument (Becton Dickinson, Mountain View, CA).

Reconstitution of SCID Mice

$9A5^+$ bone marrow cells, isolated by cell sorting from C3D2F1 mice, were injected intravenously into adult SCID hosts (5.0×10^5 cells). After ten to fifteen weeks the animals were sacrificed and cells from the bone marrow, lymph node, thymus, and spleen were analysed by immunofluorescence for expression of the donor haplotype ($H\text{-}2^k$). Alternatively, donor cells from C57Bl/6 (Thy1.1 congenic) mice were injected intraperitoneally into neonatal SCID mice, and tissues were analysed 8-12 weeks afterwards for donor cells bearing the Thy-1.1 allelle or $H\text{-}2^b$ haplotype.

CFU-S Analysis

$9A5^+$ and $9A5^-$ or $9A5^+Mac\text{-}1^-$ and $9A5^+Mac\text{-}1^+$ bone marrow cells from BDF1 mice were isolated by fluorescence activated cell sorting, and injected intraperitonially into irradiated BDF1 hosts (1100 Rads). Animals were sacrificed eight, ten, or thirteen days later and splenic surface colonies were counted. Spleens were fixed, parafin embedded and stained with hematoxylin and eosin by the Tissue Procurement Facility at the University of Alabama at Birmingham.

In vitro Differentiation on the S17 Stromal Cell Line

S17 stromal cells (Collins et al. 1987) were grown in RPMI 1640 media supplemented with MEM amino acids, MEM nonessential amino

acids, MEM sodium pyruvate, MEM vitamin solution, L-asparagine, L-serine, L-glutamine and 2-ME (Gibco/BRL, Grand Island NY), and 10% FBS (Hyclone) in 75cm^2 tissue culture flasks (Costar, Cambridge, MA) at 37^{o}C in a 5% CO_2 chamber. Adherent cells were removed by treatment with trypsin-EDTA (Gibco/BRL), resuspended in media, irradiated (1000 Rads) and incubated in ninety-six well tissue culture plates (Costar, Cambridge, MA) at 2.0x10^4 per well 24 hrs prior to addition of isolated bone marrow cells. 9A5$^+$Mac-1$^-$ and 9A5$^+$Mac-1$^+$ bone marrow cells from BDF1 mice were isolated by fluorescence activated cell sorting, added to cultures of S17 stromal cells at 8.0x10^4 per well, and after three days incubation, were analysed for differentiation to B lineage cells by immunofluorescence surface staining with the 14.8 and BP-1 antibodies and for cytoplasmic IgM (cμ). IL-7 was added to the cultures (10 ng/ml, Pepro Tech. Inc., Rocky Hill, NJ) every three days and after nine days cells were analysed for expression of 14.8, BP-1 and cμ as before and in addition for expression of the 9A5 antigen.

Enzyme Analyses

For glycophosphatidylinositol-anchor analysis of the 9A5 antigen, SCID thymoma cells (1.0x10^6) were treated for 45 min at 37^{o}C with phosphatidylinositol-specific phospholipase C (PI-PLC; Sigma Chemical Co., St. Louis, MO) in Hanks balanced salt solution (HBSS, pH 7.4), 10 mM Hepes and 1.0% BSA. Treated and control cells (incubated in buffer only) were analysed for antigen expression by immunofluorescence.

Trypsin (Sigma Chemical Co., St. Louis, MO) treatment of SCID thymoma cells was accomplished by incubation with concentrations up to 200 μg/ml PBS (pH 7.4) for 30 min at 37^{o}C. Control and test samples were

washed with soybean trypsin inhibitor (50 μg per ml PBS), before immunofluorescence analysis for the 9A5 antigen.

Results

Production of the 9A5 Antibody

Lymph node cells from wild mice, *Mus spretus*, hyperimmunized with fetal thymocytes from inbred C3D2F1xDBA/2J mice, were fused with a myeloma variant, and the resultant hybridomas were screened for production of antibodies reactive with donor fetal thymocytes (E15-E17). Out of a panel of eighteen hybridoma clones selected by this screening strategy, the antibody product of one, 9A5, was selected for further characterization. The 9A5 antibody typed as IgMκ was found to react with thymocytes from *Musculas musculus domesticus* (Mus biochemical group 1) but not with species of mice representing Mus groups 2, 3, or 4a, or with *Mus caroli.*

TABLE 2: Expression of the 9A5 antigen on different mouse species

Species	Mus group	Strain	9A5 Expression
M. m. domesticus	1	BIK/g	+
		DBA/2	+
		Balb/c	+
		C57BL/6	+
		C3H/He	+
		AKR	+
M. m. musculus	2	MYL	-
Mus spretus	3	Spret-1	-
		SEI	-
M. macedonicus	4a	XBS	-
Mus caroli		Caroli	-

Table 2 lists the inbred mouse strains from Mus group 1 that were tested for thymic expression of the 9A5 antigen and in all cases 30%-50% of cells were positive. The thymus from strains representing other species, MYL (Mus 2), Spret-1 and SEI (Mus 3), XBS (Mus 4a) and Caroli, did not contain $9A5^+$ cells.

Tissue Distribution and Tumor Cell Expression of the 9A5 Antigen

Tissues from fetal, neonatal, and adult DBA/2J mice were analysed by immunofluorescence for binding of the 9A5 antibody. Bone marrow, lymph node, spleen, thymus, and peyers patch from adult mice (6-8 weeks), fetal liver (E12-E17) and thymus from fetal to adult ages were tested. Fetal liver on E12- E13 contained 5% of cells bearing the 9A5 antigen while during later gestional ages (E14-17), presence of $9A5^+$ cells is not detectable. The results for adult hematopoietic tissues are shown in Figure 10. Data for bone marrow (10%), lymph node (5%), and spleen (15%) represent the frequency of $9A5^+$ cells within the nylon wool non-adherent populations. This corresponds to ~15%, 7%, and 6%, respectively, for total tissue suspensions. The adult thymus contains 30-50% 9A5 bearing thymocytes, and cellular suspensions from peyers patch lacked the 9A5 antigen.

Because of the pattern of expression in adult tissues, a panel of B- and T-cell lines were tested for expression of the 9A5 antigen (Table 3). The pre-B and B-cell lines were negative as were five of the six representatives of T-lineage cells. The one $9A5^+$ thymoma cell line (isolated from a SCID mouse) displayed an immature thymocyte phenotype ($Thy\text{-}1^+CD3^-CD4^+CD8^+$), while another immature cell, Yac-1 ($Thy\text{-}1^+CD3^-CD4^-CD8^-$), was negative. The mature T cell lines tested did not express the 9A5 antigen.

Figure 10. Immunofluorescence analysis of the tissue distribution of the 9A5 antigen. Viable cells from DBA/2 mice were incubated with biotinylated 11B5 mAb or an isotype matched control mAb and cell-bound antibodies were detected by Av-PE. Analysis was by automated flow cytometry.

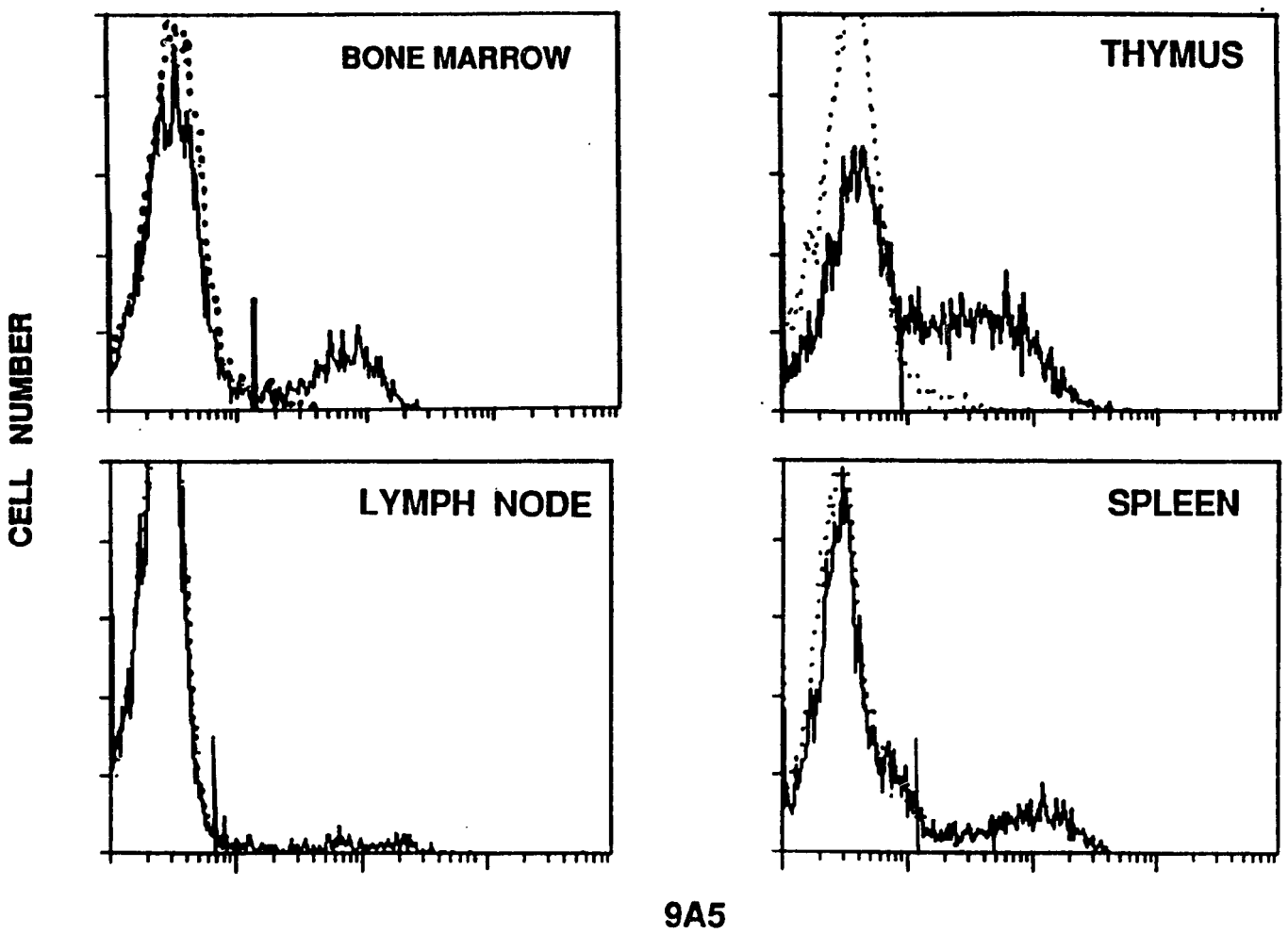

BONE MARROW
THYMUS
LYMPH NODE
SPLEEN
CELL NUMBER
9A5

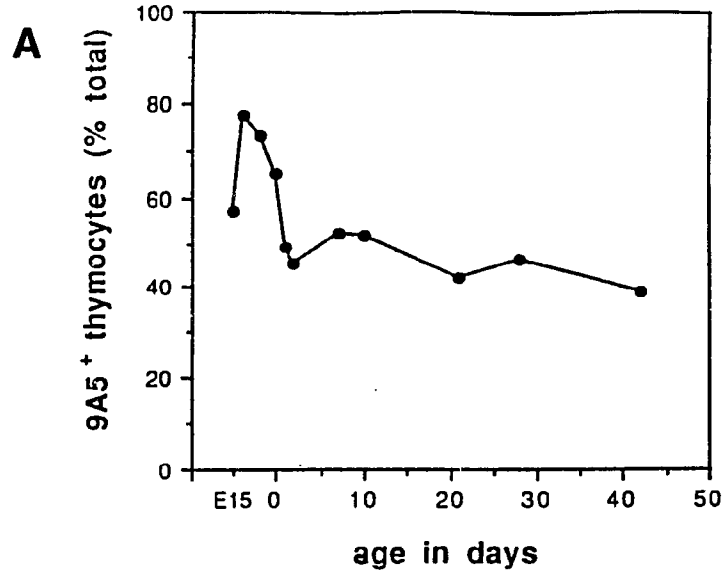
TABLE 3: Tumor cell expression of the 9A5 antigen

