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Characterization and analysis of a hematopoietic progenitor population detected using AB8.

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CHARACTERIZATION AND ANALYSIS OF A HEMATOPOIETIC PROGENITOR POPULATION DETECTED AB8

by

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A DISSERTATION

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The sequence of development of B lymphocytes has been described in the differentiation of pro-B cells to mature B cells. However, many steps in the process of hematopoietic cell differentiation occurring at the transition from a stem cell to a committed progenitor cell are poorly understood. We asked the following questions: Are there committed B cell progenitors that are not B220 positive as defined in contemporary schemes of mouse B cell differentiation, and are there alternate pathways in early B cell development apart from the ordered progression currently accepted? In an attempt to identify these populations, monoclonal antibodies were generated against cultured pre-B cells. One such antibody, AB8, appears to define a population of lymphoid cells intermediate in phenotypic and functional characteristics between the stem cell population and the earliest currently defined committed lymphoid cells.

In adult bone marrow, AB8+ cells are divided into two populations, one of which costains with Ter119. The AB8+/Ter119neg cells do not include hematopoietic stem cells, and they do not yet express characteristic lineage commitment markers. When this AB8+/Ter119neg population is sort purified and analyzed in in vitro stromal assays, this

population progresses into a B lineage pathway in a stepwise manner. Additionally, a similar population was detected in fetal liver with AB8. As in bone marrow, AB8+ cells in fetal liver do not have stem cell activity, but they do have the potential to give rise to myeloid and lymphoid colonies. As found in bone marrow, AB8+ cells from fetal liver progress through defined stages of B lineage differentiation.

These studies have (i) identified a monoclonal antibody and corresponding molecule that is associated with an early, probably noncommitted progenitor population in adult bone marrow and fetal hematopoietic sites, (ii) demonstrated that these populations do not have stem cell potential but can generate myeloid and lymphoid type colonies in limiting dilution assays, (iii) demonstrated that these populations can generate B lineage cells, and (iv) illustrated the potential use of this antibody to isolate a transitional progenitor to address questions of lineage commitment and differentiation.

DEDICATION

For my brother, Martin

ACKNOWLEDGMENTS

The work presented in this dissertation represents the successful portion of the work undertaken during my graduate school career. I would like to acknowledge my mentor, John Kearney, for supporting this work and all the other efforts during my time in his laboratory. In this laboratory, the freedom for independent ideas and study was encouraged. His support for participation in scientific courses and meetings was instrumental in permitting a graduate student to learn by observation not only what is expected and accepted presentation practices but also how scientific colleagues interact at large.

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DISCUSSION

LIST OF ABBREVIATIONS

CFU-s	Colony forming unit-spleen
scid	Severe combined immune deficient
DNA	Deoxyribonucleic acid
Rh123	Rhodamine 123
Lin-	Lineage marker negative
dpc	Days post coitus
mRNA	Messenger ribronucleic acid
PCR	Polymerase chain reaction
AGM	Aorta, gonads, mesenephros region
IL	Interluekin
ES	Embryonic stem
NK	Natural killer
Ig	Immunoglobulin
Ca^{2+}	Calcium
SCF	Stem cell factor
RT-PCR	Reverse transcriptase-polymerase chain reaction
FL	Ligand for flk2/flt3
PBS	Phosphate buffered saline
TGF-β	Transforming growth factor beta
neg	Negative
FCS	Fetal calf serum
M	Molar
FL	Fluorescein

LIST OF ABBREVIATIONS (Continued)

PE	Phycoerythrin
bio	Biotin
APC	Allophycocyanin
mAB	Monoclonal antibody
Cy5	Cychrome five
BSA	Bovine albumin
SA	Strept-avidin
cDNA	Cellular DNA
PBS	phosphate-buffered saline
PI-PLC	phosphatidylinositol-specific phospholipase C
CFU-c	Granulocyte-macrophage colony
BFU-e	Erythroid burst colony
CFU-GEMM	Pluripotent mixed colony
IgM	Mu immunoglobulin complex

INTRODUCTION

The differentiation of a stem cells to mature hematopoietic lineage cells has been a primary focus of research in developmental immunology. These studies have resulted in determining the bone marrow as the major source of adult hematopoiesis, the yolk sac and paraaortic-splanchnoplura as sources for embryonic stem cells as well as the division of lymphoid cells into B and T cells. Although hematopoietic cells with distinct morphological features, such as erythroid, megakarocytes and myeloid cells, were described early and methods for their detection and function were developed, characterization of lymphocytes was not as easily defined. The division of the functions of lymphocytes in the immune system has been suggested for some time. In the early 1900s, Chase and Landsteiner showed in a series of experiments that particular immunological functions could be transferred with either cells or serum substances. Studies of human immunological defects, such as Brutons X linked a gamma globulinemia, described by Ogden C. Bruton, to animal models of mice and chicken defined functions unique to each lineage as well as giving clues as to their origin. Experiments in chickens demonstrated that the removal of the bursa of Fabricius early in development resulted in a severe diminution of the ability to produce antibody but did not alter T cell functions (1). Similarly early thymectomy in mice, rabbits, and chickens resulted in the abolishment of T cell response but did not drastically alter antibody responses (2-5).

More recent studies have generated means of identification and isolation of stem cells and early lymphocyte lineage committed progenitors based on cell surface expression of certain cell surface molecules. In addition, in vitro culture and in vivo transfer models have been developed to characterize progenitor populations and to evaluate the requirements needed for growth and differentiation as they progress from undifferentiated states to cells committed to particular lineages. To assess cells in the hematopoietic cascade, a variety of clonal in vivo and in vitro culture systems have been used to isolate and define committed lineage progenitors. The tracing of B and T cells as lineages has elucidated the differentiation steps, receptor-mediated selection processes, proliferative clonal expansion processes, and, more recently, signal transduction pathways active at many of the distinct stages during this process. However, it is clear that many transition states and regulatory mechanisms governing this process are unknown.

A critical step in the process of hematopoietic cell differentiation occurs at the transition from a stem cell to a committed progenitor cell and is poorly understood. The work presented in this dissertation was originally focused on the question of whether there are committed B cell progenitors that are not B220 positive as defined in contemporary schemes of mouse B cell differentiation. Additionally, are there alternate pathways in early B cell development apart from the ordered progression currently accepted? During the progression of this work, the focus changed to include an early, perhaps noncommitted, progenitor-type population.

Stem Cells

Stem cells possess the abilities both to self-renew and to produce committed progenitors for all hematopoietic lineages. Initially, stem cell activity was analyzed by the ability of precursor cells to produce colonies in 12 d colony forming unit-spleen (CFU-s) assays (6, 7). In this assay, cells are transferred into a host animal and, after 8-16 ds, macroscopic colonies present on the spleen of the host animal are assessed. However, the CFU-s assay was determined not to adequately predict long-term hematopoietic reconstitution (8, 9) because more than only stem cells are active in colony formation. Other types of in vitro culture assays have been used and optimized to determine stem cell activity. Initially, agar cultures were used to determine the potential to form mixed colonies (10), and, more recently, long-term methylcellulose cultures with defined factors (11) that support stem cell potential have been developed. In vivo models to examine the stem cell potential of a population were developed, taking advantage of mutations such as severe combined immune deficient (SCID) mice to assess long-term reconstitution after sublethal irradiation of recipients or sublethatally irradiated animals and competitive reconstitution experiments (12).

Although characteristics of longevity and self-renewal have been assumed to be properties of stem cells for some time, the extent of these properties has been questioned. Two models have been proposed of how stem cells generate hematopoietic cells over the lifespan of the animal. One is that life-long hematopoiesis is maintained by a succession of short-lived clones (13-16). The second model is stable extended clonal maintaince of the hematopoietic system (17-20). An informative series of experiments to address these

issues are from Keller and Snodgrass (21), in which murine stem cells were marked by unique retroviral integration sites that could be traced in their progeny in recipient animals after long periods of time. Initially, these authors reported clones which persisted for 5 months after transfer and clones in which the parental clone did not consistently produce all lineage type progeny. In subsequent experiments, when marked clones were followed for longer periods of time, stable clones which were functional for 15 months were detected, and a given clone was shown to expand itself while maintaining its committed lineage generation potential (21).

Recent evidence indicates a mechanism by which stem cells can retain their self-renewing capacity throughout the life span of the host animal. It has been previously proposed that there is an absolute limit to the number of divisions that somatic cells can undergo (22-24). More recent findings suggest that telomere loss is associated with cellular senescence (24, 25) and that the determining factor limiting division of somatic cells is telomere length (26). To protect against the nibbling of DNA sequences after each cell division, vertebrates have telomere sequences consisting of noncoding tandem repeats at the end of chromosomes (27, 28). Telomerase is a ribonucleoprotein complex that adds telomere sequences during each round of DNA replication to stabilize telomere length. Thus it would be predicted that long-lived cells with self-renewing capacity would express telomerase to protect their telomeres. Recent work has suggested that telomerase is expressed by most hematopoietic stem cells from murine bone marrow and fetal liver (29). Also, telomerase activity was detected in a fraction of pro-B, pro-T, and splenic

marginal zone cells--all populations with self-renewing capability. No telomerase activity was seen in cells with no self-renewal capacities, such as differentiated myeloid cells.

A major advance in the study of stem cells was the development of procedures for their isolation by physical properties such as cell size-density, specific dye uptake, or by the use of antibodies to cell surface molecules. A means of isolating stem cells prior to identification with cell surface markers was counterflow centrifugal elutriation, which fractionates cells based on their size and density (30, 31). In this manner, a subpopulation of cells was isolated. These size- and density-isolated cells were contained in a larger fraction of cells that had previously been described as stem cells based on their activity in 12 d CFU-s assays. When the counterflow centrifugal-elutriation-isolated population and the larger cell fraction were compared in engraftment experiments of irradiated recipients, each showed a difference in its ability for long-term engraftment of all lineages (32), indicating that more cells than pure stem cells are responsive in 12 d CFU-S assays.

Stem cells from murine bone marrow can be fractionated by staining with rhodamine 123 (Rh123) and divided into $Rh123^{low}$ (resting) and $Rh123^{high}$ (activated), which, when used in CFU-s assays, and repopulation assays, demonstrate different potentials for lineage regeneration. Rh123 is a fluorescent vital dye which preferentially accumulates in mitochondria membranes and is useful as a probe to indicate mitochondria and, hence, cellular activity. The $Rh123^{low}$ cells are highly enriched for long-term hematopoietic repopulating cells in in vivo transfers (30, 33), as well as in limiting dilution cultures (34). Furthermore, this $Rh123^{low}$ population can be divided by the

expression of c-kit, a tyrosine kinase which was determined to be encoded for by the W locus (35). Mice with a mutation in the dominant white spotting locus, or the W locus, are denoted W/W mice (36). All of the long-term repopulating activity, 12 d CFU-s, and cells providing radioprotection fall in the c-kit positive $Rh123^{low}$, lineage marker negative (Lin-), Ly6A+, fraction (37). The $Rh123^{high}$, c-kit+, Ly6A+/- populations are more representative of short-term hematopoietic repopulating cells; furthermore the Ly6A- population was described as containing committed progenitors because of its characteristic responsiveness to a panel of cytokines (38, 39). It has been reported that modulation of c-kit expression affects the proliferative response of stem cells. Transforming growth factor beta can modulate the responsiveness of murine hematopoietic progenitors to steel factor by downregulating expression of its ligand c-kit (40). The tyrosine kinase c-kit is but one tyrosine kinase expressed by hematopoietic stem cells that may function to regulate their ability to remain in a primitive undifferentiated state. Another tyrosine kinase that is expressed by hematopoietic stem cells is flk-2 (41). The flk-2 kinase was isolated by enriching for hematopoietic stem cells, then isolating the mRNA for use in PCR-based amplification using degenerate oligonucleotide mixtures encoding conserved amino acids. It was described as a novel tyrosine kinase expressed by fetal liver hematopoietic stem cells and primitive uncommitted progenitors associated with the W locus gene product c-kit.

Many past and current studies identify and isolate stem cells based on their cell surface phenotype. Stem cells have been reported to be $Thy\text{-}1^{low}$, lin- (42), as well as sca-1(Ly6A)+ (43). A current study of stem cells in murine bone marrow determined

that they are CD38+, which can be divided into $CD38^{bright}$, which contains the most primitive hematopoietic stem cells, and $CD38^{dim}$, which has colony-forming potential in CFU-S assays at d 12 but does not have long-term reconstitution potential. The $CD38^{bright}$ cells produce colonies in in vitro liquid culture assays, CFU-S assays, and have the ability to give rise to long-term reconstitution in transfer experiments (44). This expression pattern of CD38 also holds for stem cells isolated from embryonic d 14 fetal liver. Differential expression of CD38 by murine stem cells differs from human bone marrow and fetal hematopoietic stem cells, which do not express CD38.

Another marker currently used is CD34. Recently, monoclonal antibodies were generated against murine CD34, and it was determined that CD34 is expressed by murine hematopoietic stem cells (45, 46).

Fetal Hematopoiesis

Hematopoietic stem cells were initially determined to originate from the embryonic yolk sac (47-49). Later, studies demonstrated that definitive adult hematopoiesis originates from hematopoietic stem cells, which form in an intraembryonic source in avian species (50, 51). Similar findings were reported in amphibians (52, 53). Basically, these avian and amphibian studies showed that progenitors from yolk sac or ventral blood islands give rise to the first wave of erythroblasts and myeloid cells and, in later studies, were shown to become less potent in lineage potential than intraembryonic hematopoietic stem cells (54, 55). Additionally, the earlier studies demonstrated that the hematopoietic stem cells originating intraembryonically emerge from the region of the dorsal aorta. Subsequently, these cells seed the embryonic rudiments where they

differentiate to all hematopoietic lineages. Current studies in the mouse model have shown that intraembryonic sites are a predominant source of hematopoietic stem cells (56-61). These sites are the paraaortic-splanchnopleura at 8.5 ds post coitus (dpc) or the aorta, gonads, and mesenephros region (AGM), which differentiates from the paraaortic splanchnopleura, at 10-11 dpc.

Based on transfer studies and ontological surveys, it was assessed that certain lymphocyte subsets could be generated only in embryonic or neonatal life. Vγ3 + T cell potential was shown to be derived from the fetal hematopoietic stem cell population but not bone marrow hematopoietic stem cells (62). Similarly, B1 type B cells preferentially develop in fetal and neonatal sites versus bone marrow (63). One explanation for these proposed differences has been microenvironmental effects (64), but another explanation has been that the stem cells in fetal life may have greater potential than adult or bone marrow hematopoietic stem cells.

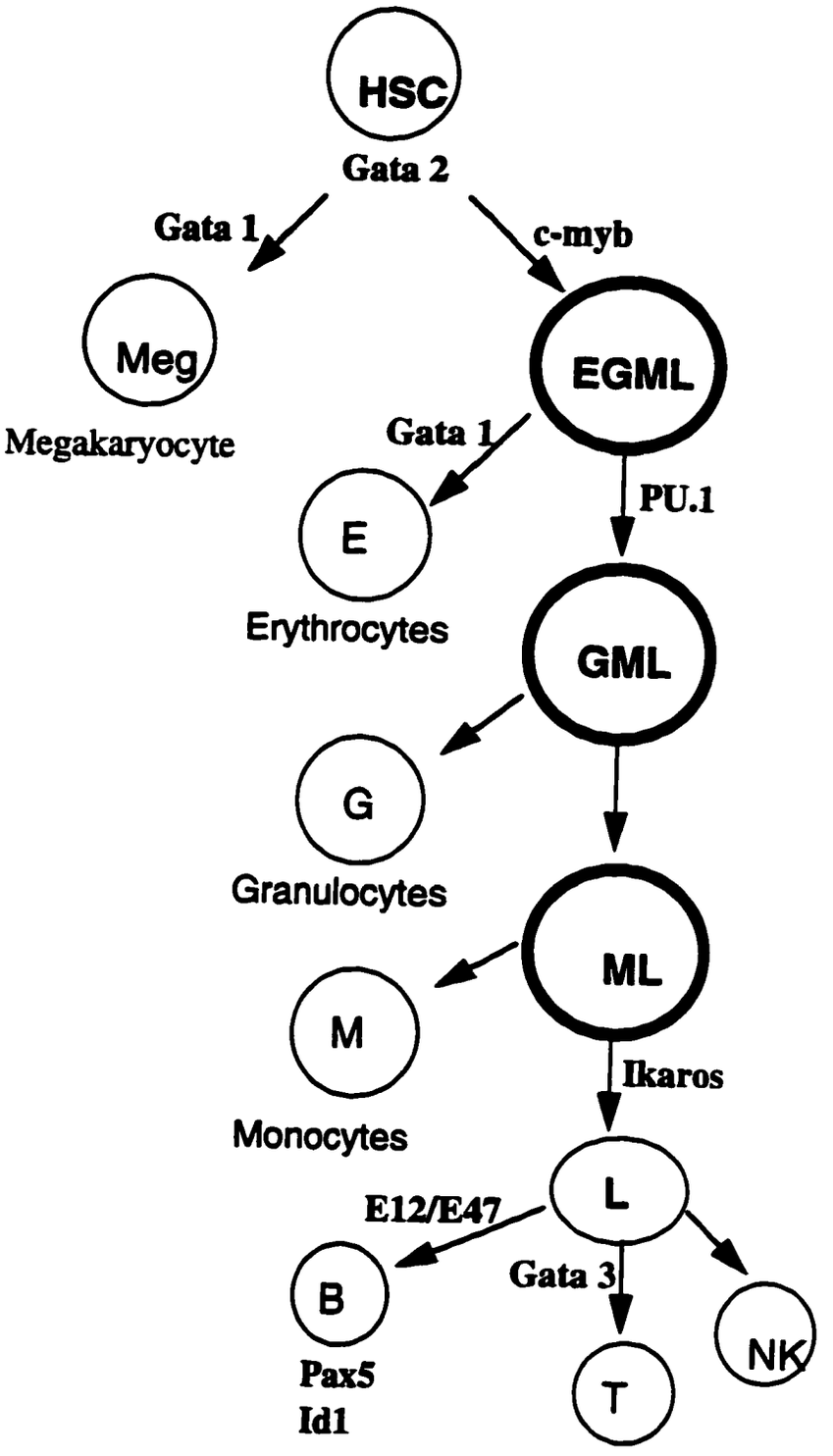
To study initial formation and development of stem cells and lineage restricted progenitors, cultured embryonic stem cells have provided a useful model. Embryonic stem (ES) cells are derived from the inner cell mass of 3.5 d blastocytes (65, 66). These cultured ES cells, when transferred into a recipient animal, have been shown to contribute to all lineages (67). Additionally, the microenvironment which these cells are transferred into will influence ES cell development (68). ES cells maintained in in vitro culture systems have been manipulated to differentiate into erythroid bodies which contain many cell types (65, 66, 69). Specifically, ES cells can be induced to differentiate from stem cells to specific lineages (59, 70). One such reported influential factor is IL-6, which was

shown to be consequential in the inductive differentiation of pluripotent ESCs toward hematopoiesis in vitro (71).