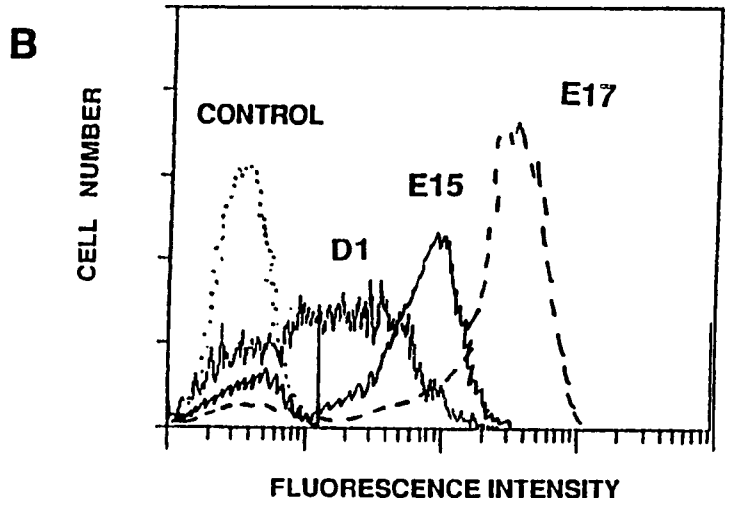
Cell line	Phenotype	9A5 Expression
38B9	Pre-B	-
18.81	Pre-B	-
L1-2	Pre-B	-
A20	Mature B	-
Yac-1	Immature T	-
SCID	Immature T	+
S49.1	Mature T	-
L6911	Mature T	-
EL-4	Mature T	-

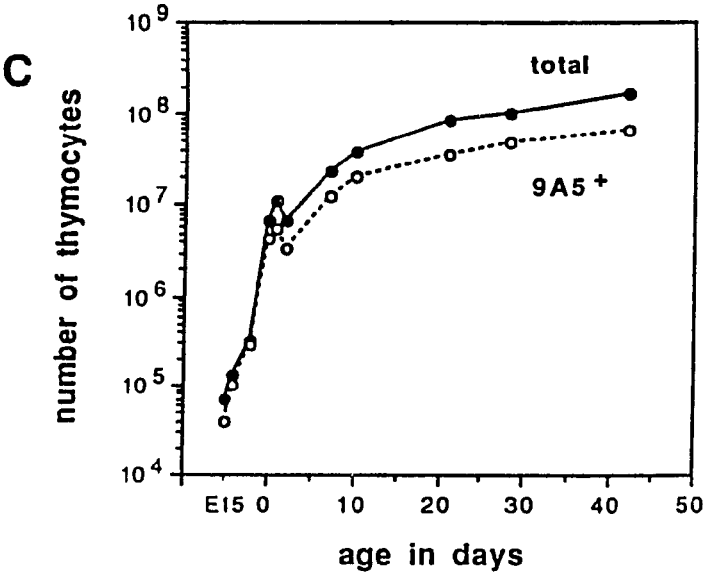
Thymic Expression of the 9A5 Antigen as a Function of Age

The immunofluorescence profiles for the expression of the 9A5 antigen in the developing thymus revealed an increase in the average frequency of $9A5^+$ thymocytes from 56% on E14 to 75% on E15 followed by a sharp decline until D5 of neonatal life when the percentage of cells increased to 50%. By D15, $9A5^+$ cells represented 30-50% of the total thymocyte population and remained at this level throughout adulthood (Fig. 11A). In addition, the levels of surface expression of the 9A5 antigen by the subpopulation of thymocytes expressing this molecule fluctuated as a function of age. Early in development (E15), when 75% of thymocytes were $9A5^+$, the peak fluorescence intensity was twenty-three times the control value. This increased to eighty-four times the background level by E17 followed by a decrease to thirteen times the background level on D1. A bright, homogeneous staining pattern was observed early in ontogeny, on E15 and E17, while broad peaks including both bright and dim populations were found at D1 and all ages thereafter (Fig. 11B). Despite the fluctuations in the frequency of thymocytes

Figure 11. Thymic expression of the 9A5 antigen as a function of age. Viable cells were incubated with biotinylated 9A5 antibody or control antibody and bound molecules were detected by Av-PE. Analysis was by automated flow cytometry. (A) Thymus from DBA/2 mice at ages E14-D42 plotted as the % of thymocytes bearing the 9A5 antigen versus the age in days. (B) Immunofluorescence analysis of 9A5-reactive thymocytes from DBA/2 mice at various ages. (C) Absolute number of thymocytes (——) compared with the total number of $9A5^+$ thymocytes (- - - -) versus age in days.


A
9A5 + thymocytes (% total)
100
80
60
40
20
0
E15 0
10
20
30
40
50
age in days


B
CELL NUMBER
CONTROL
E17
E15
D1
FLUORESCENCE INTENSITY


C
number of thymocytes
10 9
10 8
10 7
10 6
10 5
10 4
total
9A5 +
E15 0
10
20
30
40
50
age in days

expressing the 9A5 antigen and the level of surface expression, the absolute number of 9A5$^+$ thymocytes increased with age (Fig. 11C).

Phenotype of 9A5$^+$ Thymocytes in Fetal and Adult Mice

The phenotype of 9A5$^+$ thymocytes in the developing embryo revealed that on E17 >85% of thymocytes bear the 9A5 antigen and 75% of these cells expressed both CD4 and CD8 (Fig. 12). In addition, the population lacking these antigens were 9A5^{hi} cells. Essentially all of the 9A5^{lo} population of thymocytes expressed both CD4 and CD8. The large subpopulation of 9A5$^+$ thymocytes which persists in the adult thymus was analyzed for expression of the CD4, CD8, and CD3 T cell antigens (Fig. 13). Most thymocytes which bear 9A5 also expressed CD4 and CD8 (40%), while 30% expressed low levels of CD3. Very few 9A5$^+$ thymocytes are found among the mature CD3^{hi} bearing cells. These data indicate that the 9A5$^+$ cell population in the thymus includes thymocytes at various stages of maturation. Analysis of CD4$^-$CD8$^-$ and CD4$^+$CD8$^-$ thymocytes, when isolated by cell sorting, revealed that 30% of CD4$^-$CD8$^-$ cells expressed the 9A5 antigen (Fig. 14) as well as 4% of the mature CD4$^+$ cells (data not shown). The presence of the 9A5 antigen on mature cells was further analyzed during thymus repopulation in sublethally irradiated mice. Three days after irradiation the thymus contained mostly mature cells. The 9A5 antigen is present on ~12% of the radiation resistant CD4$^+$ thymocytes but was not expressed on CD8$^+$ cells (Fig. 15).

Analysis of the nylon wool non-adherent lymphoid cells in the spleen revealed that 9A5$^+$ cells are Thy-1$^+$CD3$^+$CD4$^+$ T-cells. CD8$^+$ T-cells do not express the 9A5 antigen nor do cells bearing the B220 B-cell antigen recognized by the 14.8 mAb (Fig. 16). Identical results were

Figure 12. Two-color immunofluorescence analysis of the 9A5 antigen expressed on fetal (E17) thymocytes. Viable cells were incubated with biotinylated 9A5 and with FITC-conjugated antibodies to CD4 or CD8. Cell bound 9A5 was detected by Av-PE.

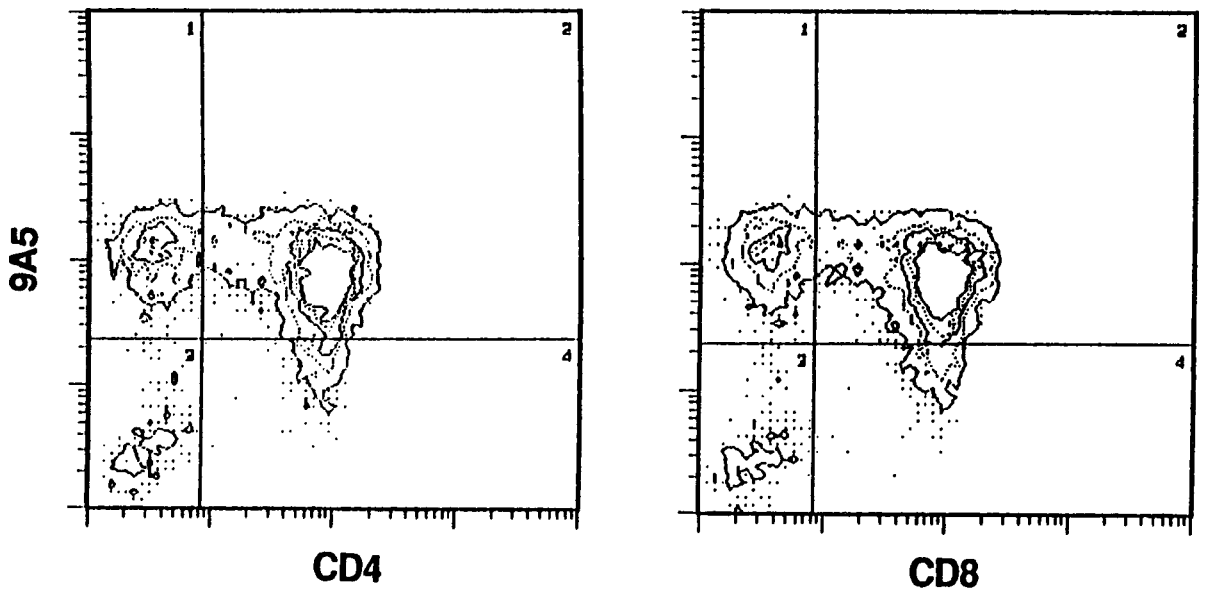

9A5
1
2
3
4
CD4
1
2
3
4
CD8

Figure 13. Two-color immunofluorescence analysis of adult 9A5 reactive thymocytes. Thymocyte suspensions were incubated with biotinylated 9A5 antibody and FITC-conjugated antibodies to CD4, CD8, or CD3. Cell bound 11B5 was detected by Av-PE.

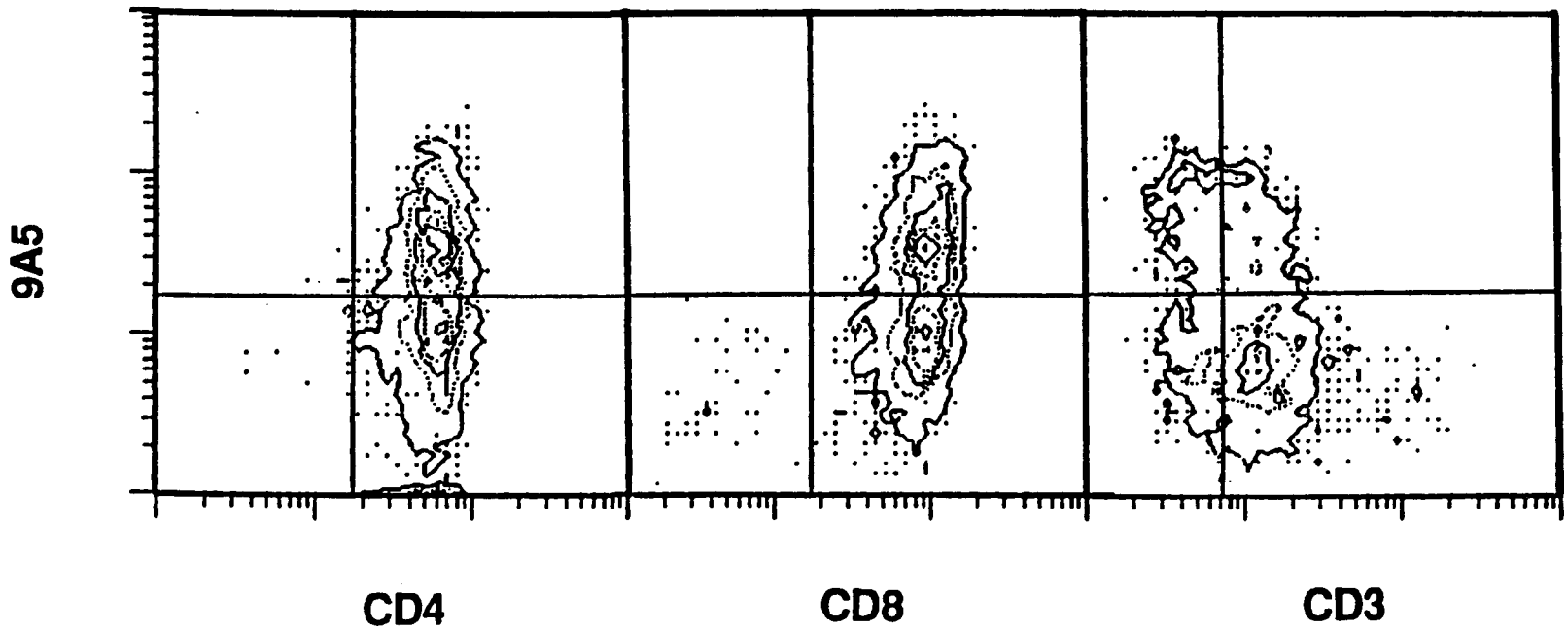

9A5
CD4
CD8
CD3

Figure 14. Expression of 9A5 on immature adult thymocytes. The $CD4^-$ $CD8^-$ thymocytes, isolated by cell sorting, were incubated with the biotin-conjugated 9A5 antibody (solid line) or isotype matched control mAb (dotted line). Cell-bound antibody was detected with Av-PE.

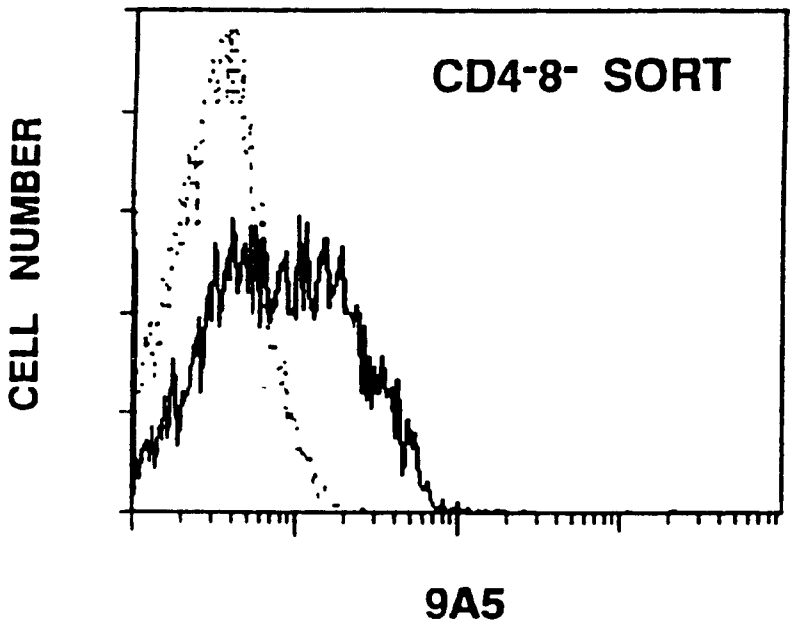
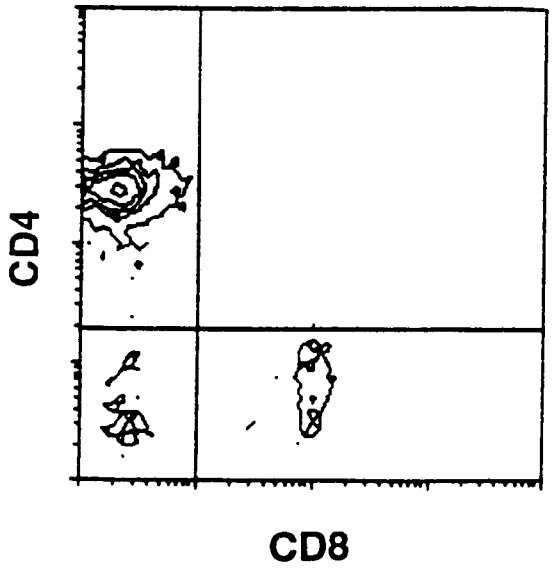
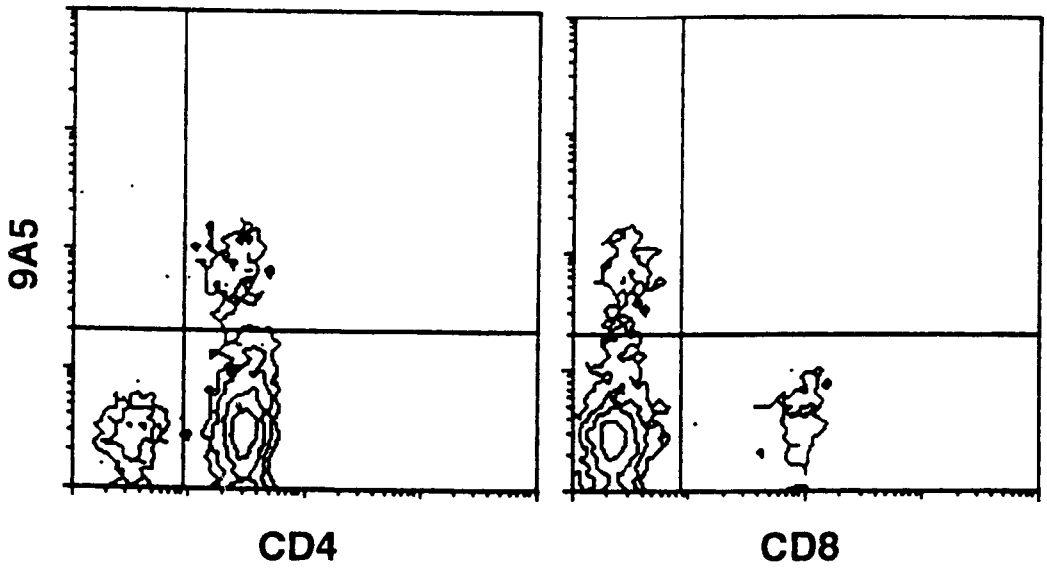

CD4-8- SORT
CELL NUMBER
9A5

Figure 15. Expression of the 9A5 antigen on mature thymocytes. The radiation resistent thymocytes present in C57Bl/6 mice three days after irradiation, analysed by two-color immunofluorescence, were incubated with biotinylated 9A5 antibody and FITC-conjugated antibodies to either CD4 or CD8. Cell-bound 9A5 was detected by Av-PE.


CD4
CD8


9A5
CD4
CD8

obtained from suspensions of lymph node cells (data not shown). The possibility that B cells removed by nylon wool were 9A5$^+$ was tested by elution of the adherent cells, followed by fluorescence analysis. The B220 and 9A5 antigens were expressed by mutually exclusive populations.

Phenotype of 9A5$^+$ Bone Marrow Cells

To determine the presence of lineage specific antigens on the 9A5$^+$ bone marrow cells, this subpopulation was analyzed by two-color immunofluorescence assays with antibodies recognizing the CD4 (T-cells), Mac-1 (myeloid cells), B220 (B-cells), and BP-2 (granulocytes) antigens (Fig. 17). 9A5$^+$ cells did not express CD4, B220, or BP-2 indicating that in bone marrow the 9A5 antigen was not present on granulocytes, B- or T-cell lineages, whereas the Mac-1 antigen is expressed by 30-50% of 9A5 bearing cells. In addition, cμ and TdT were not evident among the 9A5$^+$ cells while a small subpopulation (10%) expressed low levels of the Thy-1 antigen (data not shown).

Progenitor Activity of the 9A5$^+$ Subpopulation in Bone Marrow

For analyses of the differentiation capability of bone marrow cells bearing the 9A5 antigen, the 9A5$^+$ and 9A5$^-$ subpopulations were isolated by cell sorting. All cells used in these experiments were >98% pure. Results from a typical separation are shown in Fig. 18.

Since a large population of thymocytes expressed the 9A5 antigen, 9A5$^+$ bone marrow cells were tested for their ability to differentiate into T-lineage cells in SCID mice. The thymus from recipients injected i.v. with 0.5×10^6 9A5$^+$ cells from C3D2F1 donor mice were analyzed ten weeks after transplantation and contained high percentages of CD3$^+$, CD4$^+$ and CD8$^+$ cells compared to control animals (Fig. 19). Two color analysis with antibodies specific for CD3 and donor class I MHC ($H2^k$) revealed that

Figure 16. Two-color immunofluorescence analysis of 9A5 reactive splenocytes. Nylon wool nonadherent spleen cells were incubated with biotin-conjugated 9A5 antibody and either FITC-conjugated antibodies to Thy-1 (A), CD3(B), B220(14.8) (C), CD4 (D) or CD8 (E). Cell-bound 9A5 was detected with Av-PE.