Transcriptional Factors

There are a series of transcriptional factors or regulators of transcription factors that have been characterized and manipulated, relevant to hematopoietic lineages. Several reviews of transcriptional factors and lineage progression try to piece together the current information available on the subject into models in which many of these factors exert their influence in lineage differentiation (72-75). A depiction of one such model is shown in Fig. 1, which is based on a model designed by Singh (73). Not described in this model are the Gata transcriptional factors. The group consists of Gata 1, Gata 2, and Gata 3. They are members of a zinc-finger subfamily and are regulators of hematopoietic gene expression (76). The transcriptional factor Gata 1 is associated with erythroid lineage cells, megakaryocytes, and mast cells. The second member of the group, Gata 2, was determined to have profound effects in all hematopoietic lineages based on the defects seen in the gene inactivated mice (77). Associated with the T-cell-specific regulation of surface molecules is Gata 3 (78, 79). The transcriptional factor c-myb is abundantly expressed in progenitors of erythroid, myeloid, and lymphoid lineages but is dramatically reduced in each as they differentiate. In c-myb gene-inactivated mice, erythropoiesis in the yolk sac is intact, but, in fetal liver, erythropoiesis, myelopoiesis, and lymphopoiesis were greatly reduced, resulting in death after 15 dpc (80). Next in the progression of the model is Pu.1 influence. Pu.1 is an ets family member and is expressed specifically in hematopoietic tissues (81, 82). There are two Pu.1 gene-inactivated mice described in the

Figure 1. A model of hematopoietic differentiation and commitment. Hematopoietic transcriptional factors are expressed and active at distinct stages in differentiation. Characterization and analysis of the gene-inactivated, or knockout, mice of these factors has produced a model of differentiation, including several proposed multipotential intermediate stages. However, these cells in these intermediate stages have not yet been identified or isolated, except for the ML intermediate in fetal liver at day 12 of gestation.



literature. The first described was a mouse in which the homozygous state of the inactivated gene was a lethal mutation at late gestational age (83). Of the hematopoietic lineages in the viable homozygous fetuses, no permutation of the megakarocyte lineage was evident. Eyrthroid progenitors were present, but the later erythroid stages seemed to be affected, and the fetuses were severely anemic by 18 ds of gestation. The most striking finding of this gene inactivation was a multilineage defect in the generation of progenitors for B, T, natural killer (NK), monocytes, and granulocytes. No immunoglobulin gene rearrangement or T cell receptor gene rearrangement was detectable in the fetal livers, nor were any progenitor-associated genes, such as Rag-1, Rag-2, mb-1, B29, or VpreB, expressed. It was later reported that the transcriptional factor Ikaros was expressed in the homozygous mice (84). In the second gene-inactivated mouse reported, the homozygous mice were born and died without antibiotic intervention within 24-48 h from septicemia (85). In these mice, the erythroid lineage did not show any defect, nor were the defects in the T, NK, myeloid, or granulocyte lineages as absolute as in the other reported gene-inactivated mouse, althought the abrogation of the B lineage was consistent in both. The results may be due to the constructs used to create the mice. Next in the developmental line is Ikaros (86). It is a zinc-finger protein related to the Drosophila gap protein Hunchback. The Ikaros factor can bind to and activate the enhancer of CD3d, and its mRNA was detected in murine fetal liver and embryonic thymus when hematopoietic and lymphoid progenitors initially colonize these sites (86). Ikaros was then described as a family of five members, which are generated by alternate splicing. In a gene-inactivation mouse model where four of the five Ikaros family members are inactivated, B, T, and NK

lineage cells and progenitors were absent, but the erythroid and myeloid lineages were intact (87). Affecting the lymphoid lineages specifically are the E12 and E47 transcriptional factors derived from the E2A gene. Transcriptional factor E47 was shown to bind to sites within the promoters of the immunoglobulin heavy chain and kappa chain genes. Additionally, in a pre-T cell line model, D-to-J immunoglobulin rearrangements were induced by E47 binding. Later, in a E12 gene-inactivated mouse, the B lineage was abrogated. In these mice, no D-to-J rearrangements were detected (88). Correspondingly, in the fetal liver of these mice, the immunoglobulin germline I_{μ}, RAG-1, mb-1, CD19, and $\lambda 5$ transcripts were significantly reduced, but the B29 and u^0 transcripts were present and only slightly reduced. When transgenic mice expressing either E12 or E47 are generated on the complete E2A knockout background, each individually promotes minimal advancement in B cell differentiation. These transgenic mice were crossed together on the E2A null background which showed E12 and E47 to be acting synergistically to promote B cell differentiation (89). The Pax5 gene encodes the transcriptional factor BSAP, which is expressed differentially from early pro-B cells to mature B but not by differentiated plasma cells. Furthermore, BSAP was reported to be expressed in murine fetal liver correlating with the onset of B lymphopoiesis (90). The promoter of the CD19 gene was suggested to be the target of BSAP, and its expression pattern was closely correlated with expression of CD19 (91). Mice in which the Pax5 gene was inactivated showed a defect in the B lineage and the posterior midbrain (92). As suggested by the normal expression pattern, the B lineage cells were blocked in differentiation at the B220+/CD43+ pro-B stage. In this pro-B pool, only minimal D-J rearrangements were detected, and CD19 was

not expressed. The Id1 gene is a member of a family of genes that are inhibitors of helix-loop-helix transcriptional factors. This family of genes is expressed in an inversely differential pattern during B cell differentiation. Id1 is expressed in early multipotential or newly committed pro-B cells, while Id2 and Id3 expression is minimal early in B cell differentiation but is upregulated during differentiation (93). Transgenic mice that constitutively express Id1 B cell development were impaired at early stages. No pre-B (B220+/CD43-) or B (B220+/IgM+) B cells were detectable (94). Reduced frequencies of V(D)J and VkJk rearrangements were detected in the B cells present in the Id1 transgenic mice, and, in addition, there was a lower than normal expression of Rag-1, Rag-2, and λ5 genes.

Multilineage Progenitors

The course of exit of hematopoietic stem cells from their long-lived totipotent state has been described as a stochastic event. Once this occurs, the mechanisms that cause a cell to commit to a particular lineage and then continue to progress differentially in that lineage are not clear. The existence of a common, committed, progenitor cell has been difficult to prove because its existence is not yet distinguishable by a specific set of surface markers. Two models for the progression of stem cell to committed progenitors have experimental data to add substance to them. One model is direct lineage commitment, with a chance of a second try for another cell type at alternative times after the first choice. The alternative model involves a stepwise commitment, with each step subsequently restricting the renewal potential of the cell in its progression. Early data to support this model came from the characterization of progenitor cell lines or ES cell lines

that could be induced to exhibit features of one lineage or another, depending on the inducing factor (69, 95-98). Also, cell lines with characteristics of two lineages were described, such as a monocytic cell line with its immunoglobulin genes in a rearranged state (99). Later work indicated that, if bone marrow cells were marked with a retrovirus integration site, some insertion patterns were seen in all lineages, indicating these were the progeny of a stem cell; it was also noted that a particular pattern of insertion could be seen in some lineages but not all lineages, indicating they were progeny of a common progenitor (100). Recently, it has been reported that in 12 d fetal thymus cells of the most immature subset that express FcγRII/ III are T lineage committed cells, but cells that are negative contain the potential to differentiate to T, B, or myeloid lineages (101).

However, the most convincing body of experimental data for a multipotential cell is the suggestion of the common B-myeloid lineage precursor. Evidence for this common progenitor has been accumulating. A monoclonal antibody, AA4 has been used to enrich for hematopoietic cells from 12 d fetal liver (97, 102) . This enriched population was found to contain cells that had the potential to give rise to multipotential or restricted B/ myeloid precursors. In a retroviral marking experiment previously addressed, a unique viral integration site was traced from a nonprimitive or nonstem cell progenitor to the lymphoid and myeloid lineages, suggesting a common multipotential progenitor (100).

In other experimental studies, factors were implicated in driving or preventing differentiation of noncommitted cells into one lineage or another. Two progenitor clones, an IL-3 (LyD9) dependent clone and a subclone of LyD9 that was IL-4 (K4) dependent, were both shown to differentiate into B/myeliod lineages on bone marrow stromal cells.

The data suggested that the K4 clone was a multipotential intermediate between the LyD9 clone and committed B or myeloid cells (103). In addition to in vitro culture differentiation and retroviral marking experiments, an interesting idea of making a family tree by using patterns of phosphorylated proteins to group and compare cell lines representative of the lineages was made (104). By correlating similar and dissimilar patterns of phosphorylated proteins, a progression was mapped from megakaryocytic, to erythroid, to neutrophils, to monocytes, and finally to B and T lymphocytes. Furthermore, an association between the B1 lineage and myeloid lineage was made using cell lines derived from fetal liver with properties of myeloid and B lineage cells which could be induced along either the B lineage pathway or the myeloid lineage pathway (105, 106).

In another retroviral marking experiment, a marked mixed population derived from ES embryo bodies, when transferred into sublethally irradiated mice, reconstituted macrophages, mature T and B lymphocytes, and plasma cells of donor origin (69). It was postulated that this mixed population may represent a population of immortalized multiprogenitors. Additionally, in experiments using the AA4 antibody and a combination of other monoclonal antibodies, a group of homogenous noncommitted cells from the fetal liver was identified that could generate both macrophages and B cells (102). Current studies of this bipotential progenitor population from fetal liver suggest that cytokines, particularly IL-11, mast cell growth factor, and IL-7, are involved with the differentiation and proliferation of the progenitor pool (107, 108).

In all of these experiments, cell lines from fetal liver, purified fetal liver, or cells derived from ES cells were used to identify multiprogenitor cells; however, no such multiprogenitor cell has been isolated from bone marrow.

B Lineage Differentiation

Location of Differentiation in Bone Marrow Structure

In an interesting series of experiments that addressed B lineage differentiation, B cells with a certain phenotype were labeled in vivo and visualized by electron microscopy in bone marrow cross sections to determine their physical relationship with other cell types. A model was proposed in which stem cells and other early progenitor cells are located peripherally near the surrounding bone. As these progenitor cells develop, they move toward the center of the extravascular tissue spaces, with close association to stromal reticular cells where apoptotic cells and other dying immature B cells are removed by macrophages. The mature B cells were then shown to undergo an intravascular maturation phase before the functional B cells were exported into the blood stream (109). Later experiments better defined that the early pro-B cells are in the periphery of the bone marrow, associated with stromal reticular cells (110).

Molecules Associated with B Cell Differentiation

The expression of certain molecules has proven extremely useful for defining differentiation from one stage to the next to describe stages in B cell differentiation. Some of these proteins have defined functions in B cell differentiation, and others, to date, do not. The first and most widely used marker is B220, an isoform of CD45 that is expressed by B lineage cells and some early progenitor cells in the bone marrow. This

molecule, CD45, has been shown to have phosphatase activity. However, its function on B cells is unclear but appears to be involved as a coactivating molecule.

CD43 is a sialoglycoprotein that is expressed on early bone marrow progenitors and on early committed B cell progenitors in mice. It is also expressed by an extended array of cells, including thymocytes, peripheral T cells, granulocytes, monocytes, macrophages, NK cells, platelets, activated B cells, plasma cells, and B1 B cells in the spleen.

CD19 is expressed specifically by B cells, it is a member of the immunoglobulin (Ig) superfamily and is expressed very early in B cell differentiation until a B cell terminally differentiates to a plasma cell (111). A function for CD19 was first described using human B cell lines, in which it augmented Ca^{2+} mobilization induced by co-crosslinking with the immunoglobulin receptor (112). Further work clarified this function: specifically, coligation of membrane CD19 with IgM decreased the threshold for antigen-receptor-dependent stimulation, compared to signaling through BCR alone (112). Further, it was demonstrated that CD19 ligation does not mobilize Ca^{2+} until the pre-B cell stage. Thus, CD19 was identified as a modulator of Ig signal induction; however, CD19 is expressed prior to intact immunoglobulin receptor expression in early B cell differentiation. In murine studies, CD19 is found to have the same modulatory role in B1 and B2 B cells, even though CD19 coligation does not activate Ca^{2+} mobilization in B1 lineage cells (113). As it might have been predicted, in CD19-deficient mice, B cells have significantly decreased proliferative responses to mitogens, as well as reduced serum immunoglobulin levels (114). It was also noted that, although there was a reduction in the

number of peripheral B cells, there was no striking effect on B lineage differentiation in the bone marrow. However, in CD19-transgenic mice, B cell differentiation in the bone marrow was impaired (115).

The λ5 and V pre-B genes are homologous to the Ig light chain genes and are specifically expressed in immature B lineage cells (116-120). The combination of the protein products of these two genes is referred to as the surrogate light chain complex. Both the λ5 and V pre-B proteins were shown to associate with the μ chain in transfection experiments and in murine and human pre-B cell lines (121, 122). B lineage differentiation in mice deficient for the λ5 gene is blocked at the pre-B cell stage, although some leakiness is seen (123). This correlates with a study which demonstrated that λ5 and V pre-B expression spans several developmental stages but that cell surface expression is limited to the pre-B cell stage (116). However, another study indicated that the surrogate light chain can be associated with a 130-kD protein on the surface of early B cell lines that are negative for the Ig μ chain, which may be a surrogate heavy chain (117). Surrogate light chain expression in bone marrow of normal mice compared to B-cell-deficient mutant mice suggested that association of the surrogate light chain with the mu chain is a critical step for the selection and amplification of cells with a functionally rearranged m-heavy chain (124). This data indicated that λ5 may be expressed by some B220-negative/ CD43-positive cells, perhaps an early progenitor population.

Class II molecules are differentially expressed on bone marrow versus fetal B cell progenitors (125, 126). Another cell surface molecule expressed at a specific stage in B

lineage differentiation is the BP-1 molecule, but, although it expresses active ectoenzyme activity, its function on the B cell is unknown (127).

Current Schemes of B Lineage Differentiation

In 1991, a scheme of sequential steps in B lineage differentiation from early progenitors to mature IgM/IgD B cells in the bone marrow was described by Hardy et al. (128). The compartments were labeled fractions A-F, A being the earliest identifiable B lineage committed cell. These fractions were described on the basis of changes in expression of cell surface markers and correspondence to the sequential Ig gene rearrangement that occurred during B cell development. It was shown that cells isolated from the early fractions progressed through the predicted fractions in an orderly manner with time when placed in in vitro cultures. The earliest fraction was determined to be the most stromal contact dependent, whereas the fractions B-C were not as dependent on stromal contact but were most responsive by proliferation to IL-7.

Hardy's group further demonstrated that these fractions characteristically expressed gene B cell associated genes, including Tdt, lambda 5, V pre-B, mb-1, and the Rag genes 1 and 2 (129).

An alternate means of defining the earliest committed B cell progenitor was based on the expression of cell surface markers B220 and CD19 (130). In this model, a small fraction of the B220 positive cells is CD19 negative, and it is within this fraction the earliest progenitors are found. This small population can be further subdivided into a NK1.1-positive population, which are NK progenitors. Additionally, a CD4-positive population is defined in the B220+/CD19 negative fraction, which is perhaps composed

of T cell progenitors, as well as a double-negative population. It is the double-negative population of cells that contains early lymphoid progenitors. The two models are not dramatically different, but the B220/CD19 model may define fraction A more precisely. Thus the current earliest defined committed B cell progenitor can be described as B220 positive and CD19 negative and AA4.1 positive and NK1.1 negative.

Cytokine Involvement

Stem cell factor (SCF) through c-kit, its ligand, synergizes with IL-7 to promote proliferation of precursor B cells in vitro (131-133). Antibodies to c-kit were shown to inhibit the growth of B cell precursors in vitro and in vivo (134) . To further elucidate the role of stem cell factor, normal mice were compared to W/W mice, mice having mutations at the dominant white spotting locus. The W/W mice are described as not expressing c-kit on the cell surface, while the hematopoietic lineages are effected but not abolished, and the mice die shortly after birth (135). Thirteen-d fetal liver precursors from either wild-type or W/W mice were transferred into Rag2 -/- mice to evaluate the role of stem cell factors in B cell differentiation. It was noted that, in the W/W transfers, the mice failed to generate donor thymocytes or myeloid type cells, but the B lineage, B2 as well as B1, was not significantly reduced (136). These contradicting experiments indicate that more informative experiments need to be done to elucidate the SCF/c-kit interaction's role in development. The stromal growth factor insulin-like growth factor 1 can promote the differentiation of pro-B cells and costimulates pro-B colony formation (137). It was determined that c-kit is a tyrosine kinase receptor which is important in the proliferation and differentiation of a number of cells, including hematopoietic lineages. In an attempt to

identify other tyrosine kinase-receptor-type proteins involved with commitment and differentiation, RT-PCR products of mRNA from fetal liver lymphoid cells were screened with degenerate primers to isolate new proteins. Flk1, flk2, and flt3 are of the same subclass III family as c-kit (138-140). The ligand for flk2/flt3 (FL) has been identified and cloned (141, 142). FL stimulates growth of hematopoietic progenitors, but not pro- or pre-B cells, either alone or in combination with SCF or IL-7 (143). When the ability of FL to promote colony formation in methylcellulose cultures with lineage-negative progenitors was analyzed, FL alone has weak activity, but FL in combination with IL-3, SCF, or IL-11, significantly enhanced granulocyte-macrophage colony formation (144). In the same study, FL in combination with IL-6, IL-11, or granulocyte-colony stimulating factor supported growth of multilineage colony formation. It has also been reported that FL in combination with IL-11 and IL-7 is sufficient to support the differentiation of uncommitted fetal progenitors into the B lineage in an in vitro system (145). These data are supported by the abnormalities seen in flk/ftl3-deficient mice. The flk2/flt3-deficient mice are generally normal, but a deficiency in primitive B lineage progenitors is detected. When bone marrow from flk2/flt3-deficient mice is used in transfer experiments, further deficiencies in the T and myeloid lineages of the donor type are found (146).

Il-7, a cytokine, has been shown to be essential for the B lineage (147). In the murine system, IL-7 can induce proliferation of B220+ B cell precursors but has not been shown to induce differentiation from one stage to another (148); also, IL-7 does not affect B220-negative precursors to differentiate into the B lineage (149). However, in humans,

IL-7 has been shown to induce the expression of CD19 on progenitor B lineage cells (150). In IL-7-transgenic mice, B lymphopoiesis in bone marrow is dramatically altered, and the number of pro- and pre-B and immature B cells is significantly increased (151). In IL-7-deficient mice, B lymphopoiesis in the bone marrow is blocked at the pre-B stage, and these abnormal pre-B type cells can migrate out to the periphery. In the thymus, while the cellularity is decreased, the normal distribution of CD4 and CD8 is retained (81). Splenic T cells are also reduced, but the B cell compartment of the spleen is most severely effected. The splenic B cell population consists of these abnormal immature B cells and a reduced but significant number of cells that express B220 and surface Ig.