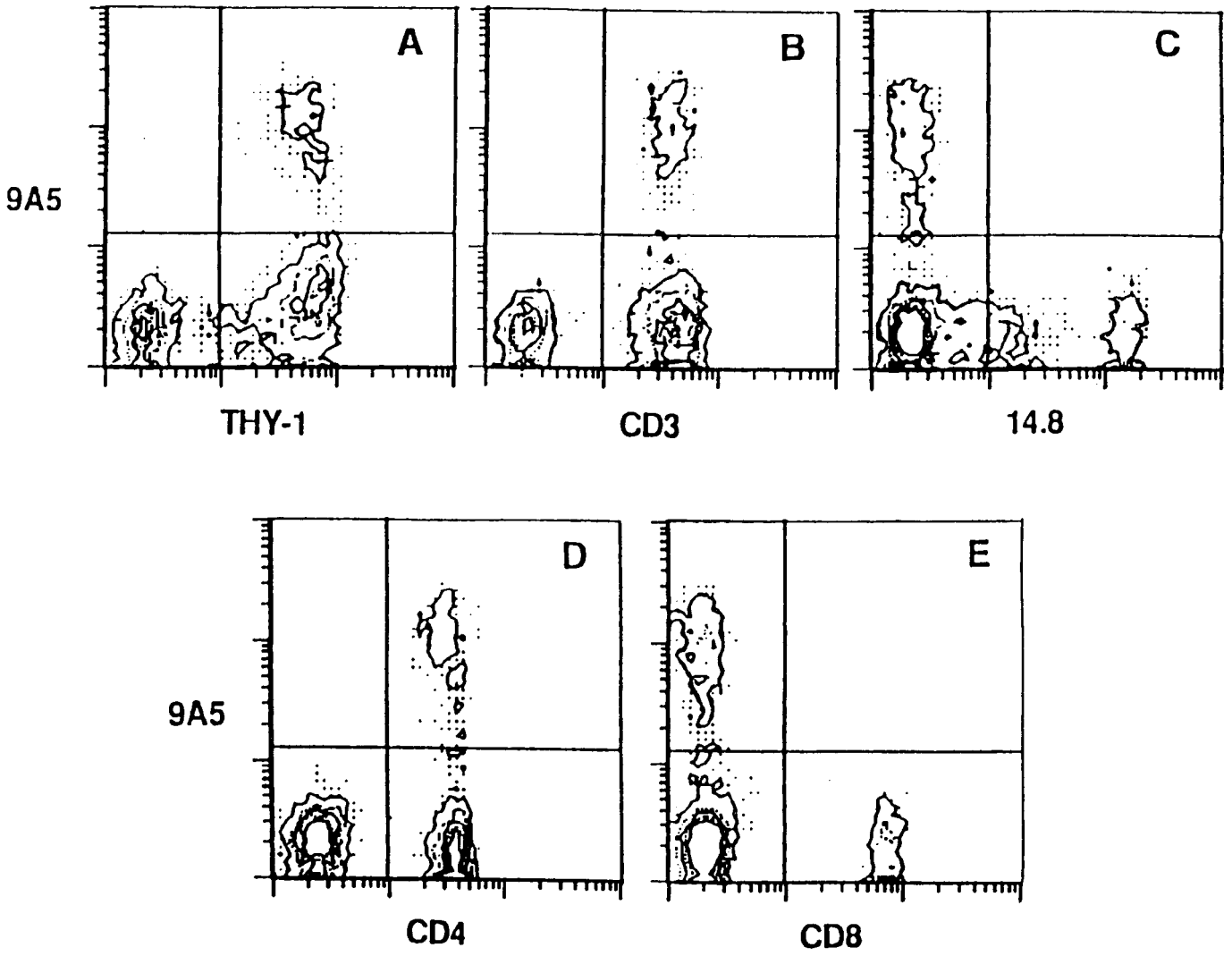

A
B
C
9A5
THY-1
CD3
14.8
D
E
9A5
CD4
CD8

Figure 17. Expression of lineage markers on 9A5$^+$ bone marrow cells. Two-color immunofluorescence analysis of nylon wool non-adherent bone marrow cells: viable cells from DBA/2 mice were incubated with biotinylated 9A5 antibody and FITC-conjugated antibodies to CD4 (A), B220(14.8) (B), BP-2 (C), or Mac-1 (D). Cell bound 9A5 was detected with Av-PE.

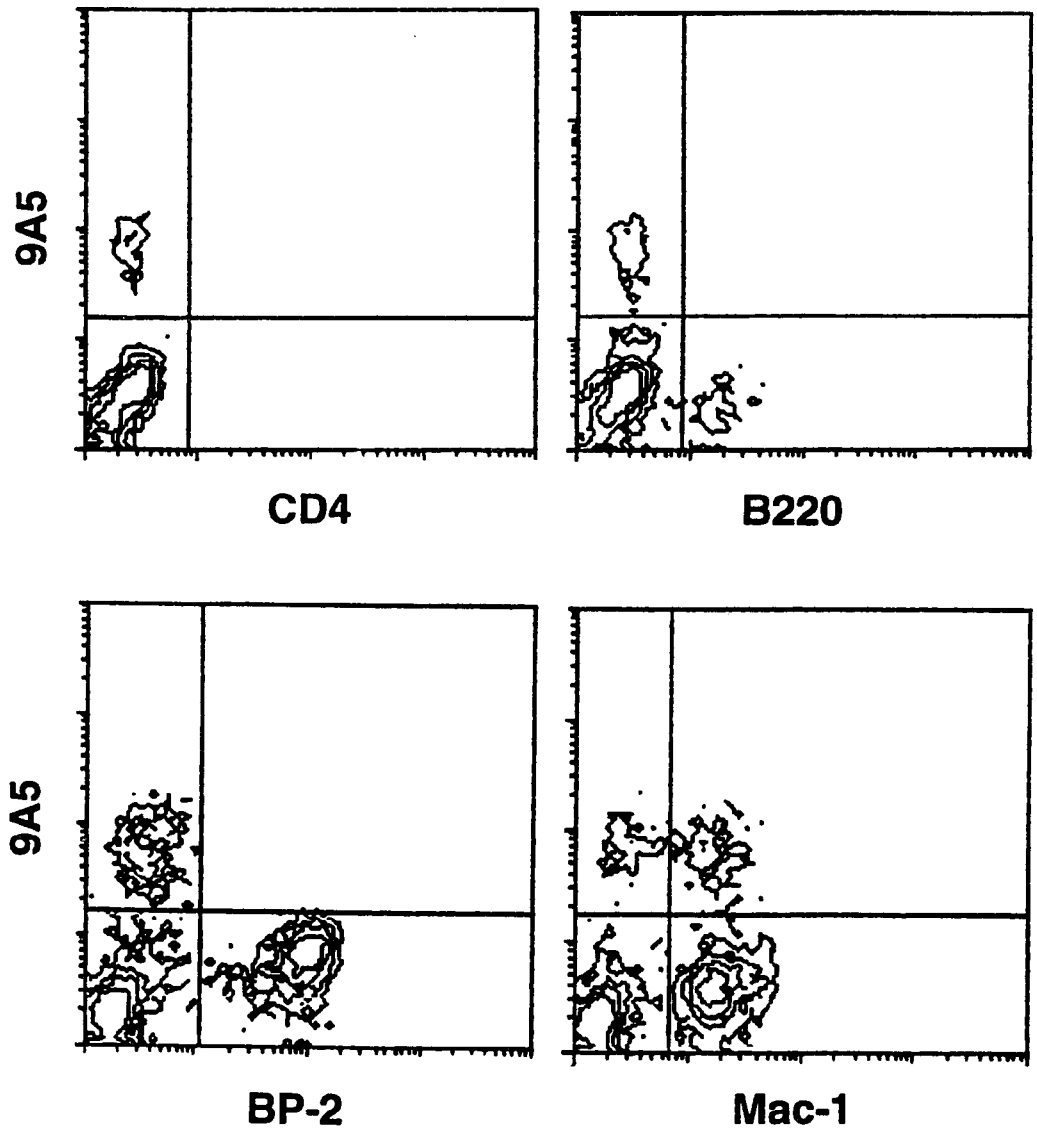
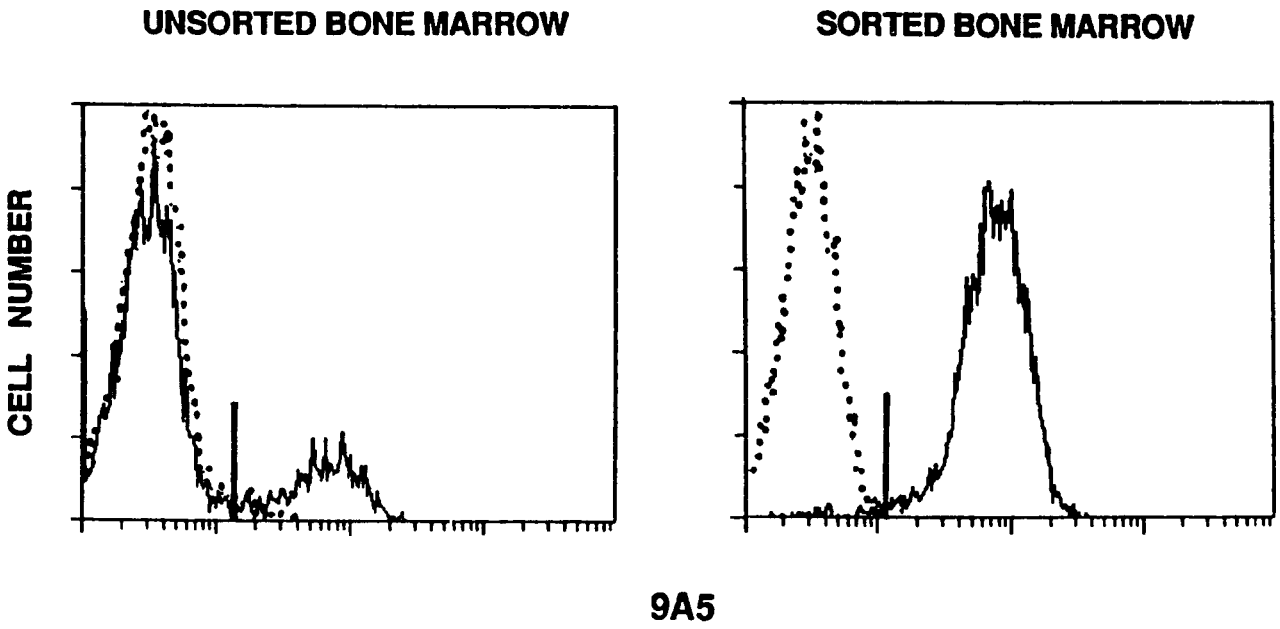

9A5
CD4
B220
9A5
BP-2
Mac-1

Figure 18. Isolation of $9A5^+$ bone marrow cells by cell sorting. The nylon wool nonadherent fraction of bone marrow was incubated with biotinylated 9A5 antibody and surface bound antibody was detected by Av-PE. $9A5^+$ and $9A5^-$ populations were separated using a FACSTAR IV instrument. Shown are the immunofluorescence profiles before and after separation. (dotted lines - control mAb; solid lines - $9A5^+$ cells).