Another cytokine implicated in B lineage differentiation is IL-2. IL-2 receptor gamma chain expression was shown to be initiated by functional Ig gene rearrangement and expression of the immunoglobulin heavy chain and is subsequently downregulated when the immature B cells express IgD (152). However, the impact of IL-2 on these cells is not clear.

Aim of Dissertation

The questions addressed in this dissertation focus on the delineation of early stages of hematopoietic differentiation. Specifically, are there committed B cell progenitors that are not B220 positive, and are there alternate pathways in early B cell development apart from the clearly ordered progression currently accepted? If one assumes that the early steps in differentiation bridging stem cells and a known committed progenitor such as fraction A in B lineage development occurs in an orderly progression of commitment events, then these steps should be identifiable based on changes of gene

expression and cell surface markers. The existence of this type of early committed progenitor, common progenitor, or an alternate B lineage commitment pathway has been difficult to prove because they have not yet been distinguishable by the specific set of surface markers used to define and isolate these stages.

In this study, a panel of monoclonal antibodies was developed in an attempt to identify cell surface molecules associated with early lymphoid development. One such monoclonal, AB8, does indeed isolate an early B220-negative progenitor cell. The analysis of the lineage potential of this population from bone marrow and fetal liver is the focus of this dissertation.

IDENTIFICATION OF A UNIQUE EARLY LYMPHOID PROGENITOR IN MURINE BONE MARROW

by

CHANTAL MORATZ, LARRY GARTLAND, AND JOHN F. KEARNEY

In preparation for *The Journal of Experimental Medicine*

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Summary

During development of B lymphocytes, sequential stages have been described which involve the transition of stem cells to a B lineage committed progenitor; however, it is not clear whether there are alternative pathways to those described in these models. In an attempt to isolate and define alternative early lymphoid progenitor cells or pathways of development, monoclonal antibodies were generated against cultured pre-B cells and screened for their potential to characterize such populations. One such antibody, AB8, appears to define a population of lymphoid cells intermediate in phenotypic and functional characteristics between the stem cell population and the earliest currently defined committed lymphoid cells. In adult bone marrow, AB8+ cells are divided into two populations, one of which costains with Ter119. The AB8+/Ter119neg cells do not include hematopoietic stem cells but also do not yet express characteristic lineage commitment markers. When this AB8+/Ter119neg population is sort-purified and analyzed in in vitro stromal assays, this population progresses into a B lineage pathway in a stepwise manner. These data suggest that, within the AB8+/Ter119neg fraction, there is a novel immature lymphoid precursor population that has B cell progenitor activity but does not express markers for early progenitors defined by currently available precursor-progeny models.

Introduction

A central theme of developmental immunology has been to delineate the pathways and commitment steps involved in the progression of a hematopoietic stem cell to mature, functional, lineage-committed cells. Analysis of stem cells, including their sites of origin, life span, turnover rate, and their use in functional reconstitution assays, has led

to a deeper understanding of their role in the development of cells in the immune system (1-4).

The defined and ordered progression of the transitional stages from B cell progenitors (pro-B) to functional newly formed B cells ready to migrate from the bone marrow to the peripheral lymphoid organs has been well documented in two independent model systems (5-8). In these analyses, lineage progression has been defined by the expression of characteristic differentiation antigens, stage-specific gene expression, immunoglobulin gene rearrangements, and auxiliary or stromal cell contact dependence.

During the process of differentiation of stem cells to B cell committed precursors, there is clear evidence for multipotentiality or lack of commitment during the earliest stages of this process (9-11). In an attempt to define more precisely these early events and to isolate early lymphoid progenitor cells, monoclonal antibodies were generated and screened for their potential to identify and characterize unique populations.

A monoclonal antibody (mAB) AB8 was found to detect a unique marker that characterizes an early progenitor cell population in the bone marrow. These cells are distinct from hematopoietic stem cells, as well as from cells which express the lymphoid marker B220 used to define the first stages of B cell commitment. We have studied the characteristics of AB8+/Ter119neg cells at a cellular and molecular level and have assessed their potential in in vitro assays to develop into B cells. Our findings show that there is a population of AB8 positive cells in adult bone marrow that constitutes a previously undescribed population of precursors capable of developing into B cells.

Material and Methods

Animals and Cell Preparation

Four- to five-week-old BALB/c and C57BL/6 mice, purchased from Charles River Laboratories (Raleigh, NC) or bred in our own animal facilities, were used in all experiments. A single-cell suspension of bone marrow was obtained by flushing the marrow cavities of the femura and tibiae with staining media (phosphate buffered saline [PBS] containing 1% fetal calf serum [FCS] [Hyclone, Laboratories, Logan, UT]) with a 26-gauge needle and 10-ml syringe of medium. The marrow plugs were gently mixed and disaggregated by repeated aspiration with a 10-ml syringe. The cell preparation was then treated with lysis buffer (0.017 M Tris, 0.16 M ammonium chloride, pH 7.2) to eliminate erythrocytes and was washed before use.

If cells used in in vitro culture experiments were to be reanalyzed by means of flow cytometry, the cultured cells were harvested by gentle pipeting or treatment with 1% EDTA if necessary and washed once before immunofluorescent staining and analysis.

Antibodies

The fluorescein (FL)-, phycoerythrin (PE)-, biotin (bio)-, and allophycocyanin (APC)-conjugated mAbs RA3.6B2 (anti-CD45), anti-CD45LCA, 7D4 (anti-CD25), S7 (anti-CD43), PK136 (anti-NK1.1), 90 (anti-CD38), anti-GR-1, anti-CD4, anti-CD8, anti-c-kit, anti-CD5, anti-Ter119, anti-Sca-1, SA-APC, Rat IgG2b control, and anti-thy1.2 were obtained from PharMingen (San Diego, CA). The hybridoma producing the rat anti-mouse AB8 (IgG2b) was isolated and characterized in our laboratory by immunizing a rat subcutaneously six times at 3- to 4-d intervals with purified pre-B cells derived from IL-7-dependent cultures of mouse bone marrow cells into the area drained by the popliteal lymph nodes. One d after the last injection, the lymph nodes were isolated and

the cells were fused to P3X63Ag8.653 cells (12). The hybridoma cells lines producing anti-CD19, Ter119, and F4/80 were obtained from Dr. Douglas Fearon, Dr. Irving Weissman, and the American Tissue Culture Collection, respectively. Antibodies purified from hybridoma culture supernatants on protein G-Sepharose columns (Pharmacia, Uppsala, Sweden) were either biotinylated or labeled with cychrome five (Cy5) (Amersham Life Science, Pittsburgh, PA) according to standard procedures. FL-conjugated goat anti-mouse IgM and SA-PE were obtained from Southern Biotechnology Associates (Birmingham, AL). The FL goat anti-rat IgG was obtained from Gibco/BRL (Paisley, Scotland).

ImmunofluorescenceStaining andFlowCytometric Analysis

Cell preparations were stained with unlabeled AB8 or F4/80 and revealed with goat anti-Rat IgG (H + L) $F(ab')^2$ FL, blocked with normal rat serum, and then stained with FL-, PE-, bio-, APC-, or Cy5-labeled antibodies in the staining buffer (1% bovine albumin [BSA] [Sigma Chemical Company, St. Louis, MO] in PBS with 10 ug/ml of aggregated human IgG) for 15 min and washed twice with staining medium. The biotin-conjugated antibodies were revealed with strept-avidin (SA) PE or SA-APC. One ug/ml of propidium iodide (Boehringer Mannheim Corporation, Indianapolis, IN) was added to the stained cells (1×10^4 or 1×10^6) to exclude dead cells before analysis on a FACScan or FACSCalibur flow cytometer with Cell Quest software (Becton Dickinson & Co., Mountain View, CA) for flow cytometric analysis.

Cell Sorting.

Bone marrow cells were stained with unlabeled AB8, revealed with goat anti-Rat IgG (H + L) $F(ab')^2$ FL, blocked with normal rat serum, and stained with Ter119 PE. A fraction of the cell-sorted populations was routinely reanalyzed for expression of

individual markers and showed 98-99% purity in all experiments. The sorted populations were used for either further immunofluorescence staining and flow cytometric analysis or for in vitro cultures. Cell sorting was performed on a FACStar Four Plus (Becton Dickinson).

GiemsaStaining

Cytocentrifuge preparations were made with 1 X 10^4 of unsorted cells or each sorted population, and the slides were air dried. The slides were then fixed in anhydrous methanol (Fisher Scientific, Pittsburgh PA), and then Wright-Giemsa (Baxter Scientific Products, McGraw Park, Il) stained, and washed with water.

Cell Cycle Analysis

Sorted populations were resuspended in calcium-free PBS and permeabilized in an equal volume of ethanol at 4°C for 30 min. Cells were pelleted, mixed with 0.5mg/ml RNAase (Boehringer Mannheim) at 37°C for 20 min and then with 0.5mg/ml pepsin (Sigma Chemical Company) at 37°C for 15 min. The samples were further treated with 40ug/ml propidium iodide (Boehringer Mannheim Corporation) at room temperature for 15 min before they were run on a FACScan and analyzed using Lysys software (Becton Dickinson) for DNA content.

Stem Cell Cultures

To initiate the cultures, 2 X 10^4 of either the unsorted population or each sorted population were added to a methylcellulose medium mixture containing erythropoietin with either IL-3 or pokeweed mitogen-stimulated murine spleen-cell-cultured-medium (Mouse Bone Marrow Stem Cell Kit from Gibco/BRL or MethoCult M3430 from Stem Cell Technologies Inc.). 1-ml aliquots were then added to each well of a 24-well plate.

The cultures were incubated in a 37°C, 5% CO_2 , 90% humidity incubator for 14 d and then analyzed for colony formation.

LimitingDilution Analysis

The T220-29 stromal line, the murine NIH 3T3 cell line transfected with the human IL-7 gene (13), was irradiated (2000 rads with a cesium source irradiator) and plated at 1 X 10^3 per well on flat bottom 96-well culture plates (Costar, Cambridge, MA) in culture media (RPMI 1640 medium [Gibco BRL, Bethesda, MD] with 10% FCS [Hyclone Laboratories, Logan, UT], 100 ug/ml penicillin streptomycin, 2mM L-glutamine, essential and non-essential amino acids , sodium bicarbonate, sodium pyruvate [Gibco], and 50 μM 2-mercaptoethanol). The next day, unsorted and sorted bone marrow populations were plated on the plates containing the irradiated stomal cells, at 25, 50, 100, 200, or 400 cells per well; 120 wells of each cell dilution for each population were plated. The cultures were then incubated at 37°C, 5% CO_2 , 90% humidity incubator for 14 d. The cultures were fed at 7 d with culture media. At 14 d the plates were examined for colony formation.

Short-Term T220-29Cultures for B CellDevelopment

24-well plates were seeded at 2 X 10^3 T220-29 stromal cells in RPMI culture media, prior to the addition of 2 X 10^4 unsorted or sorted bone marrow cells per well. At the designated time points, the cultures were harvested by gentle pipeting or treatment with 1% EDTA if necessary and washed once. The clumps of stromal cells in the harvested samples were dissociated by repeated gentle aspiration in a 1-ml syringe in cold staining-buffer. The samples were stained with immunofluorescent mAbs and run on a FACScan or FACSCalibur (Becton Dickinson) for flow cytometric analysis. To exclude

dead cells, 1 ug/ml of propidium iodide (Boehringer Mannheim Corporation) was added to the stained cells. The stromal contaminants, as well as dead cells, were excluded from the analysis by an exclusion gate.

Isolation of mRNA and RT-PCR

To assess the presence or absence of mRNA transcripts, mRNA for cDNA synthesis was prepared from unsorted and sorted bone marrow populations by the TriReagent method (Molecular Research Center, Inc., Cincinnati, OH), which is based on guanidinium thiocyanate/acid phenol/chloroform extraction. After ethanol precipitation, the RNA pellet was dissolved in 20 µl of water and stored at -20°C. For first strand cDNA synthesis, 4 µl of RNA was used to convert to first strand cDNA with oligo (dT)18 primers and reverse transcriptase. One twentieth of the first strand cDNA product was subjected to PCR amplification of β-actin, λ5, and mb-1 primers. Each amplification reaction underwent 30 cycles of denaturation at 95°C for 1min, annealing at 55°C for 1 min, and extension at 72°C for 1 min for amplification and a final extension at 72°C for 10 min. The primers used were β actin (CCTAAGGCCAACCGTGAAAAG -TCTTCATGGTGCTAGGAGCCA), λ5, (CTTGAGGGTCAATGAAGCTCAG AAGA-CTTGGGCTGACCTAGGATTG), and Mb-1, GCCAGGGGGTCTAGAAG C-TCACTTGGCACCCAGTACAA).

Results and Discussion

AB8 Expression in Bone Marrow

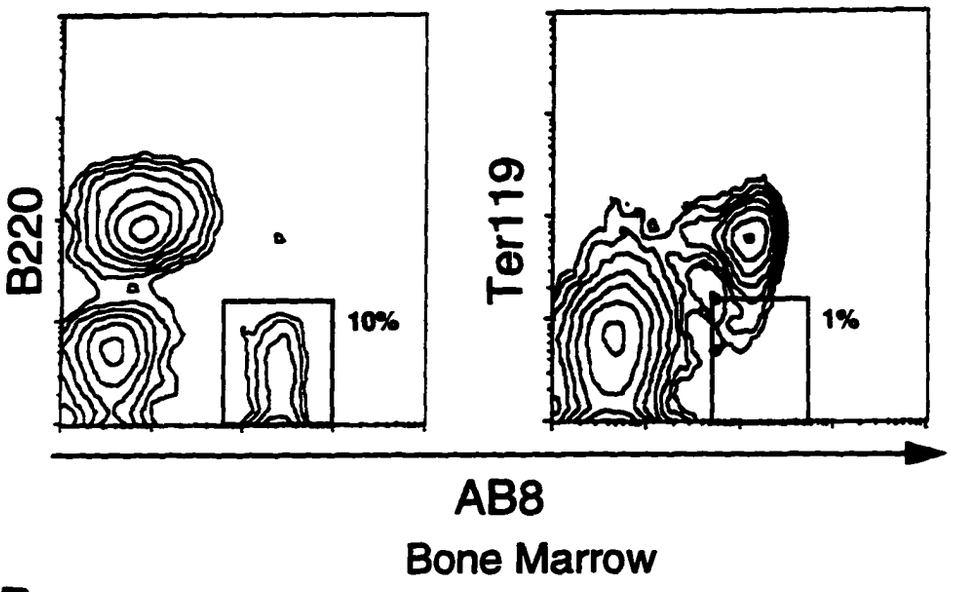
The monoclonal antibody AB8 was isolated after immunization of a rat with adult bone marrow B cell progenitors derived from T220-29 stromal cell cultures. Screening of AB8 on the cultured bone marrow cells, as well as normal bone marrow,

spleen, peripheral and mesenteric lymph nodes, thymus, and peritoneal cavity cells, showed that AB8 reacted with all the cultured bone marrow cells but only with a small percentage of cells in bone marrow (10%) and a smaller percentage of splenic cells (5%). No binding was detected in any other tissues. Secondary screening of AB8 versus antibodies to other lineage markers in the bone marrow and spleen indicated that AB8 was not expressed by B or T cells, macrophages, or granulocytes. In the spleen, AB8 binds a fraction of the NK1.1-positive, CD38 dull cells (data not shown). In the bone marrow and spleen, AB8 positive cells are divided into two populations by Ter119 binding, an erythroid marker (14) (Fig. 1A). In bone marrow, AB8 did not detectably costain B220+ cells but did bind the cultured bone marrow cells that had a pro- or pre-B cell phenotype. In cell line screening, AB8 did bind some but not all B220 positive pro- and pre-B cell lines, particularly IL-7-dependent cell lines, as well as more mature cell lines (See Table 1).

The small fraction of cells that were AB8+/Ter119neg was further analyzed for lineage markers as shown in Fig. 1B. It can be seen that this population also stains weakly for CD45LCA and CD38 but is negative for other lineage markers. CD45LCA is the common leukocyte marker, while CD38 has been shown to be expressed by hematopoietic stem cells, as well as other early progenitor cells (15). We next determined whether the AB8+/Ter119neg fraction was contained in early progenitor populations defined in other schemes of B cell development (5-7). The AB8+/Ter119neg fraction was not contained within fraction A-C (Fig. 2A). Nor is this population contained in the B220 positive/CD19 negative fraction that has been recently reported to contain NK progenitors, CD4 positive cells, as well as a fraction of cells that have lymphoid potential (Fig. 2B). When this negative fraction was sorted and restained for AB8 versus other

Figure 1. Flow cytometry of bone marrow demonstrating characteristics of AB8+ cells. Immunofluorescence staining and flow cytometric analysis of bone marrow for AB8 binding and lineage marker expression with reagents specific for B220, Ter119, CD45LCA, CD38, Gr-1, CD4, and IgM. (A) The AB8+ cells in bone marrow can be separated into two populations, both of which do not express B220. (B) AB8 binding was compared to B220, CD45LCA, CD38, Gr-1, CD4, and IgM expression in bone marrow after elimination of Ter119+/CD19+ and dead cells by use of an exclusion gate.

A.



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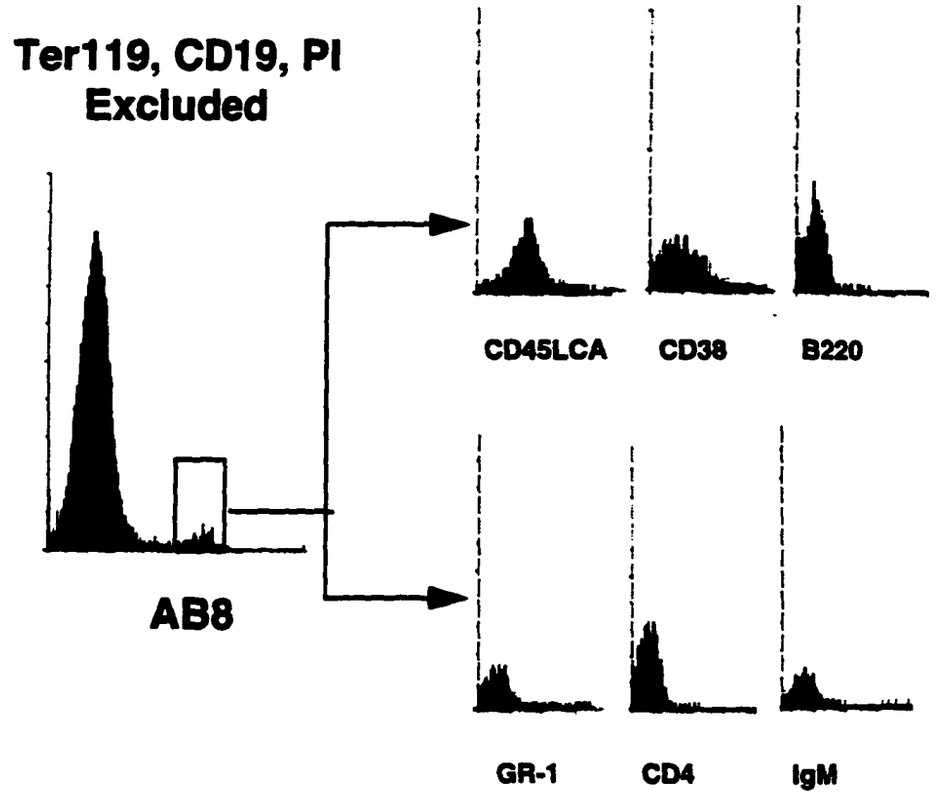


Figure 2. Comparison of the AB8+/Ter119neg bone marrow fraction to defined B lineage schemes. The binding of AB8 to bone marrow cells was compared to other early progenitor fractions by costaining with immunofluorescent reagents specific for B220, CD19, CD43, and Ter119. (A) Bone marrow fractions A-C, identified by B220 and CD43 expression, were assessed for AB8 binding. (B) The B220+/CD19neg and the B220/CD19/Ter119 negative fractions of bone marrow were assessed for AB8 binding. The B220/CD19/Ter119 negative fraction was isolated by cell sorting and subsequent immunofluorescent staining and flow cytometric analysis for AB8, as well as with reagents specific for NK1.1, CD4, CD43, SCA-1, Thy1.2, c-kit, CD38, and CD45LCA.

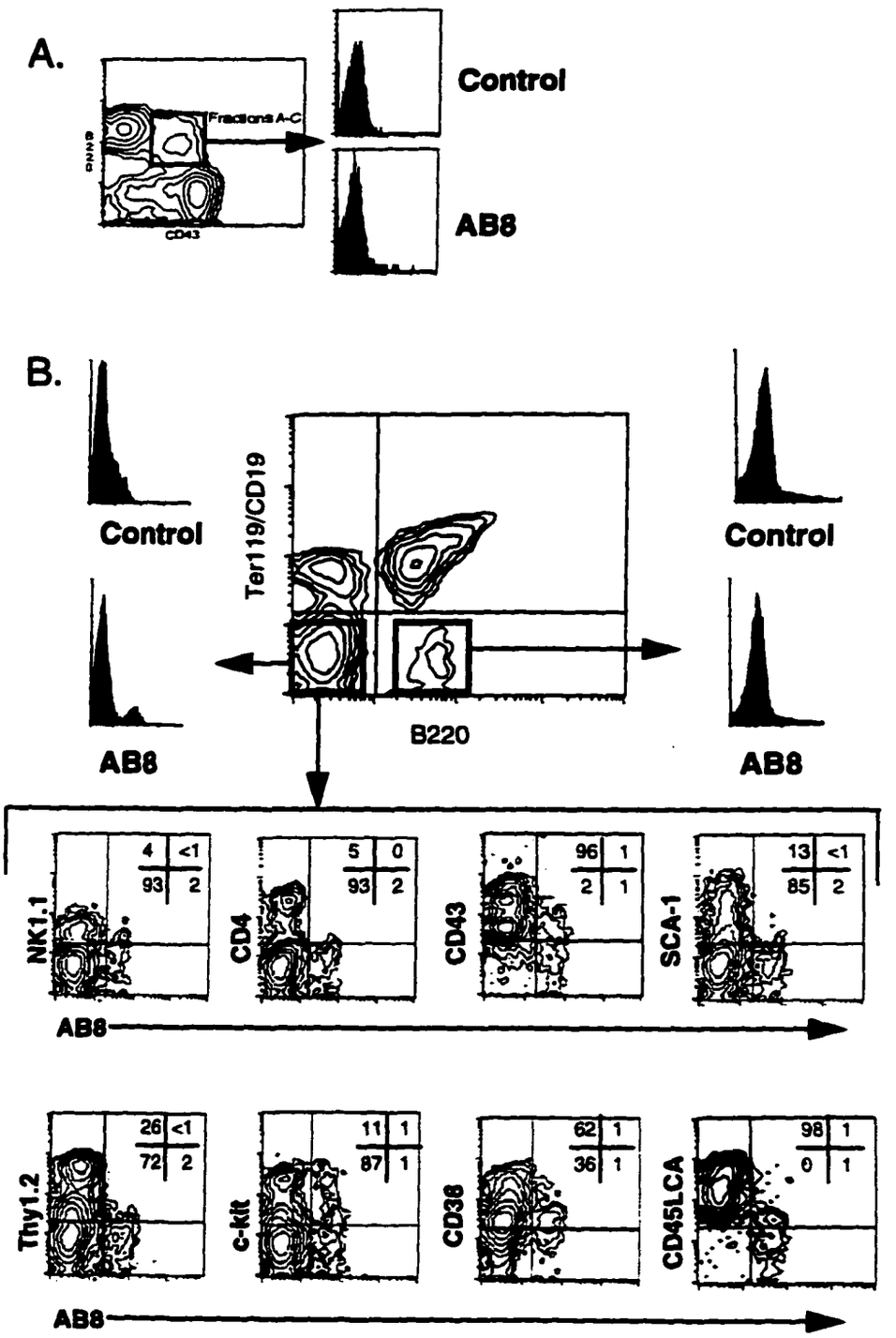


Table 1. *Analysis of AB8 Binding of Cell Lines*

Cell line	Cell type	AB8 expression
38B9	Pro-B	+
Dif-9	Pro-B	+
1881	Pro-/Pre-B	Dim
40E1	Pre-B	-
WEHI 231	"	-
WEHI 279	"	-
CH12	"	Dim to negative
CD31	"	Dim to negative
A20	"	++
SIA	T cell	++
EL-4	"	+
Yac-1	"	+
TK-1	"	-
P815	Megakaryocyte	+
WEHI 3	Myeloid	Dim
HA1.6	"	-
HTX-1	"	Dim
HATF	"	Dim
MEL	Erythroid	++
NIH3T3	Fibroblast	-
T220-29	Stromal line	-
PA6	"	-
FLST2	"	-

lineage markers, a fraction of the AB8 positive cells appeared to stain weakly for NK1.1, as well as CD43, which is expressed by many early progenitor populations at varying intensities. Neither SCA-1 nor Thy1.2 stem cell markers (16, 17) are coexpressed by this population, even though this fraction is CD38 dim. The AB8 population is split by c-kit expression.

The AB8+/Ter119neg fraction of the bone marrow represents approximately 1% of the bone marrow; this population is not contained in present schemes of early B cell progenitor populations nor in defined stem cell populations as assessed by cell surface phenotype. However, this population does express hematopoietic progenitor markers, such as CD45LCA, CD43, CD38, and c-kit. Additionally, AB8 reacts with cell lines of various hematopoietic lineages, as well as cultured bone marrow cells. This phenotype supports the proposition that the AB8+/Ter119neg cells in bone marrow include an as-yet-undefined B cell progenitor population.

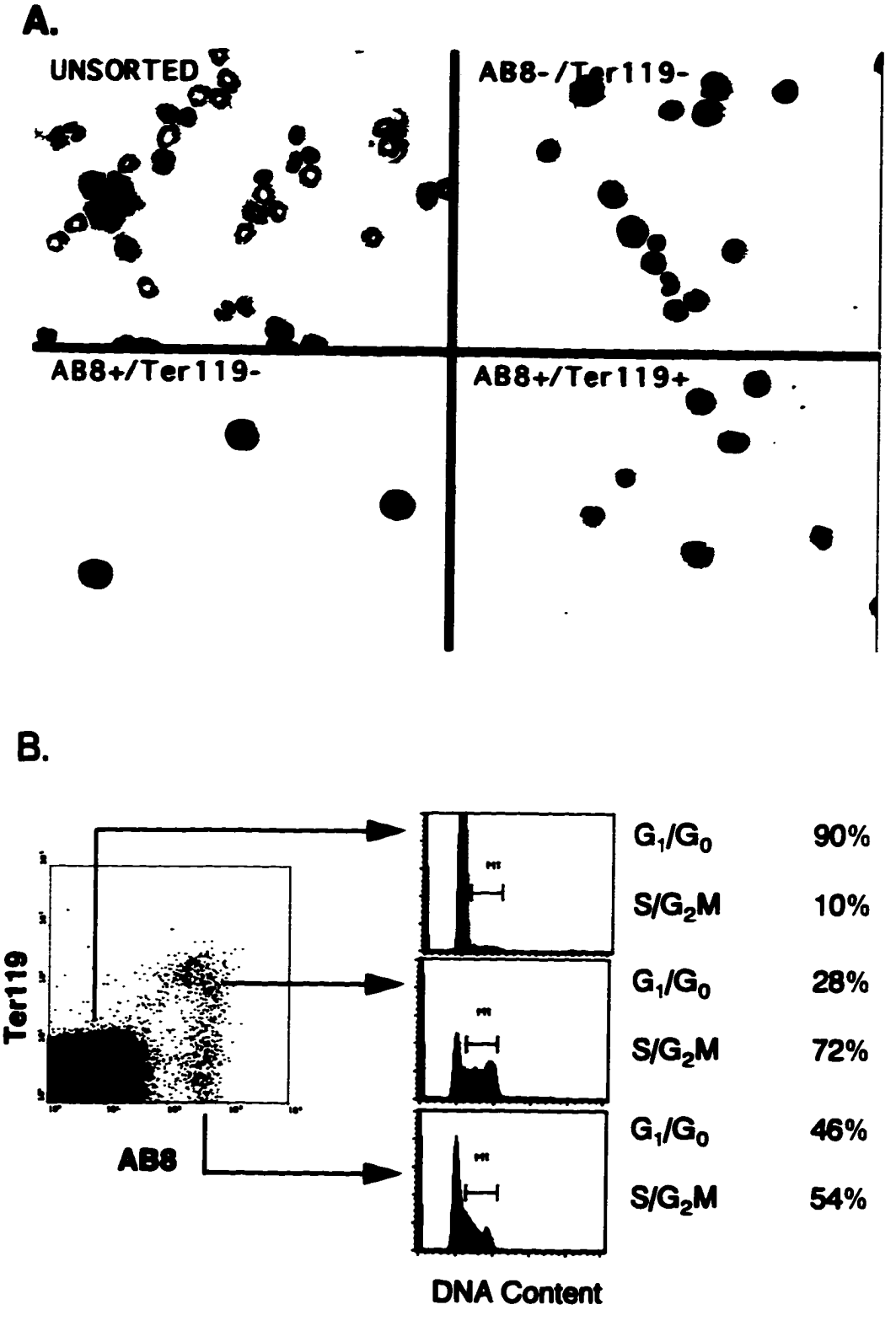
Cell Cycle and Morphology of the AB8 Sorted Populations from the Bone Marrow

Normal bone marrow was stained for AB8 and Ter119, and the sorted double negative, AB8+/Ter119+, AB8+/Ter119neg fractions were used for Wright-Giemsa staining or propidium iodide incorporation to determine morphology and cell cycle status. As shown in Fig. 3A, the AB8+/Ter119neg sorted fraction consists of large lymphoblastic cells with a circular nucleus that encompasses most of the cell mass. Analysis of the AB8+/Ter119neg fraction for propidium iodide incorporation as a measure of cell cycling showed that a significant portion of the cells are in cycle as shown in Fig. 3B. As expected, the AB8+/Ter119+ fraction contains very actively cycling cells, which would correlate with proliferating erythroid progenitors.

Hematopoietic Potential of AB8+/Ter119neg Bone Marrow Cells

The potential of the AB8+/Ter119neg fraction to develop into hematopoietic cells was next examined by in vitro assays to determine their stem cell, lymphoid, and particularly B cell potential. In each approach, bone marrow cells were sorted based on the expression of AB8 versus Ter119. The, AB8+/Ter119+, AB8+/Ter119neg double-

Figure 3. Morphological and cell cycle analysis of the AB8+/Ter119neg fraction of bone marrow. (A) Unsorted, AB8neg/Ter119neg, AB8+/Ter119+, and AB8+/Ter119neg sorted cells were used to make cytocentrifuge slides. These slides were air dried, fixed, and stained with a Wright-Giemsa stain to evaluate their cellular morphology. (B) Each of the sorted bone marrow fractions, as well as unsorted bone marrow, was assessed on a FACScan for analysis of DNA content. The percentage of cells determined to be in G_0/G_1, S, or G_2/M for each fraction in bone marrow is indicated for comparison.



AB8+/Ter119neg fractions, as well as unsorted bone marrow, were used in the cultures to evaluate the progenitor activity of each fraction.

In the first series of experiments, in vitro stem cell culture systems were used to determine the stem cell potential of the sorted fractions as tabulated in Table II. As expected, based on the flow cytometry staining, the AB8+/Ter119neg fraction does not give rise to CFU-c, BFU-e, or CFU-GEMM colonies. The failure of the AB8+/Ter119+ fraction to form BFU-e or erythroid burst colonies was likely due to the analysis of these cultures at 14 d so that colonies initiated by these cells may have died out by that time and would not have been counted. As expected, the double-negative and unsorted bone marrow formed all three types of colonies characteristic of stem cell activity.

Table 2. *Phenotype of colonies from In vitroStem Cell Cultures*

	Colonies per 2 X 10^5 Cells		
	CFU[a]	BFU[b]	CFU-GEMM[c]
Bone Marrow			
Unsorted	25	16	22
	37	10	20
Negative sort	18	11	18
	33	7	10
AB8+/Ter119+	6	3	3
	5	1	0
AB8+/Ter119neg	2	0	0
	0	0	0

a: Granulocyte-Macrophage colony (flat, non-hemogobinized, translucent cells)
b: Erythroid burst colony (densely packed orange to dark red hemoglobinized)
c: Pluripotent mixed colony (compact hemoglobinized center, periheral flat lawn of large or small cells)

In the next series of experiments, we asked if the AB8+/Ter119neg fraction of the bone marrow has lymphoid potential. This question was addressed by in vitro limiting dilution cultures using the irradiated T220-29 stromal line (14). Again, each of the sorted fractions, as well as unsorted bone marrow, was placed in the culture. As expected, the unsorted and double-negative sorted fractions were proficient in forming lymphoid colonies, forming either adherent nonuniform large myeloid-type colonies, small circular homogeneous lymphoid-type colonies, or mixed colonies. The AB8+/Ter119neg sorted fraction formed mixed phenotype colonies at all cell dilutions plated, and the AB8+/ Ter119+ sorted fraction did not form colonies (Fig. 4) .

Since the AB8+/Ter119^{neg} population in the bone marrow appeared to express leukocyte potential in the limiting dilution assays, it was next determined whether they specifically had B lineage potential. An in vitro culture system was used to address this question using a stromal line known to support B lineage differentiation. The sorted AB8+/Ter119neg faction give rise to B lineage cells on the T220-29 stromal cell line, as illustrated in Fig. 5. As expected, in these cultures, the unsorted and the negative sorted bone marrow also generates B220+ B lineage cells in culture. The AB8+/Ter119+ sorted fraction was not productive on this stroma and did not give rise to cells which could be reanalyzed for phenotypic markers. Fig. 6 shows the percentage of cells at each defined stage of B cell differentiation that were generated after culture from each bone marrow fraction. In summary, the AB8+/Ter119neg fraction generates cells in culture representing each stage of early B cell differentiation. The pro-B fraction represents only about 2% of the cultured cells, but this is not strikingly different from the unsorted or the negatively sorted fraction. The percentages of late pro-B/Pre-B fraction, as well as the

Figure 4. Limiting dilution analysis of sorted bone marrow fractions grown on irradiated T220-29 stromal line. The bone marrow sorted AB8+/Ter119neg and AB8+/Ter119+ fractions were used in a limiting dilution assay to calculate the frequency of cells with leukocyte potential in each fraction. Each data point represents 240 individual cultures. A representative colony formed by the AB8+/Ter119neg fraction is shown.

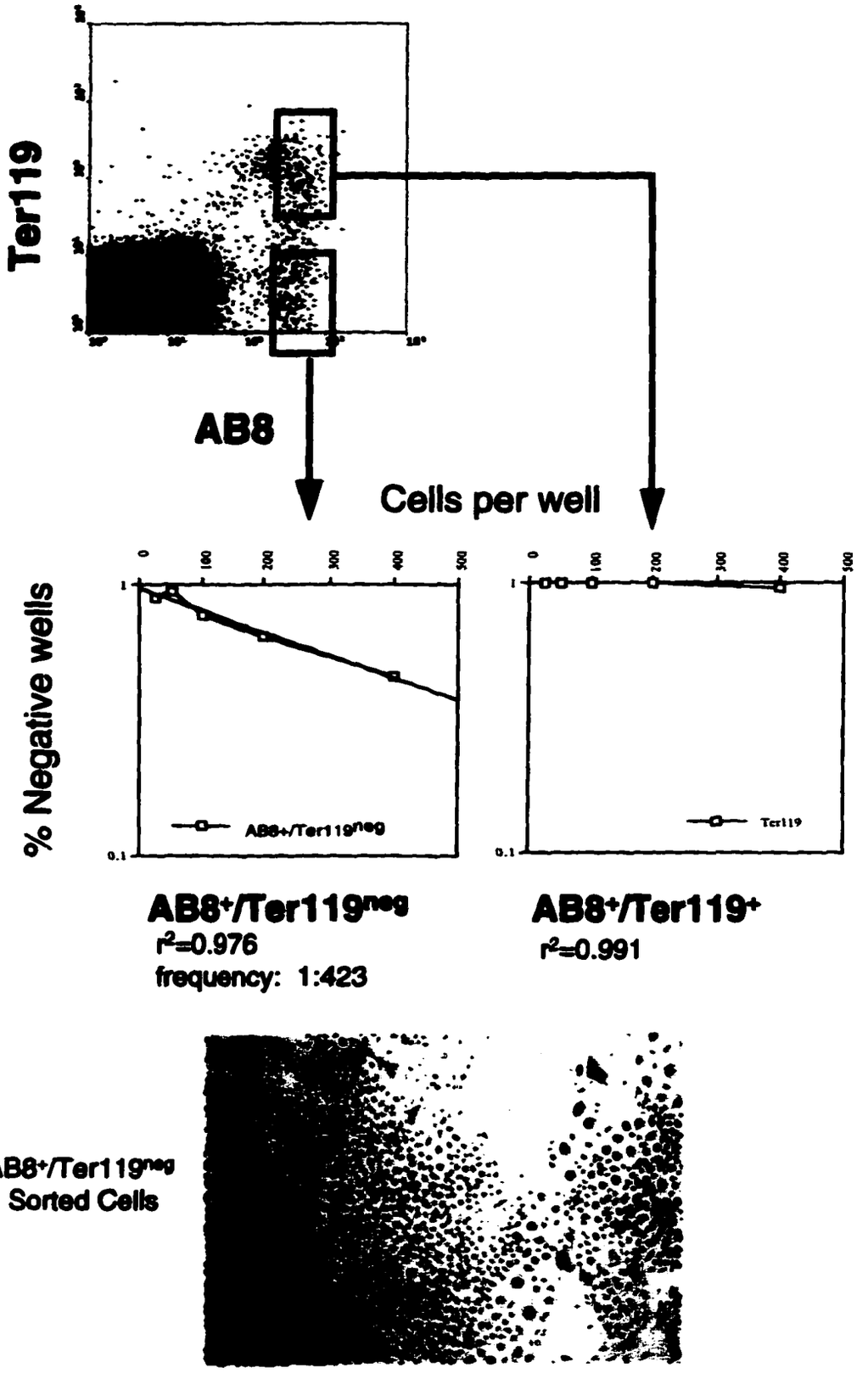


Figure 5. Postculture phenotype of cells derived from T220-29 cultures. Unsorted bone marrow, as well as sorted bone marrow fractions, was cultured on the T220-29 stromal line. At 6 d in culture, the cells from each population were harvested and analyzed by flow cytometry. Antibodies specific for CD43, B220, CD45LCA, BP-1, and IgM were used to determine the percentage of cells in the cultures at each B lineage stage. Forward scatter versus side scatter, as well as propidium iodine uptake, was used to exclude remaining stromal cells and dead cells, respectively.