UNSORTED BONE MARROW
SORTED BONE MARROW
CELL NUMBER
9A5

thymocytes were of donor origin since $CD3^{hi}$ expressing cells displayed the donor haplotype (Fig. 20). In addition, ~25% of lymph node cells expressed the $H2^{k+}CD3^{+}$ donor phenotype.

The low level expression of class I MHC antigens made it difficult to prove that all $CD3^{+}$ cells were of donor origin. To address this, neonatal SCID mice were injected i.p. with 1 x 10^5 $9A5^{+}$ bone marrow cells from C57BL/6-Thy-1.1 congenic mice. Eight weeks after transplantation the SCID thymus contained Thy-1.2^{+} thymocytes of SCID origin and Thy-1.1^{+} donor cells (Fig. 21). Analyses of peripheral lymphoid organs revealed that all lymphoid cells in the lymph node and ~10% in the spleen were $CD3^{+}H2^{b+}$ T-cells which expressed the Thy-1.1 antigen (data not shown).

CFU-S Analyses

After injection of bone marrow cells, the formation of colonies on the surface of the spleen in irradiated mice is an indication of progenitor cell activity within the donor population (Till and McCulloch 1961; Magli, Iscove and Odartchenko 1982). To determine if $9A5^{+}$ bone marrow cells could differentiate into myeloid and erythroid lineages, varying numbers of this subpopulation (1-2 x 10^5) were tested for generation of CFU-S eight days after transplantation. In all experiments, $9A5^{+}$ bone marrow cells generated more CFU-S (6-15) than the same number of $9A5^{-}$ (0-2) or unseparated bone marrow cells (1-2). This indicated that the $9A5^{+}$ cells were enriched in a precursor cell population. All recipients receiving $9A5^{+}$ or total bone marrow cells survived for eight days, while four of six recipients of $9A5^{-}$ cells died before testing. Hemotoxylin and eosin staining of spleen sections revealed that precursors within the $9A5^{+}$ subset can differentiate into myeloid, erythroid, and mixed colonies,

Figure 19. Reconstitution of adult SCID mice with 9A5$^+$ bone marrow cells. The thymocytes from a SCID mouse ten weeks after reconstitution with C3D2F1 donor cells were stained with biotinylated 9A5 antibody or FITC-conjugated antibodies to CD4 or CD8. Cell bound 9A5 was detected with Av-PE.

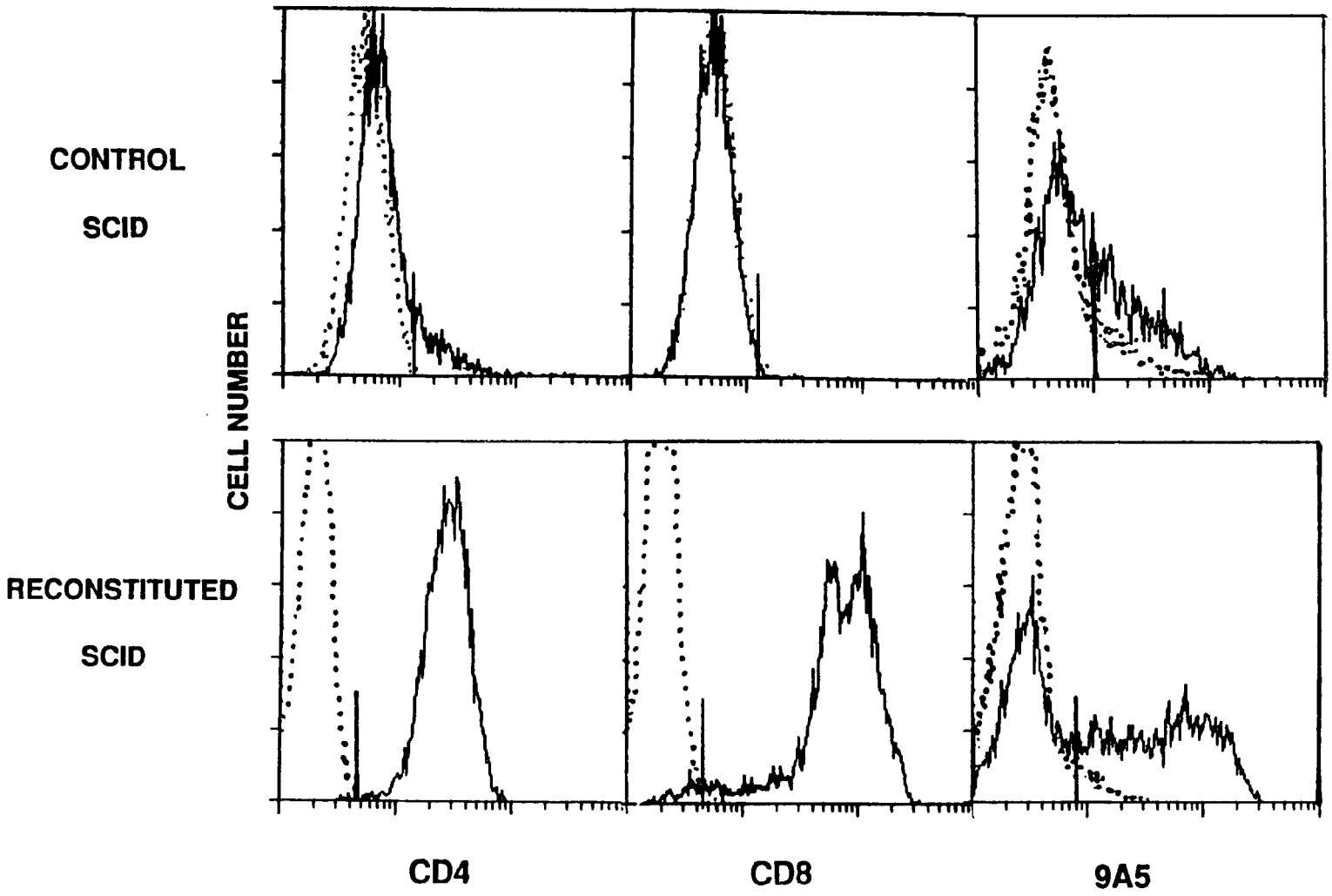

CONTROL
SCID
RECONSTITUTED
SCID
CELL NUMBER
CD4
CD8
9A5

Figure 20. Presence of $CD3^+$ cells in reconstituted SCID thymus. Thymocytes from the SCID mice from Figure 19 were incubated with FITC-conjugated mAb to CD3 (solid line) or were analysed by two-color immunofluorescence with CD3-FITC and biotin conjugated antibody to the donor MHC class I haplotype ($H\text{-}2^k$). Cell bound antibody was detected with Av-PE.

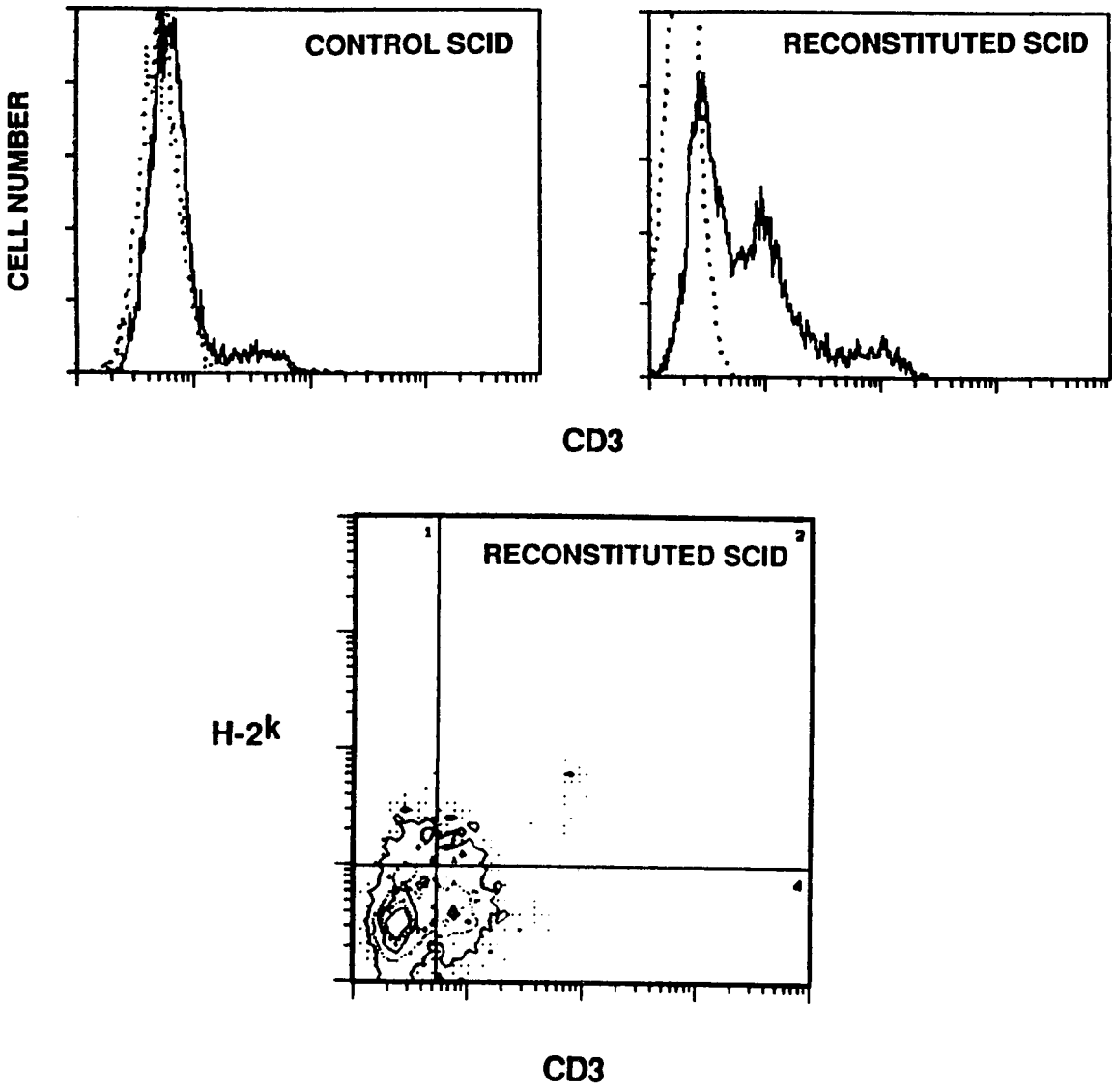
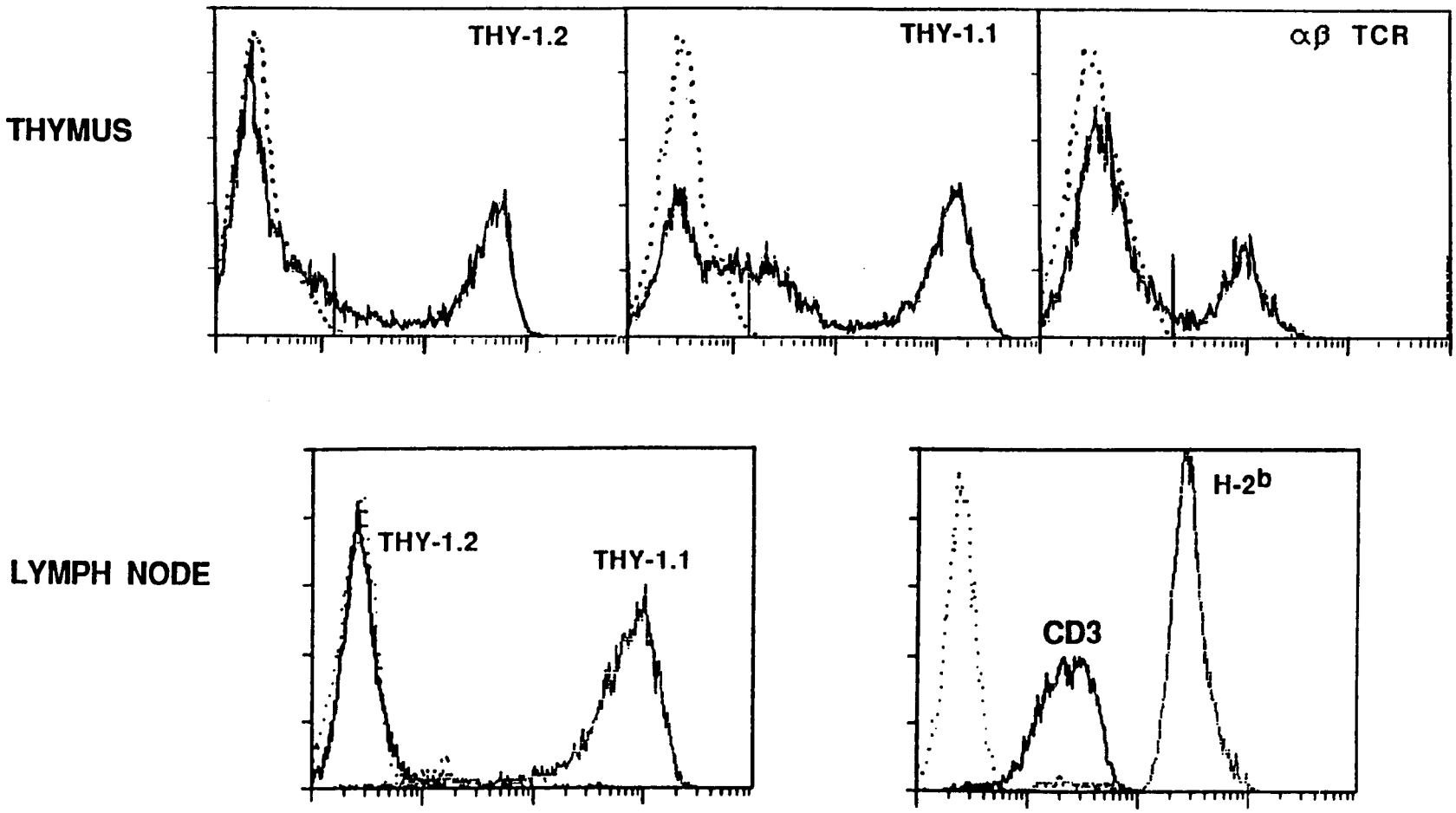