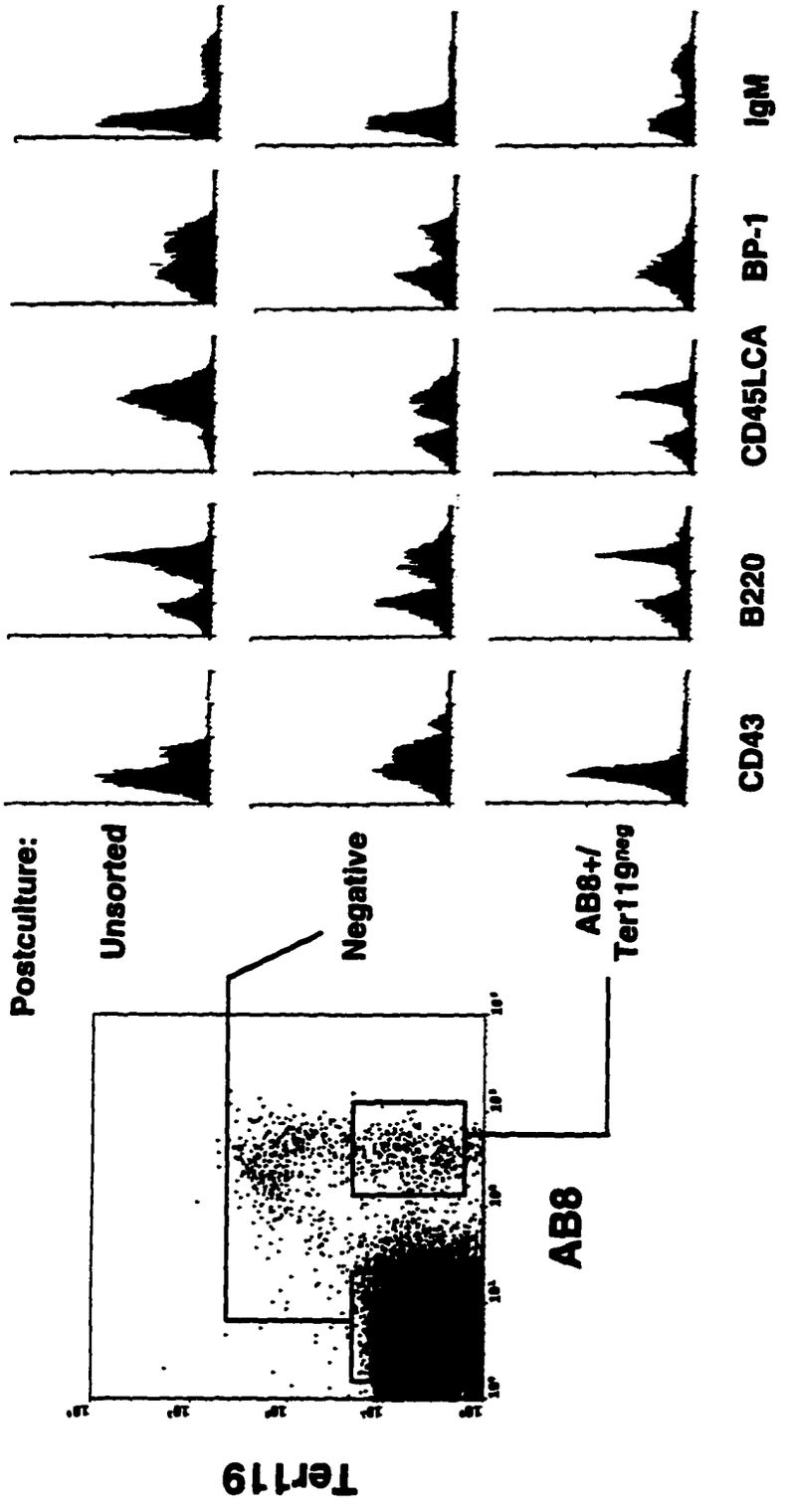
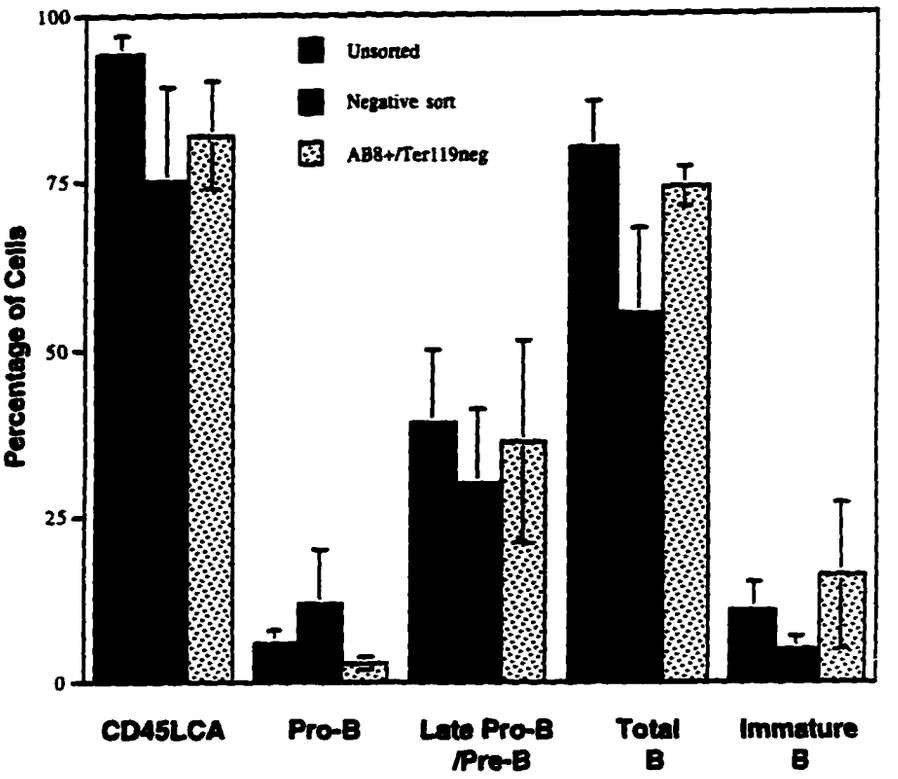


Figure 6. Postculture percentages of cells in B lineage differentiation stages. Phenotype of lymphocytes derived from T220-29 cultures unsorted bone marrow, as well as sorted bone marrow fractions, were cultured on the T220-29 stromal line. At 6 d in culture, dual color combinations of antibodies specific for CD43, B220, CD45LCA, BP-1, and IgM were used to determine the percentage of cells in the cultures at each B lineage stage. Forward scatter versus side scatter, as well as propidium iodide uptake, was used to exclude remaining stromal cells and dead cells, respectively.



total percentage of B lineage cells from any of the fractions, were not remarkably different. Recovery of surface mu immunoglobulin (IgM) positive cells varies from experiment to experiment and appears to correlate with the strain of mice used.

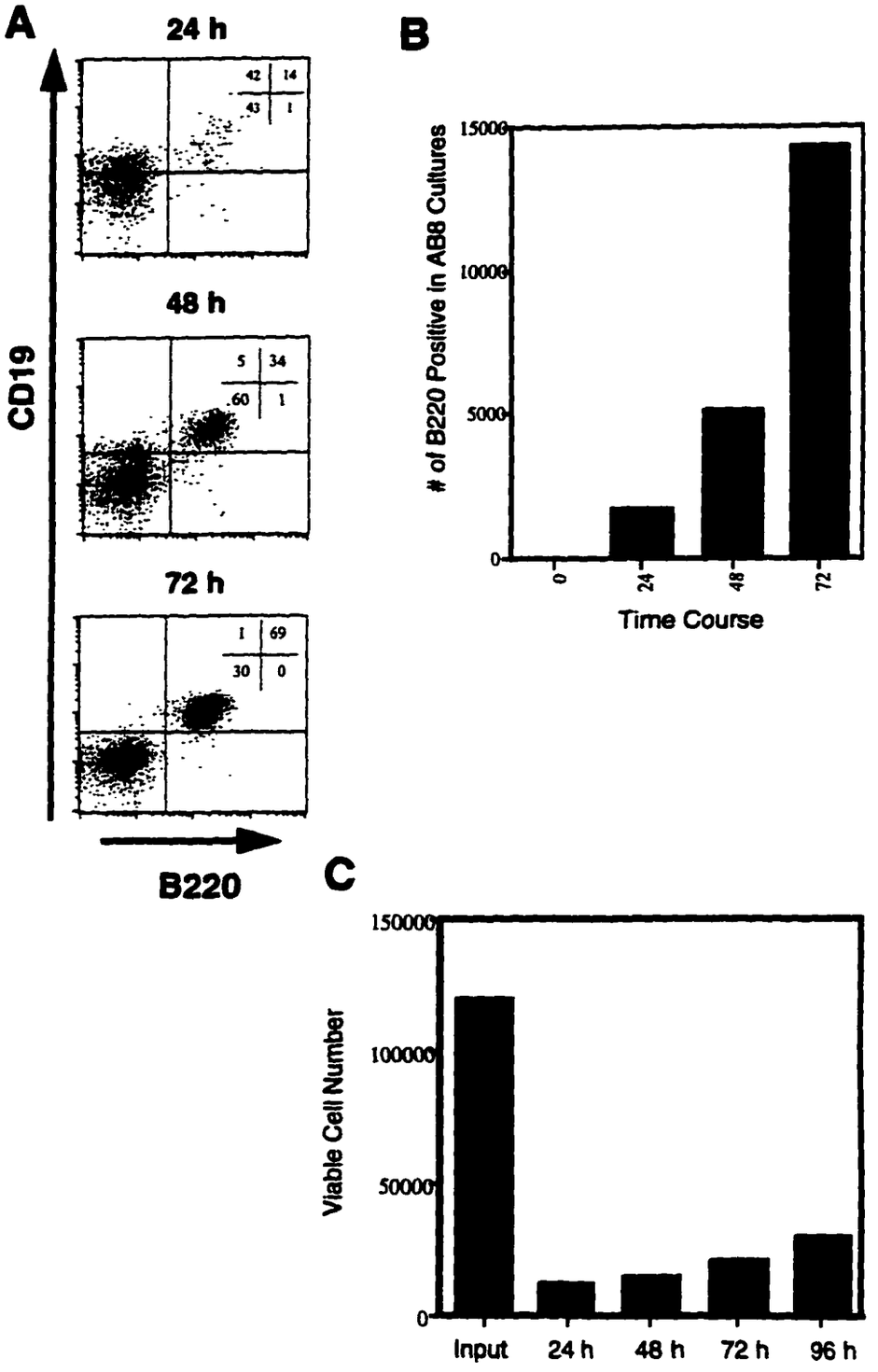
Kineticsof The Progression into the B Lineage

Experiments directed at determining the kinetics of the transition from AB8+/Ter119neg-stage cells to cells expressing B220 and CD19 demonstrated that the AB8+/Ter119neg population was temporally similar to that shown for the progression of fraction A to immature B cells after 4 d in culture (5) (Figure 7). After 24 h in culture, only a few cells begin to express B220 and CD19, but by 72 h, 70% of the cells in culture are B220+/CD19+ (Fig. 7A). This cannot be accounted for solely by the expansion of B220+ cells present after 24 h in culture as shown in Fig. 7B. There is a seven-fold increase in the number of B220+ cells after 48 h of culture, corresponding to a decrease in the number of AB8+ cells in culture. However, this increase in cells expressing B220 and CD19 is not matched by a corresponding increase in cell numbers in the cultures over time. Additionally, the determination of cell viability at each time point examined revealed that the majority of AB8+ cells placed in culture die in the first 24 h; after this initial 24 h, the number of viable cells in the cultures does not increase significantly for an additional 24 h (Fig. 7C). This combination of data indicates that the cells within the AB8+/Ter119neg population with B cell potential survive and differentiate for the first 72 h of culture before a significant amount of proliferation is seen.

Comparison of Early Progenitor Populations

We next attempted to address the efficiency with which the AB8+/Ter119neg cells give rise to B cells by comparing the efficiency of them to another early progenitor

Figure 7. Phenotypic changes of the AB8+/Ter119neg cells of bone marrow after 24, 48, and 72 h in culture. (A) Flow cytometry analysis of cultured cells stained with immunofluorescent mABs specific for B220 and CD19 are presented as dual color dot plots with the percentage of cells in each quadrant calculated. (B) The number of cells expressing B220 at each time point is shown. (C) The number of viable cells harvested at each time point is compared to the number of cells used to start the cultures.

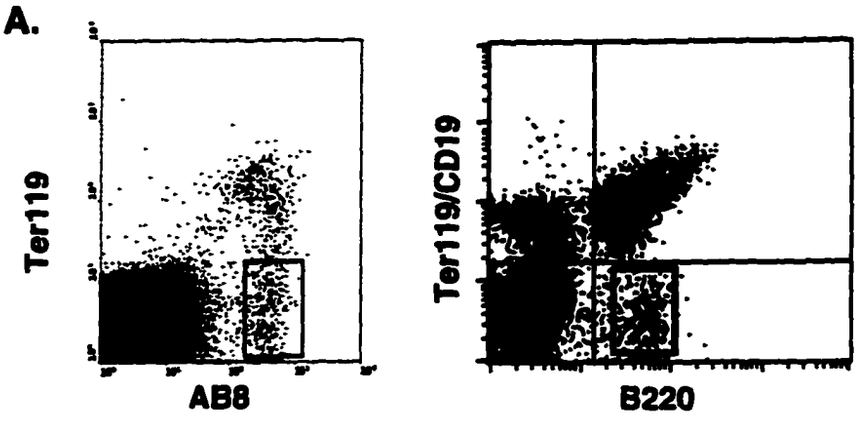


population, the B220+/CD19neg fraction of bone marrow (Fig. 8A). Even though the B220+/CD19neg is a mixed population, it does contain lymphoid progenitors at the earliest defined committed B lineage stage. The sorted fractions were placed on the T220-29 stroma for 6 d, harvested, and analyzed by flow cytometry for B lineage markers. The AB8+/Ter119neg fraction was not more efficient than the B220+/CD19neg sorted fraction in giving rise to more mature B lineage cells, as demonstrated in Fig. 8B. It was not markedly slower than the B220+/CD19neg fraction of bone marrow in comparison of percentage of culture, nor was there a dramatic difference in the number of cells in each B cell stage from either culture.

Gene Expression in Unsorted and Sorted Bone Marrow Fractions

The sorted fractions were analyzed for the expression of mb-1 and λ5, molecular markers characteristic of the earlier stages of B cell commitment (Fig. 9). The mb-1 gene encodes Ig-α, is associated with the immunoglobulin complex (18), and is expressed very early in differentiation (19). Transcripts for mb-1 were detected in both unsorted and the negative sorted bone marrow fractions, as expected. No transcript was detected in the AB8+/Ter119+ fraction, but transcripts were detected in the AB8+/Ter119neg sorted fraction. The AB8+/Ter119neg fraction also expresses λ5, which is reported to be expressed earlier in differentiation with a 130-kD protein (20, 21). Staining for Tdt expression in the AB8+/Ter119neg sorted fraction was negative (data not shown); thus it is expected that no transcript for Tdt will be present. These results are additional evidence indicating that the AB8+/Ter119neg fraction may contain an early B cell progenitor population.

Figure 8. Comparison of early progenitor populations on T220-29 cultures. The bone marrow sorted AB8+/Ter119neg and B220+/CD19neg fractions were cultured for 6 d on the T220-29 stromal line. After 6 d of incubation, the cells derived from each sorted fraction were assesed by immunofluorescence staining and flow cytometric analysis with antibodies specific for CD43, B220, CD45LCA, BP-1, and IgM to determine the percentage of cells in the cultures at each B lineage stage. Forward scatter versus side scatter, as well as propidium iodide uptake, was used to exclude remaining stromal cells and dead cells, respectively.



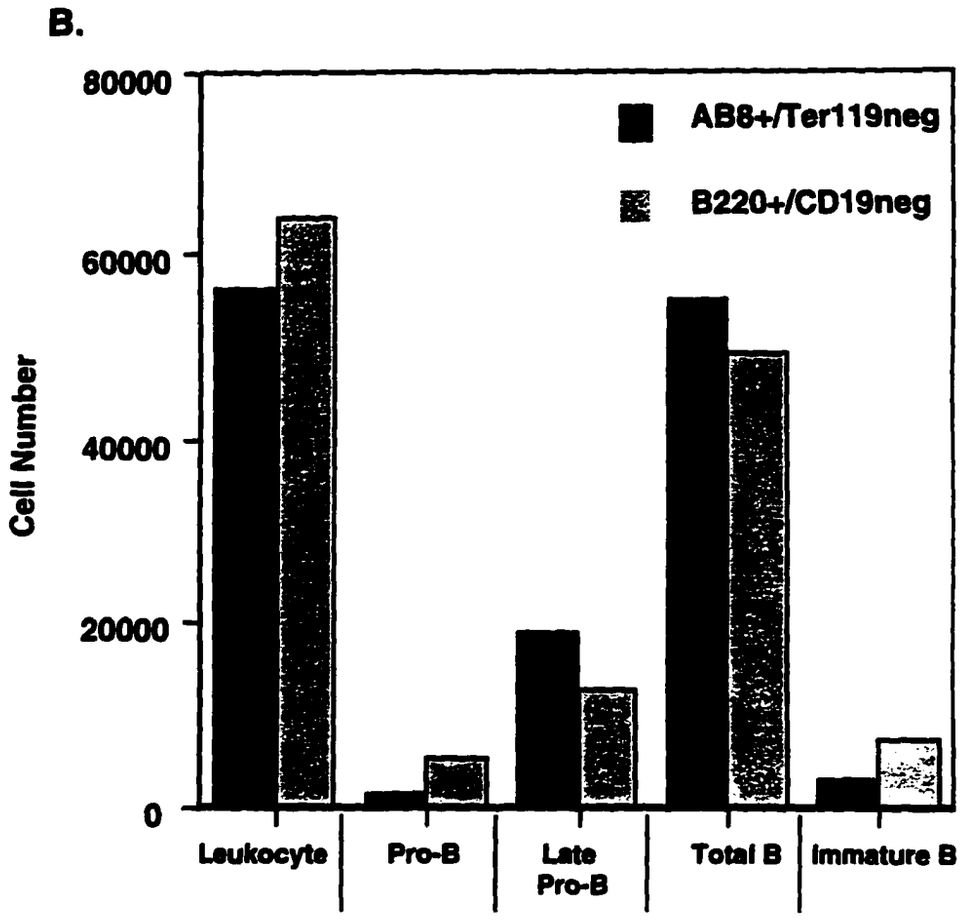
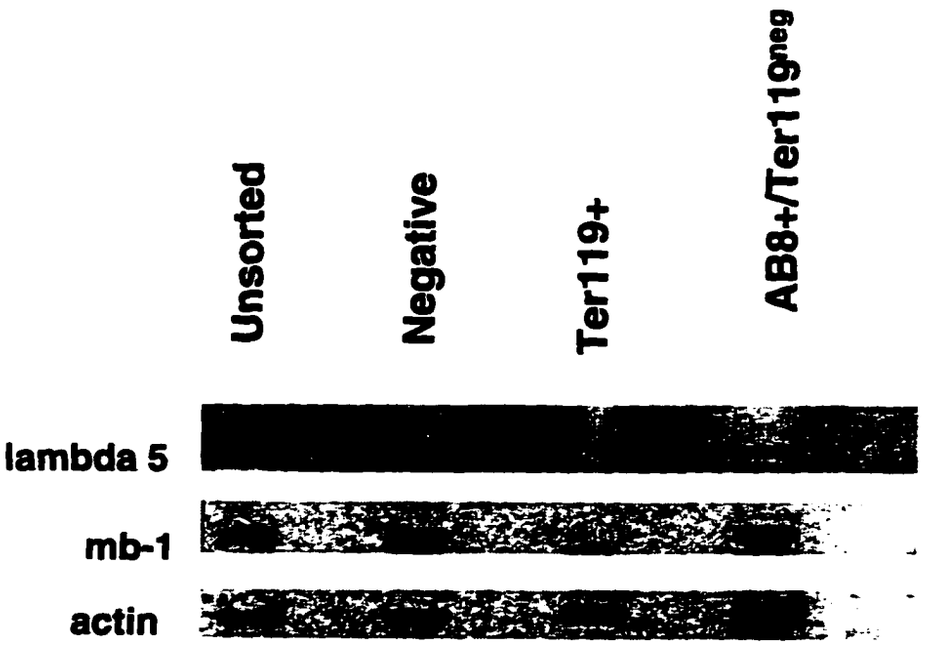


Figure 9. Gene expression patterns. Representative ethidium bromide stained gels of PCR transcript amplifications of unsorted and sorted bone marrow fractions are shown. From unsorted bone marrow and each sorted fraction, cDNA was generated and used to detect gene expression of β-actin, lambda 5, and mb-1.



it is expected that no transcript for Tdt will be present. These results are additional evidence indicating that the AB8+/Ter119neg fraction may contain an early B cell progenitor population.

Discussion

AB8 binding is restricted to subpopulations of lymphocytes in the adult bone marrow, where it appears to define a transitional progenitor population. In addition, AB8 binds to populations of fetal hematopoeitic cells (manuscript in preparation). Clearly, the AB8+/Ter119neg population in bone marrow is not contained in fraction A-C, nor in the B220+/CD19neg population. The AB8+/Ter119neg cells are not stem cells. However, this progenitor may give rise to more than one lineage, as suggested by the morphology of colony types formed in the limiting dilution assays. The T220-29 culture assays clearly show that AB8+ cells can generate B cells in culture, with B cells representative of each characteristic stage. Additionally, the AB8+/Ter119neg fraction appears to move as quickly through the stages of B cell differentiation in culture as does the B220+/CD19neg population. The preliminary RT-PCR data adds evidence to indicate that these cells in bone have progenitor activity.

The predominant type of colony seen in the limiting dilution assays with the AB8+/Ter119neg fraction consisted of adherent, large, nonuniform, myeloid-type cells with clusters of non- or loosely adherent homogenous circular lymphoid-type cells on and interdigitating with the adherent myeloid-type cell layer. Similar types of colonies were seen in limiting dilution culture assays using sorted 12 d fetal liver cells. These sorted 12 d fetal liver cells were reported to be multipotential progenitor cells for the B and myeloid lineages (11, 22, 23). Also retroviral marking experiments of ES cell cultures and bone marrow progenitors have suggested a common progenitor for these lineages (9,

10, 24, 25). But to date, no such population has actually been phenotypically identified or isolated from adult bone marrow.

As noted above, the T220-29 culture assays clearly show that AB8+ cells can generate B cells at all characteristic stages in culture. This is intriguing because it implies that the AB8+/Ter119neg fraction progresses through the steps of B cell differentiation. Also, this population may be more efficient in putting together an immunoglobulin complex than the negative sorted population, which contains mostly committed B lineage cells. A possible explanation for this is that the AB8+/Ter119neg fraction, when placed in culture, may differentiate without the regulatory restrictions normally found in the bone marrow; thus all cells with a complete immunoglobulin rearrangement expand in these cultures. However, the negative-sorted fraction contains committed B lineage cells that have been exposed to the regulatory environment of the bone marrow and may not differentiate further but are capable of proliferating on the stroma while they might otherwise be deleted in the bone marrow. Support for this idea comes from the data showing B cell progression from the B220+/CD19neg fraction compared to the AB8+/Ter119neg fraction. Again, the B220+/CD19neg fraction contains an early B lineage cells, which, in culture, would differentiate without the regulatory restrictions normally found in the bone marrow, and does seem to move as quickly as the AB8+/Ter119neg fraction through B cell differentiation steps in culture.

However, another explanation may be that the AB8+/Ter119neg fraction represents a progenitor population which is an alternate lineage commitment pathway that is more permissive for the B1 lineage. Furthermore, this seemingly unregulated efficient progression throughout B cell differentiation may be reflective of the normal differentiation of B1 lineage cells that is normally overlooked in bone marrow. However,

experiments to address this have not given conclusive evidence for an alternative pathway to date.

In these T220-29 cultures, more than 1 in 423 of AB8+/Ter119neg cells, the frequency calculated from the limiting dilution assays, survives and contributes to the B lineage cells seen in the short-term T220-29 cultures. The most convincing evidence of this is data obtained in the kinetic studies of the AB8+/Ter119neg cells on the T220-29 stromal cell line. In these cultures, approximately 10% of the culture survives, and the majority go on to differentiate into B220+/CD19+ cells, which is approximately a 1 in 10 frequency for survival and 1 in 30 for B lineage potential. These estimated frequencies do not match the 1 in 423 estimated frequency from the limiting dilution culture assays. This suggests that, under the right microenvironmental conditions, these surviving cells can be pushed into the B lineage pathway. One reason for this difference may be the stroma conditions. Even though the same stromal line was used, in the limiting dilution cultures the stroma is irradiated, while in the short term T220-29 cultures, it is not. Second, in the limiting dilution cultures, myeloid-type cells do grow out, which may inhibit more mature B cell generation, leaving no dual potential cells to be detected. Additionally, in the short term T220-29 cultures, the cells that rapidly progress into the B cell lineage do so and may die out by 2 wk, when the long term cultures are assessed.

The RT-PCR data provides preliminary additional support that the AB8+/Ter119neg fraction is an early B cell progenitor population. Further investigation of genes expressed other than those that are B cell-specific should help to confirm the AB8+/Ter119neg population as a multipotential progenitor population.

From the data presented, it is suggested that the AB8+/Ter119neg cells are a transitional progenitor population between a stem cell and a committed lineage cell. This

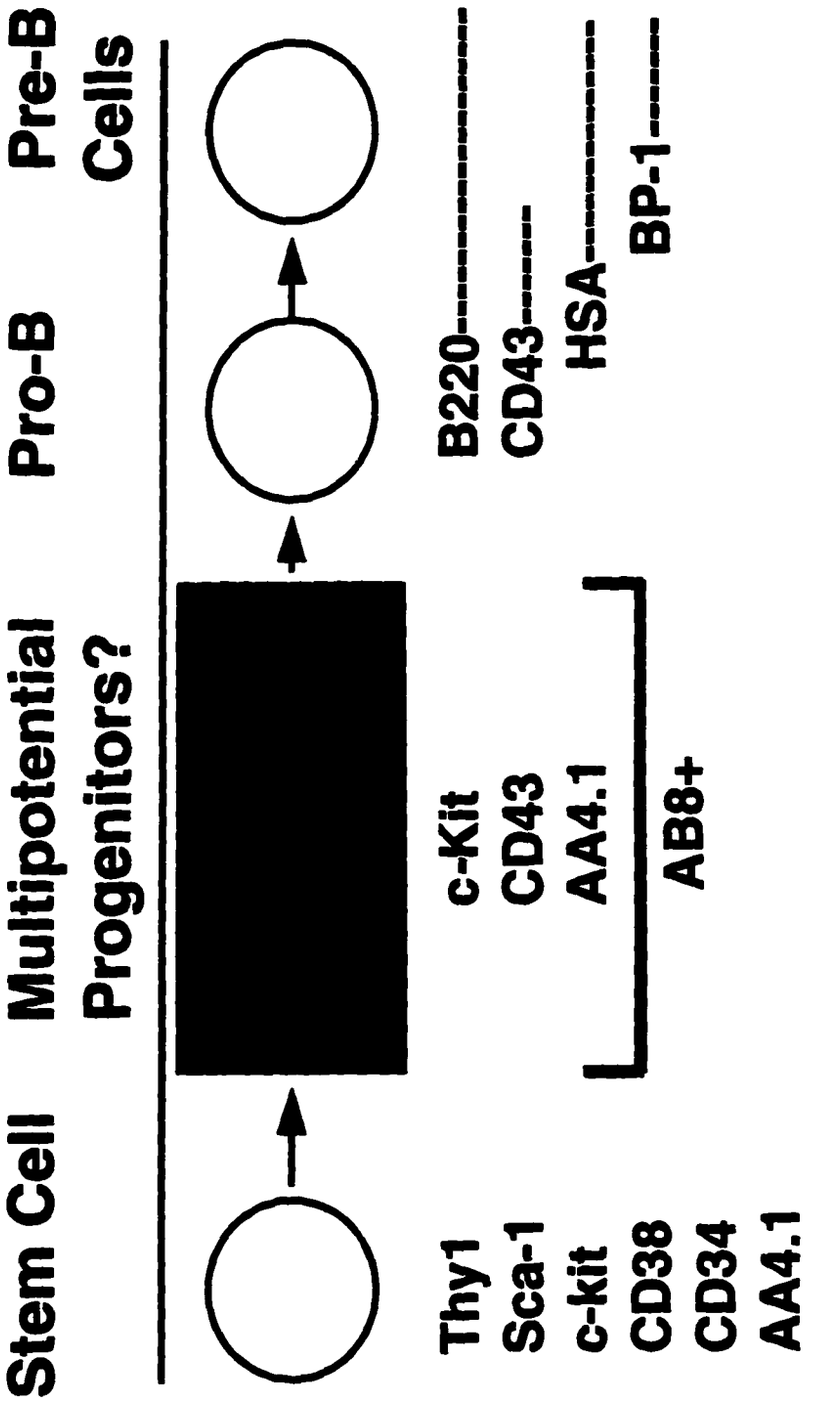
population would not have been identified in stem cell studies because it does not express markers used to isolate stem cells. The B220 marker used to identify the earliest progenitors of B lineage cells is absent or present at undetectable levels; so again, this population would not have been considered in the described B lineage schemes.

This transitional progenitor population is approximately 1% or less of the bone marrow. Thus, without a definitive, specific marker for this restricted transitional step, the AB8+/Ter119neg population would be easily missed. The monoclonal AB8 is unique in that it marks this transitional step: it does not mark the stem cell or committed lymphoid progenitors. Other cells in the bone marrow which are detected by AB8 can be easily excluded by erythroid markers and do not survive in lymphoid culture systems, so they do not obscure the results obtained.

As diagrammed in Fig. 10, the data presented here suggest that AB8 can be used to isolate a) early progenitors with B lineage potential, b) possibly early progenitors with myeloid and B lineage potential, and c) most erythroid lineage cells by costaining with Ter119. The ability of these populations to generate other hematopoietic lineages is untested. This transitional progenitor population may be a universal step in lineage progression or an alternate pathway of lineage commitment, perhaps of B1 lineage progression. This could explain the relatively low frequency of B1 cell production in adult bone marrow. However, experiments to address this issue in the bone marrow have not been definitive. In this model, AB8 would be expressed soon after a stem cells moves out of the stem cell pool and is retained until commitment to a lineage occurs, except in the erythroid lineage.

In conclusion, AB8 has enabled a transitional progenitor population from the bone marrow to be identified, characterized, and isolated. Initial studies with this isolated

Figure 10. AB8 expression by early precursors in bone marrow. A model of lineage potential is postulated based on the data presented in this report of where the AB8+/Ter119neg population fits into the scheme of hematopoietic differentiation.



population suggest that it may be a multipotential potential progenitor. Further analysis of the lineage potential and gene expression of the AB8+/Ter119neg population in the bone marrow will allow issues of lineage commitment and differentiation to be addressed that could not be previously addressed because there have been no existing ways to identify and isolate these cells.

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ISOLATION AND CHARACTERIZATION OF AN EARLY TRANSITIONAL PROGENITOR POPULATION IN MURINE FETAL LIVER

by

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In preparation for *European Journal of Immunology*

Format adapted for dissertation

Summary

During fetal development, B cells appear in a programmed pattern in relation to the order and timing of hematopoietic cell seeding. Although the progression of events within the B cell lineage is similar in fetal liver and adult bone marrow, there are differences associated in progenitors or molecular mechanisms operating at each ontological time. We have used a monoclonal antibody, AB8, to identify, isolate, and characterize an early progenitor cell population present in fetal liver. AB8+ cells do not have stem cell activity but do have the potential to give rise to myeloid and lymphoid colonies. When placed on a stromal line that is efficient in supporting differentiation of B cells, AB8+ cells progress through defined stages of B lineage differentiation. Additionally, cells of the B1 phenotype are generated from the AB8+ population in these stromal cultures. These data suggest that multipotential progenitor cells exist within the AB8+ population and that AB8 detects a molecule that is restricted in its expression on a cell undergoing transition from the limited or multipotential progenitor to a unipotential cell.

Introduction

Sites of hematopoietic origins in fetal development are multifocal, each varying in the cell lineages that can be generated from each site. The lineage capacities of these hematopoietic stem cells may be affected by a varity of factors, including microenvironment. Hematopoietic stem cells were initially determined to originate from the embryonic yolk sac (1-3). Later studies demonstrated that, although stem cells from the yolk sac do seed intraembryonic tissues, definitive adult hematopoiesis originates

from hematopoietic stem cells that form in an intraembryonic source in avian species (4, 5). Similar findings were reported in amphibian studies (6, 7). Basically, these avian and amphibian studies showed that progenitors from yolk sac or ventral blood islands give rise to the first wave of erythroblasts and myeloid cells. Later studies showed that the yolk sac-derived stem cells are less potent than intraembryonic hematopoietic stem cells in proliferative capacity and differ qualitatively in their differentiative potential (8-10).

Current studies in mice have shown that intraembryonic sites are the predominant source of hematopoietic stem cells (9-15). These sites are the paraaortic splanchnopleura at 8.5 days post coitius (dpc) or at 10-11 dpc in the aorta, gonads, and the mesenephros (AGM) tissues.

Hematopoietic stem cells are seeded into fetal tissue rudiments at various times, which start after blood circulation is initiated (16, 17). The liver is first colonized at 10 dpc, with erythropoiesis seen at 11 dpc, while myelopoiesis and lymphopoiesis begin at 12 dpc (18-20). The fetal liver continues to be the major site of hematopoiesis in fetal life and continues this activity until approximately 1 wk after birth (21), although there is evidence that the liver may resume hematopoietic stem cell activity in adult life under certain circumstances (22). The thymus is colonized by hematopoietic stem cells at 11 dpc, and significant lymphopoiesis begins at 12 dpc (23, 24). The cells present at 12 dpc in the thymus retain the ability to differentiate into the T, B, or myeloid lineages (25). A second wave of hematopoietic cells seed the thymus at 13-14 dpc. The second wave of cells to seeds the thymus migrates from the fetal liver and is more restricted in lineage potential (26). Progenitors of B and T cells are detected in the omentum by 13 dpc (27-

30). The origin of these cells and information as to whether these are hematopoietic stem cells or more committed progenitors is undetermined. The mesentery at this time and later also contains similar cells, which is not suprising, considering that these two tissues arise from a common source and are contiguous with each other. The spleen is colonized at 15 dpc; initially erythropoiesis, then myelopoiesis and lymphopoiesis ensue. The spleen is seeded again in ontogeny by thymus and fetal liver -derived precursors. In adult animals, the spleen is seeded by bone marrow and thymus and is a peripheral lymphoid organ (14, 31). Finally, bone marrow is seeded by hematopoietic stem cells at 16 dpc and becomes the predominant site of hematopoiesis in the adult (16, 31).

Experimental evidence for poststem cells, but still multipotential progenitor cells, is more abundant in fetal development than in adult bone morrow. These multipotential cells have been shown by analysis of progenitor cells marked with retroviral markers in embryoid bodies that are derived from cultured embryonic stem cells or bone marrow (32, 33). Evidence for these multipotential cells was also obtained from in vitro culture assays of 12-dpc fetal liver, in which certain populations maintain the capacity to differentiate into both B or myeloid lineages (33-35). In these in vitro culture experiments, reagents specific for c-kit, AA4.1, and Sca-1 were used to fractionate the fetal liver cells to show that the multipotential capacity was restricted to a particular, small subpopulation. However, these markers are expressed over several differential stages of hematopoiesis, including stem cells. It was not demonstrated that this sorted population did not have stem cell capacities.

In this report, the antibody AB8 is used to identify a population of progenitors within the fetal liver that has multipotential ability, including specifically B lineage potential, but clearly does not have the capacity of a hematopoietic stem cell.