CONTROL SCID
RECONSTITUTED SCID
CELL NUMBER
CD3
RECONSTITUTED SCID
1
2
4
H-2k
CD3

Figure 21. Reconstitution of neonatal SCID mice with 9A5$^+$ bone marrow cells. Thymocytes from a SCID mouse 8 weeks after injection with cells from C57Bl/6 Thy-1.1 congenic mice were incubated with biotinylated antibody to Thy-1.1 or FITC-conjugated antibody to Thy-1.2 or anti-αβ TCR culture supernatant, detected by FITC conjugated goat anti-hamster Ig antibody. Cell-bound Thy-1.1 was detected by Av-PE. Dotted lines indicate binding of control mAb.


THYMUS
THY-1.2
THY-1.1
αβ TCR
LYMPH NODE
THY-1.2
THY-1.1
CD3
H-2b
FLUORESCENCE INTENSITY

whereas the few colonies formed from 9A5$^-$ cells were erythroid in nature. In addition, a large number of mature erythrocytes were present in spleens of mice receiving the 9A5$^-$ subpopulation (Fig. 22).

A comparison was made between isolated 9A5$^+$Mac-1$^+$ and 9A5$^+$Mac-1$^-$ bone marrow cells with respect to their ability to generate CFU-S at eight, ten, and thirteen days post transplantation into irradiated recipient mice. The ratio of D13 to D8 CFU-S generated fron $5X10^4$ injected cells was thirty-seven for the 9A5$^+$Mac-1$^-$ subpopulation, four for the 9A5$^+$Mac-1$^+$ cells, and the D13/D8 ratio for total bone marrow cells was fifteen (Table 4). The greater ratio generated by 9A5$^+$Mac-1$^-$ bone marrow cells indicated the presence of uncommitted progenitor cells within this subpopulation.

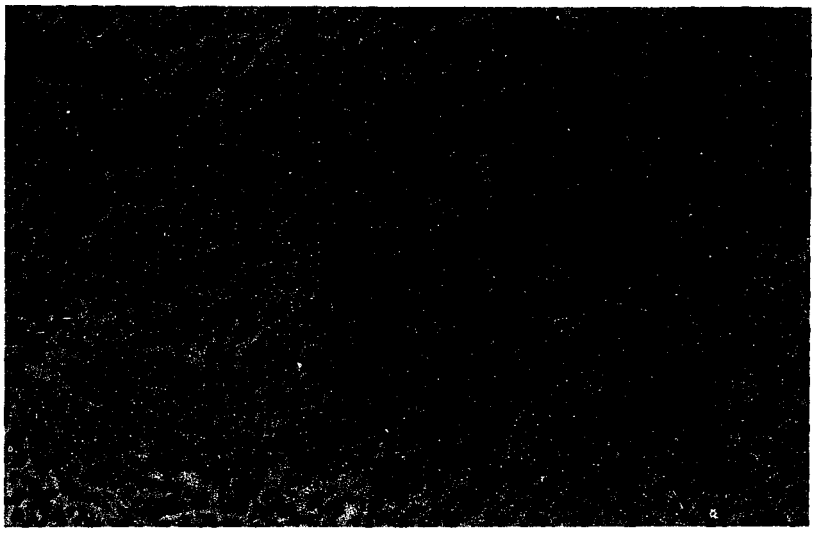
TABLE 4: CFU-S analysis of 9A5$^+$ bone marrow cells

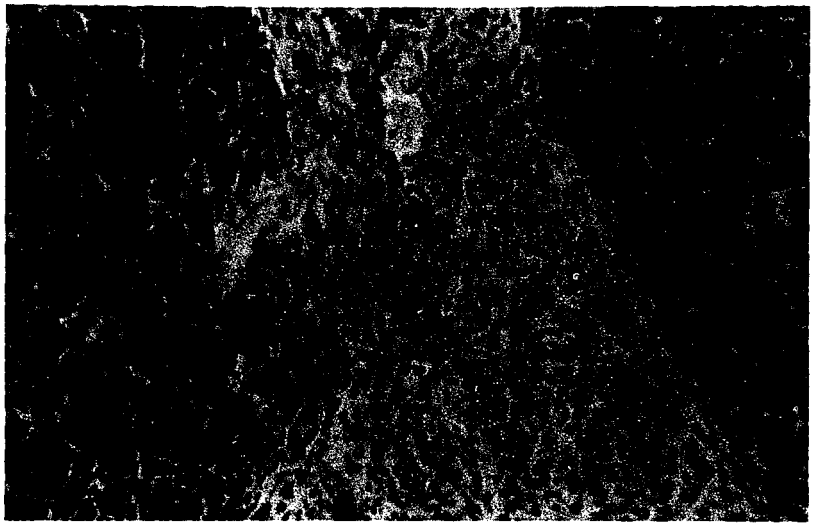
	Number of splenic foci after injection with:		
Days after transplantation	Total bone marrow cells	9A5$^+$Mac-1$^+$ bone marrow cells	9A5$^+$Mac-1$^-$ bone marrow cells
8	0	0	0
10	3	0	5
13	15	4	37

In vitro Differentiation of 9A5$^+$ Bone Marrow Cells

The *in vivo* experiments demonstrated the ability of 9A5$^+$ bone marrow cells to differentiate into myeloid, erythroid, and T-lineage but not B-lineage cells. The ability of this subset to develop into B-cells was

Figure 22. Histochemical analysis of CFU-S. The Spleen from irradiated BDF1 mice eight days after injection with unseparated bone marrow (top), $9A5^-$ (center) or $9A5^+$ bone marrow cells (bottom) were paraffin embedded, sectioned and stained with hemotoxylin and eosin (magnification 50X).





tested by culturing 9A5$^+$Mac-1$^+$ and 9A5$^+$Mac-1$^-$ subpopulations with the S17 stromal cell line (Fig. 23). While most of the Mac-1$^+$ cells died after three days in culture, the 9A5$^+$Mac-1$^-$ cells expressed the B220 B-lineage antigen and 2% expressed cμ. Following the addition of IL-7, the cell number increased three-fold, >99% were 14.8$^+$BP-1$^+$ B cells, and ~70% were cμ$^+$. In addition, after B220 expression occurred, the 9A5 antigen was no longer present on the cell surface. These data demonstrated that B-cell precursors reside within the 9A5$^+$Mac-1$^-$ subpopulation of bone marrow.

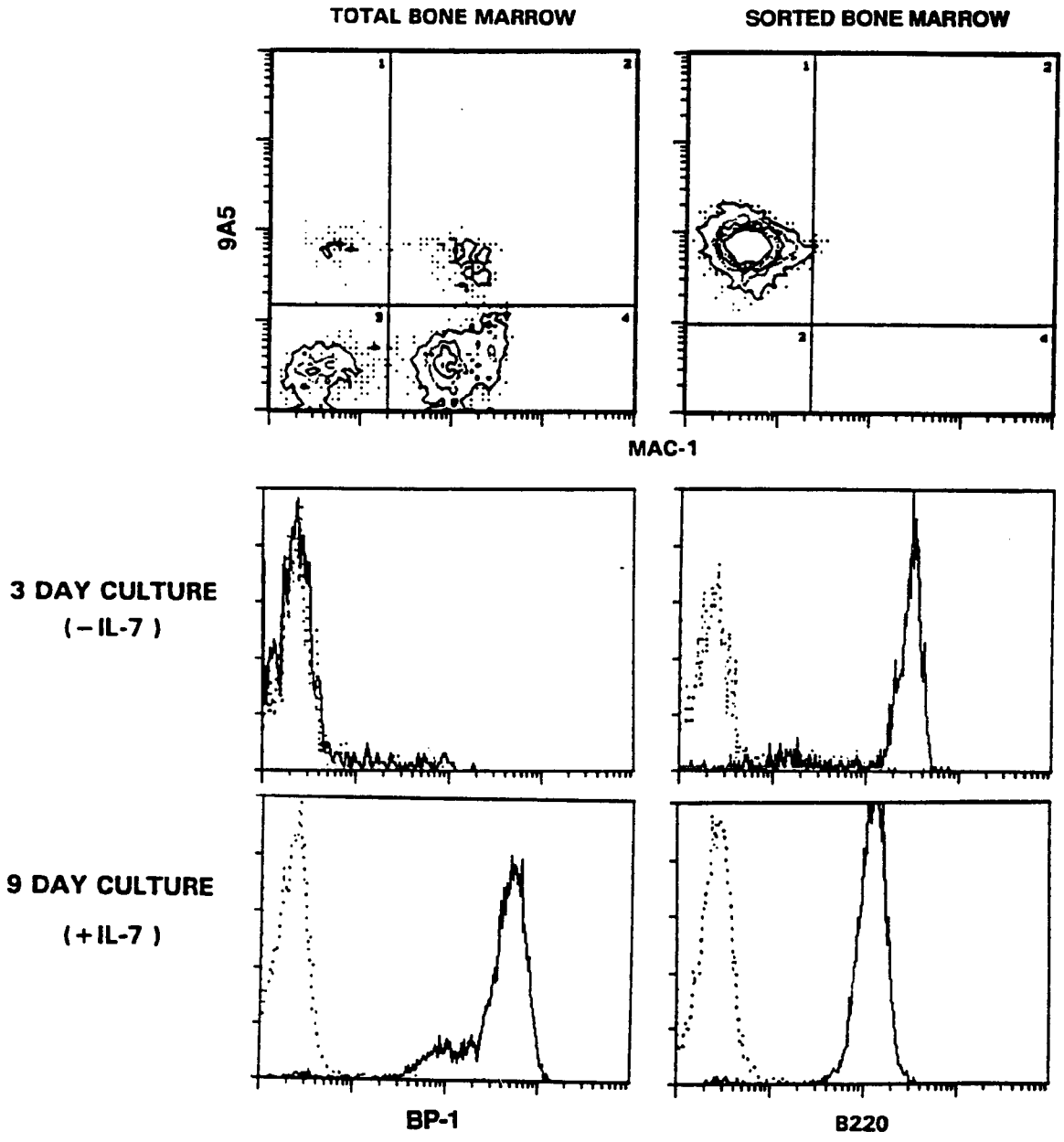
Biochemical Analysis

The molecular weight of the 9A5 antigen could not be determined due to the inability of the 9A5 antibody to precipitate a molecule in immunoprecipitation experiments. In order to determine the protein nature of the antigen, SCID thymoma cells were subjected to treatment with the proteases, trypsin and pronase. Immunofluorescence analysis of trypsin treated cells revealed that a concentration of 25 μg/ml removed >99% of the 9A5 antigen from the cell surface (data not shown). Similar results were observed after pronase treatment. The antigen was not effected by treatment with PI-PLC indicating that it is a transmembrane molecule.

Discussion

The 9A5 monoclonal antibody recognizes a transmembrane protein found on the surface of a subpopulation of thymocytes from all strains of laboratory mice tested as well as CD4$^+$ T-cells. In addition, the 9A5 antigen is expressed on a minor subpopulation of cells in bone marrow and fetal liver. The differentiation capacity of the fetal liver cells bearing the 9A5 antigen is not known, although 9A5$^+$ bone marrow cells have the capacity to differentiate into the myeloid, erythroid, T- and B-cell lineages.

Figure 23. Differentiation of $9A5^+$ bone marrow cells into B-cells. $9A5^+Mac\text{-}1^-$ bone marrow cells isolated by cell sorting were cultured on a layer of S17 stromal cells. After three days in culture IL-7 was added. Cells from three day (without IL-7) and nine day cultures (plus IL-7) were incubated with biotin conjugates of BP-1 or 14.8. Cell bound antibody was detected with Av-PE. Also shown are the two-color immunofluorescence analysis of cells incubated with 9A5 and anti-Mac-1 as in Figure 8, and the contour plot analysis of the isolated cells.


TOTAL BONE MARROW
SORTED BONE MARROW
9A5
MAC-1
3 DAY CULTURE
(−IL-7)
9 DAY CULTURE
(+IL-7)
BP-1
B220

This bone marrow subpopulation is negative for expression of most lineage markers except Mac-1 which is present on ~50% of the 9A5$^+$ subset. In addition, ~10% of 9A5$^+$ bone marrow cells express low levels of the Thy-1 antigen.

The 9A5 antigen is expressed early in thymocyte development and is retained by most of the developing cells through the CD4$^+$CD8$^+$ stage, and by a subpopulation of mature CD4$^+$ cells in thymus, spleen, and lymph nodes, but not in peyers patch. The function of the antigen on mature T-cells is unknown. It appears unlikely that this molecule is a homing receptor such as MEL-14 (Gallatin et al. 1983) since it is restricted to a subpopulation of CD4$^+$ T-cells and is not present on CD8$^+$ T-cells or B-cells. It is possible that 9A5 antigen expression is restricted to memory or virgin T-cells similar to CD45 expression (Lee et al. 1989). Alternatively, this antigen may designate TH1 or TH2 helper T-cells which differ in their patterns of cytokine production (Mosmann and Coffman 1987).

Approximately 55% of cells in the E14 thymus express the 9A5 antigen. The frequency of positive cells increases until E17 followed by a decrease until after birth. The level of 9A5 antigen expression on thymocytes is homogeneous during early ontogeny (E14-17) and then changes to a broad heterogeneous expression including 9A5^{hi} and 9A5^{lo} populations after birth. The presence of 9A5^{hi} cells expressing both CD4 and CD8 on E17 thymocytes suggests that 9A5 expression is lost as the CD4$^+$CD8$^+$ cells mature.

The subpopulation of bone marrow cells which bear the 9A5 antigen is very interesting in lieu of the recent reports on the phenotype of hemopoietic stem cells (Spangrude, Heimfeld and Weissman 1988;

Heimfeld and Weissman 1992; Muller-Sieburg, Martina and Wineman 1992). Several criteria must be met in order to identify a stem cell population: the ability to rescue a recipient from lethal irradiation, the capacity to differentiate into all blood cell lineages, and the ability to self-renew and continue to repopulate a host animal in long term reconstitution studies. The $9A5^+$ bone marrow cells satisfy the first criteria, since animals injected with this population or unseparated bone marrow survived whereas 66% of recipients given $9A5^-$ cells died. The ability to differentiate into myeloid and erythroid lineage cells was demonstrated by CFU-S analyses. The six fold increase in the number of D8 CFU-S generated by 9A5 bearing bone marrow cells compared to unseparated bone marrow indicated an enrichment of myeloid and erythroid progenitor cells within the $9A5^+$ subset. In subsequent experiments when $9A5^+$Mac-1^+ and $9A5^+$Mac-1^+ cells were tested, the D13/D8 ratios were 2.5 fold greater in the $9A5^+$Mac-1^- subset as compared to bone marrow injected control animals. It has been reported that colonies which appear at D8-10 in CFU-S analyses are derived from committed precursors and D12-14 colonies are generated from uncommitted progenitor cells (Magli, Iscove and Odartchenko 1982). Based on this observation, our data indicate that the $9A5^+$ subpopulation contains both immature and committed progenitor cell populations and most CFU-S activity resides within the $9A5^+$Mac-1^- subset.

The ability to differentiate into T-lineage cells was demonstrated by reconstitution of the thymus in adult and neonatal SCID mice injected with the $9A5^+$ cells isolated from bone marrow. Differentiation into cells of other lineages could not be demonstrated in these mice. The reasons for this are unknown, but we can speculate that a subpopulation of $9A5^+$

cells is either multipotent or already committed to the T-lineage in the donor and migrates to the host thymus. It is unlikely that a bone marrow environment in the host is necessary for T cell maturation to continue, yet cells of other lineages, especially B cells, require this environment before development along this lineage can occur. It is possible that the large 9A5 antibody bound to the injected cells interferes with seeding of precursor cells to the proper nitch, thus preventing differentiation along the B-lineage pathway. The potential of $9A5^+$ bone marrow cells to differentiate into B-cells *in vitro* was demonstrated by culturing $9A5^+Mac\text{-}1^-$ cells with an adherent layer of S17 stromal cells thereby inducing this subpopulation of bone marrow cells to differentiate into $9A5^-B220^+c\mu^+$ B-cells. In addition, as these $9A5^+Mac\text{-}1^-B220^-c\mu^-$ cells enter the B-cell pathway the 9A5 antigen is no longer expressed on the cell surface. This is consistant with the lack of 9A5 expression by B-cells in spleen and lymph nodes.

It is surprising that all thymocytes on E14 do not express the 9A5 antigen since adult $9A5^+$ bone marrow cells develop into T-cells, and the $9A5^+$ cells in fetal liver may function similarily. It is possible that once the $9A5^+$ progenitor cells reach the thymic microenvironment, the antigen is initially down-regulated and the cells enter a resting state. When induced to differentiate by thymic growth and differentiation factors, these immature progenitor cells may up-regulate the expression of surface 9A5 before continuing along the T-lineage pathway.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

Whenever an individual begins a fishing expedition, which in science is the equivalent to looking for the interesting and the unknown, the possibility of catching a big fish is extremely rare. In reality, the greatest possibility is that one's labor will produce nothing. This research project began with the goal to produce a monoclonal antibody specific for the mouse CD3 complex of proteins, which was not available at the time. While this goal was not achieved, I have no regrets because the 11B5 and 9A5 antibodies recognize antigens which are most certainly big fish.

The immunization strategy used to produce these two mAb was utilized in order to identify differentiation antigens expressed during thymocyte development. The 11B5 and 9A5 mAb were chosen from a panel of eighteen hybridoma clones on the basis of reactivity with fetal thymocytes but not on cells in adult lymphoid organs other than the thymus as is the case for the 11B5 mAb or due to a reactivity with thymocytes as well as a subpopulation of bone marrow cells, characteristics of the 9A5 mAb. Analysis of 11B5 antigen expression on thymocytes from the wild mouse strain used in this study (*Mus spretus*: Mus group 3) revealed that the 11B5 antigen is present on a subpopulation of adult thymocytes at a frequency comparable to that found in laboratory strains of mice (Mus group 1). The isolation of such an autoantibody was not expected. The limited expression of the 11B5 antigen, on thymocytes and on epithelial

cells lining the gut lumen, isolates this antigen from the antibody-producing B-cell compartment, and introducing fetal thymocytes into these areas during immunization may have been the reason for the production of an autoreactive antibody.