Material and Methods

Animals And Cell Preparation

BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Raleigh, NC) and bred in our own animal facilities for use in all experiments. Pregnancies were detected by daily examination for vaginal plugs, with the plug date being 0 dpc. The pregnant mice were sacrificed, and the fetal tissues were harvested. Fetal tissues were prepared by gentle deaggregation followed by aspiration and expulsion through a 16-gauge needle and syringe. These preparations were washed, resuspended, treated with lysing buffer (0.017 M Tris, 0.16 M ammonium cloride, pH 7.2), washed, and passed over glass wool, resulting in single cell suspension, which were then used for flow cytometric analysis or in vitro culture experiments.

If cells from in vitro culture experiments were to be analyzed postculture by flow cytometric means, they were harvested by gentle pipeting together with treatment with 1% EDTA if necessary and washed prior to immunofluoresecent staining and analysis.

Antibodies

The fluorescent (FL)-, phycoerythrin (PE)-, biotin (bio)-, or allophycocyanin (APC)-conjugated mAbs RA3.6B2 (anti-CD45), anti-CD45LCA, 7D4 (anti-CD25), S7 (anti-CD43), PK136 (anti-NK1.1), 90 (anti-CD38), anti-GR-1, anti-CD4, anti-CD8, anti-c-kit, anti-CD5, anti-Ter119, anti-Sca-1, Strept-avidin (SA)-APC, and Rat IgG2b control

were obtained from PharMingen (San Diego, CA). A hybridoma producing the rat anti-mouse AB8 (IgG2b) was isolated and characterized in our laboratory by immunizing a rat subcutaneously six times at 3- to 4-d intervals with purified pre-B cells dervied from IL-7-dependent cultures of mouse bone marrow cells into the area drained by the popliteal lymph nodes. 1 d after the last injection, the lymph nodes were isolated, and the cells were fused to P3X63Ag8.653 cells (36). The hybridoma cells lines producing anti-CD19, Ter119, and F4/80 were obtained from Dr. Douglas Fearon, Dr. Irving Wiessman, and the American Tissue Culture Collection, respectively. Antibodies purified from hybridoma culture supernatants on protein G-Sepharose columns (Pharmacia, Uppsala, Sweden) were either biotinylated or labeled with cychrome five (Cy5) (Amersham Life Science, Pittsburgh, PA) according to standard procedures. FL-conjugated goat anti-mouse IgM and SA-PE were obtained from Southern Biotechnology Associates (Birmingham, AL). The FL goat anti-rat IgG $F(ab')_2$ was obtained from Gibco/BRL (Paisley, Scotland).

Immunofluorescence Staining And Flow Cytometric Analysis

Cell suspensions were stained with either unlabeled AB8, F4/80, or control rat IgG2b and revealed with goat anti-Rat IgG (H + L) $F(ab')_2$ FL, blocked with normal rat serum, and then stained with FL-, PE-, bio-, APC-, and/ or Cy5-labeled antibodies in staining medium [1% BSA in PBS with 10 μg/ml of aggregated human IgG (Southern Biotechnology Associates)] for 15 min and then washed twice with staining medium. The biotin-conjugated antibodies were revealed with SA-PE or SA-APC. To exclude dead cells, 1 μg/ml of propidium iodide (Boehringer Mannheim, Corporation, Indianapolis, IN) was added to the stained cells (1×10^4 or 1×10^6) before the stained cell

preparations were analyzed on a FACScan or FACSCalibur with Lysys or Cell Quest software for flow cytometric analysis (Becton Dickinson).

Cell Sorting

Fetal liver cell preparations were stained with unlabeled AB8, revealed with GαRat IgG (H + L) $F(ab')_2$ FL, blocked with normal rat serum, and stained with Ter119 PE. A fraction of the sorted populations was routinely reanalyzed and was shown to be 97-99% pure in all experiments. The sorted populations were used for in vitro cultures. Cell sorting was performed on a FACStar Four Plus (Becton Dickinson & Co., Mountain View, CA.).

Stem Cell Cultures

To initiate the cultures, 2×10^4 of the unsorted population and of each sorted population was added to a methylcellulose medium mixture containing erythropoietin and PWM-stimulated murine spleen culture medium (MethoCult M3430 kit, Stem Cell Technologies, Inc.); 1-ml aliquots were then added to a well in a 24-well plate (Costar, Cambridge, MA). The cultures were incubated in a 37°C, 5% CO_2 , 90% humidity incubator for 14 d. The cultures were then analyzed for colony formation.

Limiting Dilution Analysis

The T220-29 stromal line, NIH 3T3 cell line transfected with the human IL-7 gene (37), was irradiated (2000 rads with a cesium source) and plated at 1×10^3 per well on flat-bottom 96-well culture plates (Costar) in culture media [RPMI 1640 medium (Gibco BRL, Bethesda, MD) with 5% FCS (Hyclone Laboratories, Logan UT), 100 μg penicillin-streptomycin, 2 mM L-glutamine, essential and non-essential amino acids,

sodium bicarbonate, sodium pyruvate (Gibco), and 50µl 2mM 2-mercaptoethanol (Sigma Chemical Company, St. Louis, Mo.)]. The next day, unsorted and sorted bone marrow populations were plated on the irradiated stroma-containing plates at 25, 50, 100, 200, or 400 sorted cells per well. For each cell dilution, 120 wells were plated. The cultures were placed in a 37°C, 5% CO_2 , 90% humidity, and the cultures were fed at 7 d. At 14 d, the plates were examined for colony formation.

Short- Term T220-29 Cultures for B Cell Development

24-well plates were seeded with 2 X 10^3 T220-29 stromal cells per well in RPMI culture media prior to the addition of 2 X 10^4 unsorted or sorted bone marrow cells per well. At designated time points, the cultures were harvested by gentle pipeting or treatment with 1% EDTA if necessary and washed once. The clumps of stromal cells in the harvested samples were dissociated by repeated gentle aspiration in a 1-ml syringe in cold staining media. The samples were stained with immunofluorescent mAbs and run on a FACSCalibur (Becton Dickinson) for flow cytometric analysis with Cell Quest software. To exclude dead cells, 1 µg/ml of propidium iodide (Boehringer Mannheim) was added to the stained cells. The stromal contaminants, as well as the dead cells, were excluded from the analysis by an exclusion gate.

Results and Discussion

AB8 Expression Pattern In Fetal Tissues

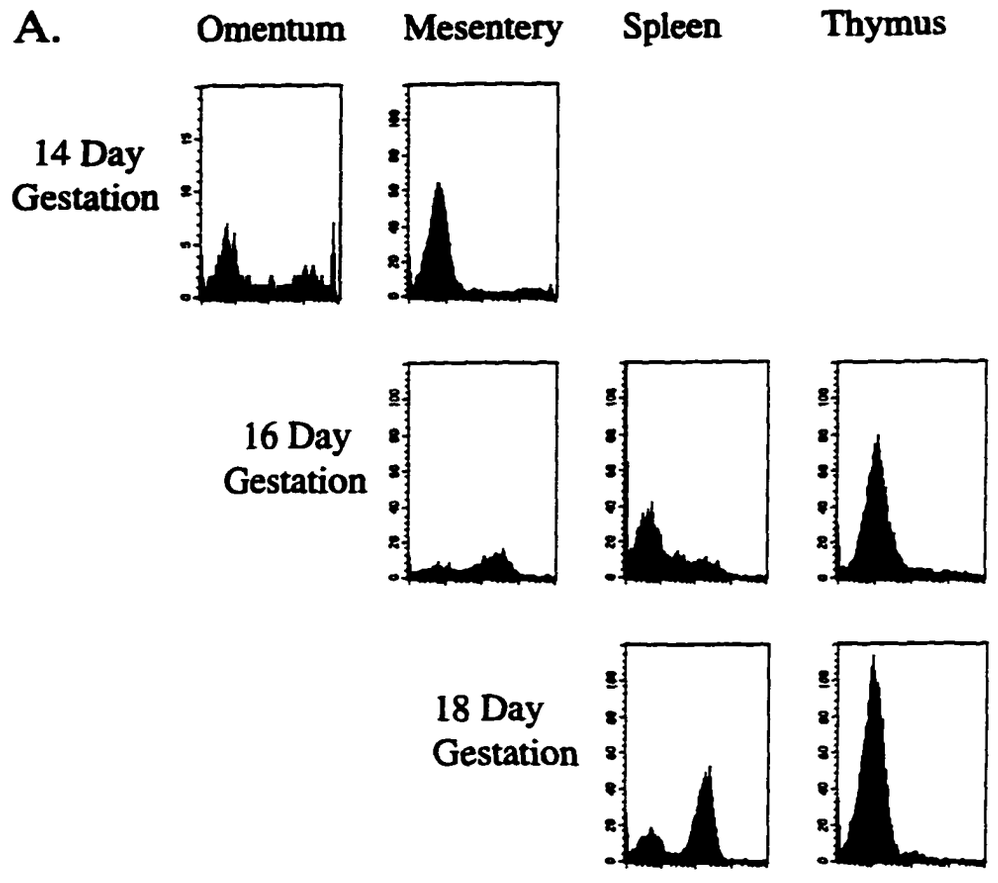
AB8 is a monoclonal antibody isolated after the immunization of a rat with bone marrow cells cultured on the T220-29 stromal line. As previouly reported, AB8 stains

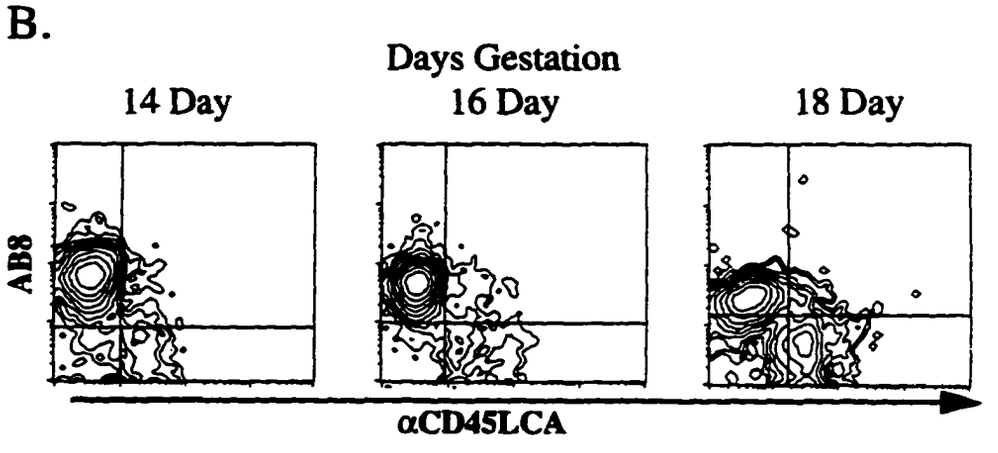
cells in adult bone marrow that seem to be in transition from stem cells to lineage committed cells (manuscript in preparation). AB8 was used to screen hematopoietic tissues during fetal development to determine whether, as in the adult, a multipotential progenitor population could be identified, isolated, and characterized. As in bone marrow, cells which costain with AB8 and Ter119, an erythroid lineage marker, seem to be erythroid progenitors. However, AB8+/Ter119neg cells were seen in a variety of fetal tissues (liver, omentum, mesentrey, thymus, and spleen), illustrated in Fig. 1A. It is also clear that the percentage of AB8+/Ter119neg cells changes dramatically during ontogeny and appears to be enriched shortly after hematopoietic seeding of each of these tissues and subsequently decreases with time. Fetal liver was chosen to evaluate the lineage potential of these cells because it is the first major site that has been shown to be seeded with hematopoietic stem cells, the accessiblity of the tissue, and the actual number of cells that could be obtained and used in in vitro culture systems. The AB8+/Ter119neg cells of the fetal liver were analyzed for their expression of CD45LCA and B220. As shown in Fig. 1B at 14, 16, and 18 dpc, a fraction of the AB8+ cells are CD45LCA dim, representing 2% of the fetal liver nucleated cells. The majority of the AB8+ cells costain with Ter119, an erythroid lineage marker.

Hematopoietic Potential Of AB8+/Ter119neg Fetal Liver Cells

The potential of the AB8+/Ter119 neg cells isolated from fetal liver at 11, 14, 16, and 18 dpc was assessed by in vitro culture assay for their stem cell, lymphoid, and B cell potential. In all of the in vitro culture assays, the fetal liver tissues were sorted based on

Figure 1. Binding pattern of AB8 in fetal hematopoietic sites. Fetal liver, omentum, mesenteric tissue, thymic, and splenic tissues at 14-, 16-, and 18-dpc were stained with reagents specific for CD45LCA, AB8, and Ter119 in combinations to determine staining patterns. (A) A time course of the appearance of the AB8+/Ter119neg population. (B) Coexpression of CD45LCA by the AB8+/Ter119neg population.





immunofluorescent staining. The sort-purified populations were then used in the culture experiments.

Evaluation Of The Stem Cell Potential Of Theab8+/Ter119neg Population In Fetal Liver

The stem cell potential of the cell-sorted fractions of fetal liver is shown in Table 1. The sorted cells were seeded into the culture medium and then assessed 14 d later for the ability to form colony-forming units (CFU-c), burst forming units (BFU-e), and pluripotent mixed colonies (CFU-GEMM). The ability to form all three of these colony types in this long-term culture assay indicates stem cell activity. In these experiments, liver from 11-dpc tissue was used because the fetal liver is seeded by hematopoietic stem cells at 10 dpc, but lymphopoiesis is not detected until 12 dpc, so if the AB8+/Ter119neg population had any stem potential , it should be detectable at this time. It can be seen that, in contrast to unsorted 11-, 14-, 16-, and 18-dpc fetal liver cells, the AB8+/Ter119neg fetal liver cells do not show stem cell activity. As expected, the AB8+/Ter119+ cells, presumably erythroid in lineage, did not produce colony numbers that were indicative of stem cell capabilities. In this culture system, it might have been expected that BFU-e colonies may have been formed in both AB8+/Ter119neg and AB8+/Ter119+ fractions, but this is a long-term culture assay, and cells which could form BFU-e from these fractions would differentiate and die out prior to evaluation of the cultures at 14 d. To evaluate their BFU-e potential, the cultures should be evaluated at 3-7 d after the initiation of the cultures. Additionally, the unsorted and negatively sorted fetal liver samples formed all three types of colonies at each time point, as expected.

Table 1. Stem cell potential of the AB8+/Ter119neg population

	Colony types in in vitro stem cell cultures colonies per 2 X 10^5 Cells		
	CFU-c[a]	BFU-e[b]	CFU-GEMM[c]
11-dpc[d]			
Unsorted	15	8	8
	15	3	2
	17	4	9
AB8+/Ter119neg	1	0	0
	0	0	0
	1	0	0
14-dpc[d]			
Unsorted	11	5	4
	9	2	4
	5	1	3
AB8+/Ter119neg	0	0	0
	0	0	0
	0	0	0
	0	1	0
16-dpc[d]			
Unsorted	13	8	11
	15	8	11
	14	5	5
	18	4	7
AB8+/Ter119neg	0	0	0
	0	0	0
	0	1	0
	0	0	0
18-dpc[d]			
Unsorted	24	4	9
	18	6	11
AB8+/Ter119neg	1	0	0
	2	0	1

a: Granulocyte-macrophage colony (flat, nonhemogobinized, translucent cells)
b: Erythroid burst colony (densely packed orange to dark red hemoglobinized)
c: Pluripotent mixed colony (compact hemoglobinized center, peripheral flat lawn of large or small cells)
d: Days post coitus

Evaluation of The Lymphoid Potential of The AB8+/Ter119neg Population in Fetal Liver

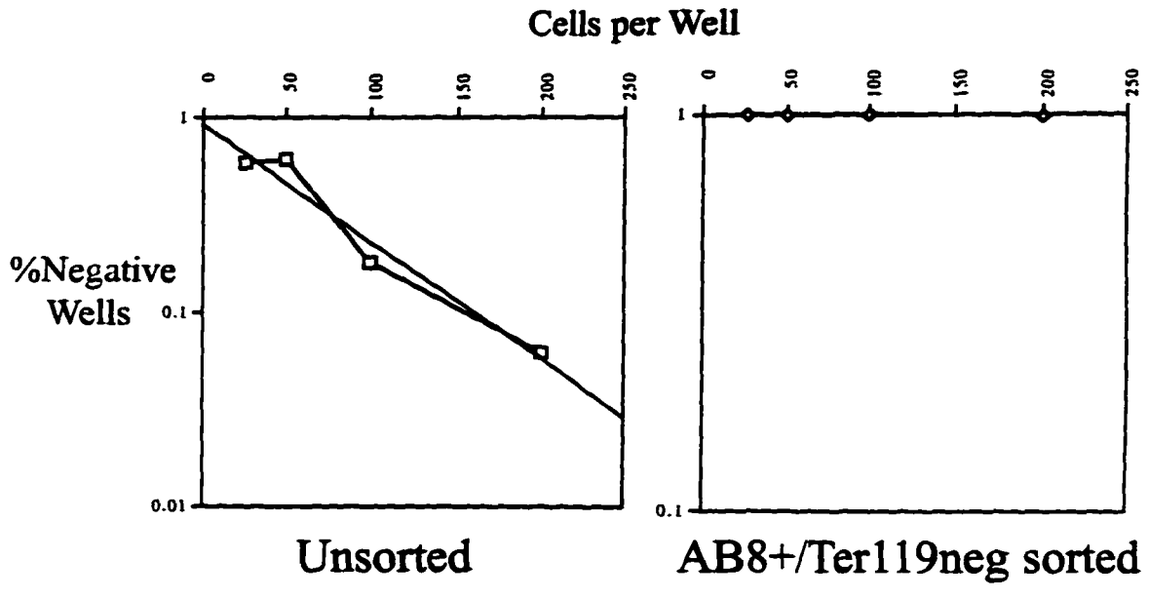
We next determined whether the AB8+/Ter119neg fraction of the fetal liver has lymphoid potential as assayed using the irradiated T220-29 stromal line as a matrix

support for the cultures. Sorted fractions from 11-, 14-, 16-, and 18-dpc fetal liver were assessed for their ability to form colonies on the stroma when evaluated at 14 d postinitation of the cultures.