The most interesting characteristics of the 11B5 antigen were its pattern of expression during fetal thymocyte development and its continued expression on a subpopulation of immature thymocytes in adult animals. The $CD3^-CD4^-CD8^-$ population of immature adult thymocytes is a heterogeneous population of cells containing at least eleven discrete subsets (Wilson et al. 1989). A hierarchy of progenitor to precursor populations within these eleven groups has not been defined, although a maturation pathway has been described in which IL-2Rα^-Pgp-1^+ cells gain surface IL-2Rα and lose Pgp-1 (IL-2Rα^+ Pgp-1^-) expression and then downregulate the expression of IL-2Rα (IL-2Rα^-Pgp-1^-) (Pearse et al. 1989). Based on this pathway, the 11B5 antigen appears to be expressed by a subpopulation of immature thymocytes which have not been previously described, namely IL-2Rα^+Pgp-1^+ cells.

The data reported in this dissertation suggests that as thymocyte development progresses, 11B5 antigen expression decreases with respect to both frequency and intensity. Based on the data presented here, it appears that stem cells which seed the thymus lack the 11B5 antigen. Thymocytes then subsequently acquire it at a high level, but then lose 11B5 antigen expression as they develop into $CD4^+CD8^+$ cells. However, the possibility that some thymocytes may lose 11B5 and return to the $CD3^-CD4^-CD8^-$ compartment cannot be eliminated. Initial studies to test this hypothesis would be to culture $11B5^+$ and $11B5^-$ thymocytes in thymic organ cultures or by intrathymic injection of isolated

subpopulations and follow the changes in expression of this antigen as cells acquire CD4 and CD8.

Another interesting observation was the small percentage of $CD3^{+}11B5^{+}$ thymocytes present within the $CD4^{-}CD8^{-}$ subset and perhaps representing γδTCR bearing cells. Retention of 11B5 antigen expression may influence the function of γδ T-cells perhaps determining their organ-specific migration patterns during early development. The obvious first step that needs to be taken is to determine if the $CD3^{+}11B5^{+}$ thymocytes are actually $\gamma\delta TCR^{+}$ and if γδTCR bearing T-cells, in skin and gut for example, bear the 11B5 antigen. If the speculation is correct, *in vitro* homing assays would be necessary to test the ability of the 11B5 antibody to block adherence of $\gamma\delta TCR^{+}$ T-cells to gut epithelial cells.

Since there were no detectable $11B5^{+}$ cells in bone marrow, the antigen may not be expressed until after thymocyte precursor cells enter the thymic microenvironment. While this may be true in the adult, the presence of a subpopulation of $11B5^{hi}$ cells in E11-13 yolk sac but not in fetal liver suggests a different pathway during fetal ontogeny. The capacity of this subpopulation to differentiate into T-lineage cells could be tested in fetal thymic organ culture or by in utero injections of $11B5^{+}$ yolk sac cells from donor mice. Regardless of the origins of pro-thymocytes, the population of cells in the thymus which express the 11B5 antigen is unique and availability of an antibody specific for this antigen will be useful in further dissecting the maturation sequence of immature thymocytes.

The antigen recognized by the 9A5 antibody is interesting in two aspects: its presence on a small subpopulation of bone marrow cells with the capacity to develop into myeloid, erythroid, B- and T-lineage cells, and

its continued expression during T-cell development. Several functional possibilities for T-lineage cells bearing the 9A5 antigen were presented in Chapter 3 and will not be discussed further here. The bone marrow population is very intriguing when compared with the recent reports on the phenotype of hemopoietic stem cells. Pluripotent hemopoietic stem cells are defined by their capacity for extensive self-renewal, their ability to rescue animals from lethal irradiation therefore allowing long-term survival, and their potential to differentiate into all blood cell lineages. The stem cell population in adult bone marrow gives rise to more restricted types of precursor cells which in turn generate lineage committed progenitors through a series of differentiation steps (Till and McCulloch 1961; Wu et al. 1968; Abramson, Miller and Phillips 1977; Till and McCulloch 1980; McCulloch 1983). In recent years, the emphasis of much experimental research has been the isolation and characterization of the hemopoietic stem cell population (Muller-Sieburg, Whitlock and Weissman 1986; Spangrude, Heimfeld and Weissman 1988; Heimfeld and Weissman 1992), although less attention has been focused on the progeny derived from activation of the hemopoietic process.

The $9A5^+$ subpopulation in bone marrow, although representing only 10% of total cells, is too large to represent a multipotent stem cell population. The cells expressing this antigen do have hemopoietic activity, since differentiation into multi-lineages was demonstrated, but this could be the result of a heterogeneous pool of committed precursors. The deciding factor for a population of cells to qualify as stem cells is the capacity for long-term survival of irradiated hosts. This may be a characteristic of $9A5^+$ bone marrow cells, although it was not demonstrated in this study. Further separation of this population is

required to fully test its potential, an obvious choice being to isolate the Thy-1^{lo}9A5^{+} cells and test their differentiative capacities.

Bone marrow cells which express the 9A5 antigen do not contain the population described by Spangrude et al. (1988) since they do not bear cell surface Sca-1. While The Thy-1^{lo}Sca-1^{+} cells are very interesting in studies to determine the undefined pathway of stem cells into morphologically recognizable lineage-restricted precursor cells, the absence of this marker on stem cells of white laboratory mice (I. Weissman, personal communication) stresses the need for a marker that is more universally expressed on most laboratory strains of mice. The 9A5 antigen satisfies this criteria. However, considering the population as a whole, I believe that the 9A5^{+} bone marrow cells represent the immediate progeny of activated stem cells, a population thought to be the most susceptable to the activity of stem cell factor (Zsebo et al. 1990). Further analysis of cell surface expression of c-kit on the 9A5 bearing cells in bone marrow is necessary before this hypothesis can be tested.

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APPENDIX A

PRELIMINARY CHARACTERIZATION OF WILD MOUSE DERIVED ANTIBODIES

A panel of eighteen monoclonal antibodies (mAb) were isolated from the hybridomas produced by fusing lymph node cells from wild mice, immunized with fetal thymocytes, with the Ag.863 myeloma fusion partner. All of these antibodies were reactive with >50% of E15-17 fetal thymocytes. In addition to the 11B5 and 9A5 mAb discussed in this dissertation, five additional members from this panel were characterized as to their biochemical properties, tissue distributions and cell lineage expression of the antigens recognized by these antibodies.

Table 5 lists the clone numbers for the five mAb, their isotypes and the apparent molecular weights of their antigens as determined by SDS-PAGE analysis under reducing and non-reducing conditions. In addition removal of oligosaccharide moieties by N-glycanase revealed a core size of 100,000 Kd for the 8C1 antigen. However, no reduction in molecular weight was observed after removal of sialic acid residues with neuraminidase or after treatment with neuraminidase plus O-glycanase indicating the presence of N- but not O-linked carbohydrates on the 8C1 molecule.

Immunofluorescence analysis of the frequency of these antigens on lymphoid tissues from adult DBA/2J mice (6-8 weeks of age)(Table 6) revealed the presence of four out of the five antigens on the surface of

cells from all the tissues tested. The one exception, 8C1, was expressed by 44% of adult thymocytes but not by cells in bone marrow, spleen, or lymph node. In addition, the 8C1 antigen was not expressed by cells in the peritoneal cavity or peyers patch but was expressed by approximately 20% of cells in E12 fetal liver and 7% of liver cells on E14. Expression of the antigen by thymocytes as a function of age revealed >80% of cells in E13-18 thymus express the 8C1 antigen. This frequency remained constant throughout the first three weeks after birth and then decreased to approximately 50% of thymocytes after six weeks of age.

Two-color immunofluorescence analysis of the 8C1 antigen versus the CD3, CD4 and CD8 antigens in adult thymus is shown in table 7. Suspensions of thymocytes from three week old DBA/2J mice were incubated with FITC- conjugated antibodies to CD4 or CD8 or anti-CD3 from hybridoma supernatants, followed by incubation with biotinylated 8C1 mAb. Bound anti-CD3 was detected by FITC-conjugated goat anti-hamster Ig, and 8C1-biotin by Av-PE. Approximately 60% of thymocytes that expressed the 8C1 antigen also expressed surface CD4 and CD8 indicating that $8C1^+$ cells are within the $CD4^+CD8^+$ thymocyte subpopulation. In addition, approximately 50% of $CD3^{lo}$ bearing cells (30% of the total) also expressed the 8C1 antigen. These data and the lack of $8C1^+$ T-cells in lymph node and spleen suggest that the 8C1 antigen is found on thymocytes within the $CD4^+CD8^+$ subset and is lost as cells differentiate into $CD4^+$ or $CD8^+$ mature thymocytes.

The phenotype of adult splenocytes that express the 18B6, 17B6 and 19D5 antigens is shown in table 8. Approximately 30% of the $Thy\text{-}1^+$ T-cells bear the 18B6 antigen (10%) and these are found within the $CD4^+$ subset, whereas the antigen is not detectable on $CD8^+$ T-cells.

Therefore, the antigen, although expressed on the majority of adult thymocytes, is retained by a fraction of CD4+ T-cells in peripheral lymphoid organs. In addition 30% of B-cells, detected by expression of surface IgM (sμ), bear the 18B6 antigen. In contrast, the 17B6 antigen with a high frequency of expression in all the adult tissues tested was expressed by all B- and T-lineage cells in the adult spleen. Both of these antigens are found on the BP-2+ (granulocyte) cells in bone marrow. The 19D5 antigen was expressed on 7% of the Thy-1+ splenocytes. Other cell lineages that bear this antigen have not yet been tested.

TABLE 5: Characterization of wild mouse antibodies

mAb		Apparent molecular weight	
Clone	Isotype	Reduced	nonreduced
8C1	IgG_{2a} κ	116	116
13C6	IgG_1 κ	65, 92	145
18B6	IgG_{2a} κ	?	?
17B6	IgG_{2b} κ	130	200
19D5	IgG_1 κ	?	?

TABLE 6: Reactivity of antibodies with adult lymphoid tissues

	Frequency of expression in % positive cells			
Clone	Bone marrow	Thymus	Spleen	Lymph node
8C1	<1	44	<1	<1
17B6	56	98	88	93
18B6	82	94	36	17
13C6	43	14	19	14
19D5	86	46	72	43

TABLE 7: Phenotype of thymocytes that express the 8C1 antigen

Phenotype	% of thymocytes
$8C1^+$	74
$CD4^+$	96
$8C1^+CD4^+$	63
$CD8^+$	89
$8C1^+CD8^+$	61
$CD3^+$	60
$8C1^+CD3^+$	30

TABLE 8: Expression of the 18B6, 17B6, and 19D5 antigens on splenocytes

Phenotype	% of splenocytes
$18B6^+$	36
$Thy\text{-}1^+$	32
$18B6^+Thy\text{-}1^+$	10
$CD4^+$	16
$18B6^+CD4^+$	6
$CD8^+$	10
$18B6^+CD8^+$	<1
$s\mu^+$	52
$18B6^+s\mu^+$	30
$17B6^+$	88
$Thy\text{-}1^+$	25
$17B6^+Thy\text{-}1^+$	25
$s\mu^+$	52
$17B6^+s\mu^+$	52
$19D5^+$	73
$Thy\text{-}1^+$	27
$19D5^+Thy\text{-}1^+$	7

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