When the AB8+/Ter119neg-sorted fractions from 11-dpc fetal liver were evaluated, no colony formation was detected, but unsorted fetal liver at 11 dpc formed colonies, as illustrated in the graphs in Fig. 2A. This implies that at 11-dpc the AB8+/Ter119neg fraction does not contain lymphoid progenitors. However, AB8+/Ter119neg fetal liver populations from 14-, 16-, and 18-dpc gave rise to colonies at 14-dpc, and the frequency of such cells slightly increased at 16-, and 18-dpc as illustrated in Fig. 2B. These results indicate that AB8+/Ter119neg cells with lymphoid potential appear between 11- and 14-dpc, and that the size of this progenitor pool expands correlating with a burst of lymphopoiesis seen after 14-dpc (38). The colonies generated at each time point from the fetal liver sorted AB8+/Ter119neg fraction are composed of two phenotypes. One contains small lymphoid-type cells (B cell-like) and the other a mixed colony phenotype of the small B cell-like cells growing on top and burrowing within clusters of adherent, large, macrophage-like cell. Both types of colonies were detected in 14-, 16-, and 18-dpc cultures of AB8+/Ter119neg-sorted fetal liver fraction as well as the negative-sorted fraction and unsorted fetal liver. The mixed-type colony was the predominant colony type in the AB8+/Ter119neg fraction, but both colony types were represented as shown in Fig. 3. The morphology of colonies from unsorted fetal liver cells from 11-, 14-, 16-, and 18-dpc fetal liver did not change with time, indicating that the predominant cells contributing to colony formation are present throughout

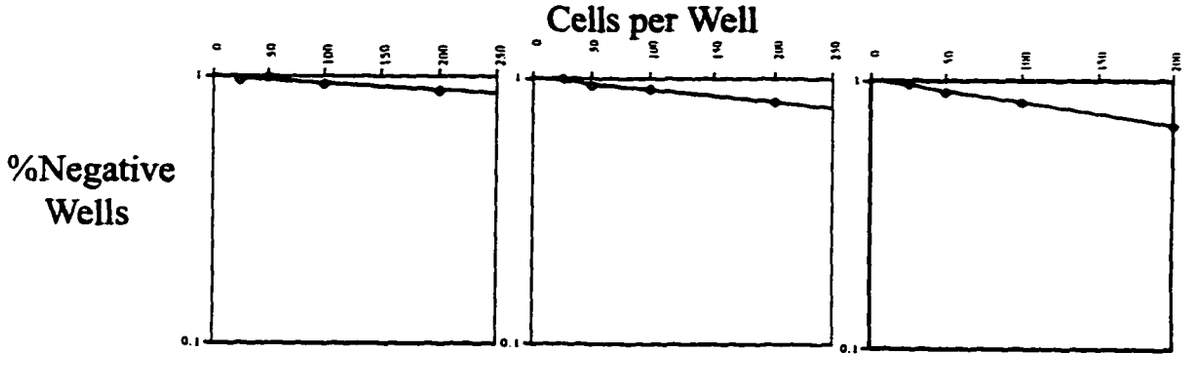
Figure 2. Limiting dilution analysis of the sorted fetal liver fractions grown on irradiated T220-29 stromal line. Timed pregnancies were used to obtain fetal liver at 11, 14, 16, and 18 dpc; each was stained and sorted into a negaitve, AB8+/Ter119+, and AB8+/Ter119neg fraction. These fractions were placed in culture, incubated for 14 d, and then evaluated for colony formation to estimate the frequency of cells with leukocyte potential in each fraction. Each data point represents 120 individual cultures. (A) The AB8+/Ter119neg-sorted fraction from 11 dpc fetal liver did not form colonies in this culture system, while unsorted 11 dpc fetal liver cells did. (B) The AB8+/Ter119neg-sorted fraction from 14, 16, and 18 dpc fetal liver formed colonies under the culture conditions.

A. 11-dpc Liver



Frequency 1:65

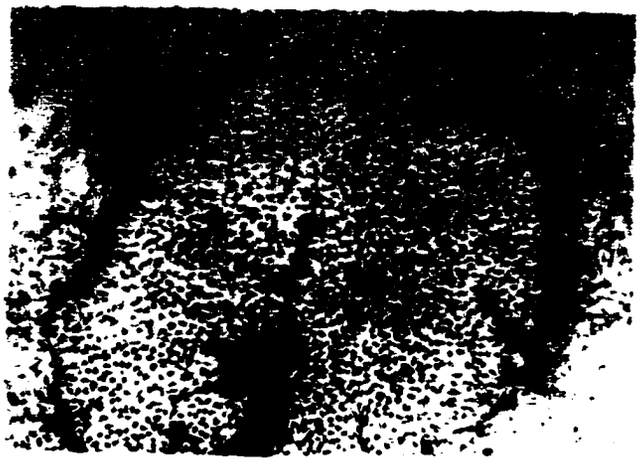
B. Fetal liver AB8+/Ter119neg-Sorted Fraction



Frequency:	1:620	1:450	1:400

Figure 3: Morphologic examination of limiting dilution cultures. Timed pregnancies were used to obtain fetal liver at 11, 14, 16, and 18 dpc; each was stained and sorted into negative, AB8+/Ter119+, and AB8+/Ter119neg fractions. These fractions were placed in culture, incubated for 14 d, and then evaluated for colony formation to estimate the frequency of cells with leukocyte potential in each fraction. Representative colonies from the AB8+/Ter119neg-sorted fraction from 18-dpc liver are shown.

18-dpc Fetal Liver AB8+/Ter119neg sort



Mixed Colony Type

Lymphoid Colony Type

ontogeny. In the AB8+/Ter119neg population, the two types of colonies observed in the limiting dilution assay were generated from relatively early to late in ontogeny (Fig. 3). However, the percentage of colonies with a lymphoid phenotype versus the mixed phenotype increases with age. As shown in Fig. 4, the percentage of B cell-like colonies derived from the AB8+/Ter119neg-sorted fetal liver fraction compared to the total number of colonies counted at each gestational age. At 14-dpc, the percentage of lymphoid colonies is low, but this percentage increases at 16-dpc and even more so at 18-dpc. This same trend of increases in the percentage of B cell-like colonies is not seen in the negative-sorted or the unsorted fractions, where, in fact, the percentage increases at 16-dpc and then decreases at 18-dpc.

Evaluation Of B Lineage Potential Of The AB8+/Ter119neg Population In Fetal Liver

Since the limiting dilution assays demonstrated that these cell could give rise to B cell-like colonies as well as mixed colonies, the phenotype of B cells generated in these cultures was next analyzed. An in vitro culture system was next used to address this question by using a stromal line which supports B cell generation. In these experiments, the T220-29 stromal line was used to analyzed the fetal liver AB8+/Ter119neg-sorted fraction in culture. Cultures of fetal liver AB8+/Ter119neg-sorted fraction from 14-, 16-, and 18-dpc all B generated lineage cells, and some of the cells which expressed surface immunogobulin were CD5-positive. In Table 2, the percentage of cells that were generated after culture at each defined stage of B lineage differentiation from each fraction is tabulated. In summary, the AB8+/Ter119neg fraction generates cells in culture which are representative of each defined stage of early B cell differentiation. The percentages

Figure 4. Percentage of lymphoid colonies formed by the AB8+/Ter119neg-sorted fetal liver in the limiting dilution cultures. The number of strictly B lymphoid-like colonies was counted from the AB8+/Ter119neg-sorted fetal liver at 14-, 16-d 18-dpc. The number of B lymphoid-like colonies is presented as percentage of total colonies formed by the sorted fraction at each time point.

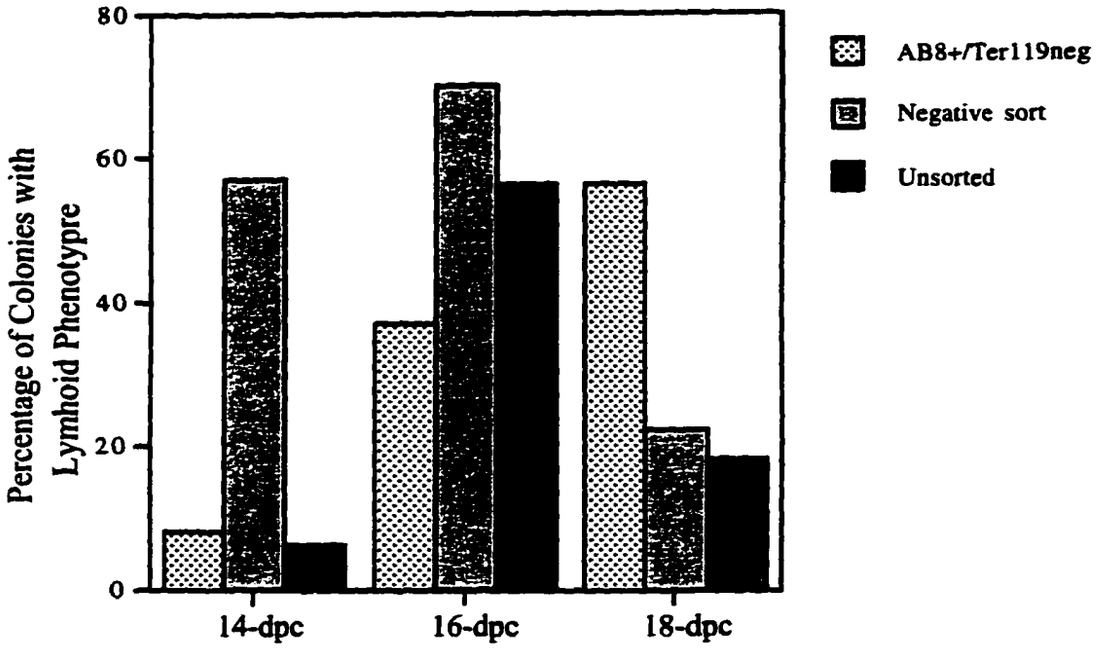


Table 2. *Phenotype Of Lymphocytes Derived From T220-29 Cultures*

	Percentage Positive Cells							
	CD45LCA	B220+ CD19neg	B220+ CD43+	B220+ CD43+ BP-1+	B220+ BP1+	B220+ CD19+	B220+ IgM+	B220+ IgM+ CD5+
14-dpc[a]								
Unsorted	97	0	38	20	--	82	<1	<1
Neg Sort	99	2	30	10	--	86	2	2
AB8+/Ter119neg	85	0	35	25	--	72	1	1
16-dpc[a]								
Unsorted	94	0	58	16	--	70	2	2
	97	2	58	--	29	83	6	4
Neg. Sort	92	0	71	20	--	60	2	2
	95	3	70	--	29	82	4	4
AB8+/Ter119neg	38	0	32	10	--	36	<1	<1
	89	3	66	--	30	80	1	1
18-dpc[a]								
Unsorted	97	3	16	--	14	72	10	7
Neg. Sort	85	2	20	--	15	79	5	3
AB8+/Ter119neg	65	2	17	--	10	44	5	2

a Days post coitus

within each B cell stage for the AB8+/Ter119neg fetal liver fraction are not strikingly different from the unsorted or the negatively sorted fractions. In cultures from each sorted fraction of the 14- and 16-dpc experiments, the majority of the cells in the cultures are in the early pro-B cell stage of B cell differentiation. After 6 d in culture very few cells are surface immunoglobulin positive from the 14-dpc samples; however, there is an increase in the percentage of immunoglobulin postive cells from fractions sorted at 16-dpc. This is consistent with the idea of a wave of B cell generation in fetal development in vivo (38, 39). In the samples sorted from 18 dpc fetal liver, the percentage of cells in these early fractions drops while there is an increase in the more mature stages. Additionally, the percentage of cells in the cultures which progress to express surface immunoglobulin increases at this time point, with a portion of these being CD5-positive B cells.

Concluding Remarks

The detection of cells by AB8 in fetal hematopoietic tissues provides a more precise marker for characterization and isolation of transitional progenitor populations. It is clear that this AB8+ fraction in the fetal liver is not a stem cell. But it does have leukocyte potential, myeloid, and B lineage potential. In this multipotential population, some cells have the potential to differentiate to both myeloid and B lineages, while others seem to have preference for lymphocyte-type colonies.

AB8+/Ter119neg cells are seen in fetal hematopoietic rudiments shortly after the times they have been reported to be seeded by stem cells. Additionally, the time at which AB+/Ter119neg cells appear in the thymus, omentum, and spleen corresponds to times

when progenitor cells in these tissues have been shown to be multipotential. The AB8+/Ter119neg cells are heterogeneous for expression of CD45LCA. This variance from the expression pattern noted in adult bone marrow may indicate that the lineage potential of the AB8 positive cells is more diverse in fetal development then in adult bone marrow development. However, an alternative explanation may be that the proportions of alternative progenitors are more abundant in the fetal microenvironment than in the adult. Apparent differences in progenitor potentials may also result from changes in the number of one type of progenitor in one environment versus another microenvironment.

As in bone marrow, the AB8+/Ter119neg cells do not have stem cell capabilities in fetal tissues. This clear division is a useful distinction when trying to study transitional progenitors versus stem cells. Markers reported to date that can be used to isolate multipotential progenitors are generally expressed over a wide range of differentiation steps, including stem cells. Thus the ability to simply distinguish stem cells from multipotential progenitors will clarify results obtained in assessing these populations. Binding of erythroid lineage cells by AB8 can be easily excluded by costaining with erythroid marker, thus leaving a restricted population.

Based on the potential of the AB8+/Ter119neg fraction in adult bone marrow, it was expected that the corresponding fetal fraction would also contain multiprogenitor cells. A multipotential population in fetal liver has been described previously, which, in limiting dilution cultures, generated mixed colonies with the phenotype of small, round B cell-like cells and plastic aderent macrophage-like cells (40). In our experiments, we wished to i) determine when in ontogeny the AB8+/Ter119neg cells are multipotential,

ii) determine if the AB8+/Ter119neg fraction in the fetal liver produced a single type colony or if varying types of colonies would be seen, to determine if this population was uniform or varied in its ability to produce colonies, and iii) compare the phenotype of the colonies formed by the AB8+/Ter119neg fraction to a reported fetal liver multipotenitial population.

Results from the limiting dilution assays suggest a dual or multipotential cell in the AB8+/Ter119neg fraction of fetal liver. In the adult, a significant number of the colonies formed in these assays had a strictly lymphoid morphology. This result suggests that, in the fetal liver, a portion of the AB8+/Ter119neg cells are more restricted in lineage potential than in the adult, while a portion of this fraction retains a multiprogenitor phenotype. These progenitor populations appear similar to those previously reported in 12-dpc fetal liver. In the results reported here, these populations are shown not to contain stem cells, and these populations persist in the fetal liver until at least 18-dpc.

In the short-term B cell cultures, the AB8+/Ter119neg cells do progress through the different stages of B cell development and give rise to B1 cells; it is not clear whether they are restricted to production of B1 B cells. The AB8+/Ter119neg fraction generates cells in culture which represent each stage of early B cell differentiation, with no striking differences from the unsorted or negatively sorted fractions. The progression of cells into the different stages of B cell differentiation and onward to express surface immunoglobulin-positive B cells in cultures of the samples is in aggreement with

published data describing a synchronous wave of B cell development in fetal liver (38, 39).

The ability to identify subpopulations in the transitional steps between stem cells and lineage committed progenitors may permit questions regarding the lineage potential of each stage and how a cell progresses through this cascade. AB8 will allow the subfractionation of such early transitional progenitors in fetal liver. The advantage of using AB8 rather than other previously defined markers is that it is restricted in expression to these transitional progenitor pools.

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SUMMARY AND DISCUSSION

The work presented in this dissertation has been an examination of adult and fetal hematopoietic progenitor cells, specifically, their potential to commit to the B lineage and progress through B cell differentiation. The objective of these studies was to determine whether uncommitted progenitors, cells after the hematopoietic stem cell stage, could be identified and be induced to differentiate into the B lineage and whether perhaps an alternative pathway of B cell differentiation that would be more permissive of B1 cell generation could be identified. This speculation was based on several lines of experimental data. First was work describing multipotential progenitors with the capacity to generate B and myeloid lineages, specifically B1 lineage B cells from fetal sites (63, 97, 102). Second, experiments in which progenitors from bone marrow or fetal tissues were marked with retoviral insertions, transferred, and the progeny of the marked cells traced in the host, which indicated common progenitors for the B and myeloid lineages (21, 69, 153).

It was reasoned that this hypothesized progenitor population of cells following an alternative pathway in B lineage development would follow an ordered progression of commitment and differentiate events that should be identifiable based on changes in gene expression and cell surface markers. One monoclonal antibody, AB8, was found to detect a unique marker that characterizes an early progenitor cell.

The experiments in the first report confirm that, indeed, AB8 can be used to identify an early progenitor population in the bone marrow, that this population can commit to the B lineage, and that it seems to progress through conventionally defined B cell differentiation stages at least according to expression of cell surface markers. Specifically, AB8 binding in the adult tissues was restricted to bone marrow and the spleen. In the spleen, AB8 costains either with Ter119, an erythroid marker (38), or approximately 1% of the CD38+/NK1.1+ cells. In the bone marrow, the majority of cells that bind AB8 costain with Ter119, leaving approximately 1% of the nucleated cells in the bone marrow to be AB8+/Ter119neg. This small population was the focus of the rest of the experiments. Giemsa staining indicated that these cells were large, uniform, lymphoblastic cells. This AB8+/Ter119neg population costained dimly with CD45LCA, CD38, and CD43, and 50% of the population expressed c-kit. All of these molecules are described to be expressed by progenitor cells. These phenotypic findings, the restricted cell number, cell morphology, and expression of characteristic progenitor marker, were suggestive of this population being a lymphoid progenitor cell type. Also, by flow cytometric analysis, the AB8+/Ter119neg cells were not shown to be contained in the earliest described committed B lineage progenitor populations (128, 130).

The majority of the experiments in the first paper were aimed at determining the hematopoietic potential of these AB8+/Ter119neg cells. They are not stem cells, determined by in vitro stem cell assays, which was not unexpected from the flow cytometric analysis. The AB8+/Ter119neg bone marrow population has lymphoid potential. Specifically, in limiting dilution assays, these cells generated colonies of

adherent large, nonuniform myeloid-type cells with clusters of non- or loosely adherent small lymphoid-type cells on and interdigitating into the adherent layer. This suggests that the AB8+/Ter119neg cells have multipotential activity for myeloid and B lineages. Such populations have been identified in fetal liver and suggested in bone marrow, but they have never been identified or isolated. However, the AB8+/Ter119neg population has not yet been definitively demonstrated to have myeloid potential. However, these cells do have B lineage potential when placed in short-term stromal cultures. The AB8+/Ter119neg cells differentiate initially to express B220 and CD19. Additionally, in these cultures, all stages of B cell differentiation are seen. It is not clear whether this AB8-marked population has a preponderance for generation of B2 versus B1 B cells. Further experiments altering the stromal conditions and cytokine additions to the cultures with subsequent mitogen stimulation would be required to adequately address the issue of B1 versus B2 potential of this progenitor population. The conclusion of the first paper is that AB8 has enabled a transitional progenitor from the bone marrow to be identified, characterized, and isolated.

The second paper examines the potential of the AB8+/Ter119neg population in the fetal liver and confirms that, as in bone marrow, this population is an early, perhaps uncommitted or transitional, progenitor population. AB8+/Ter119neg cells are seen in fetal liver, thymus, omentum, mesentery, and spleen shortly after each tissue is initially seeded in ontogeny. The appearance of the AB8+/Ter119neg cells corresponds to times when cells in these tissues are reported to be multipotential.

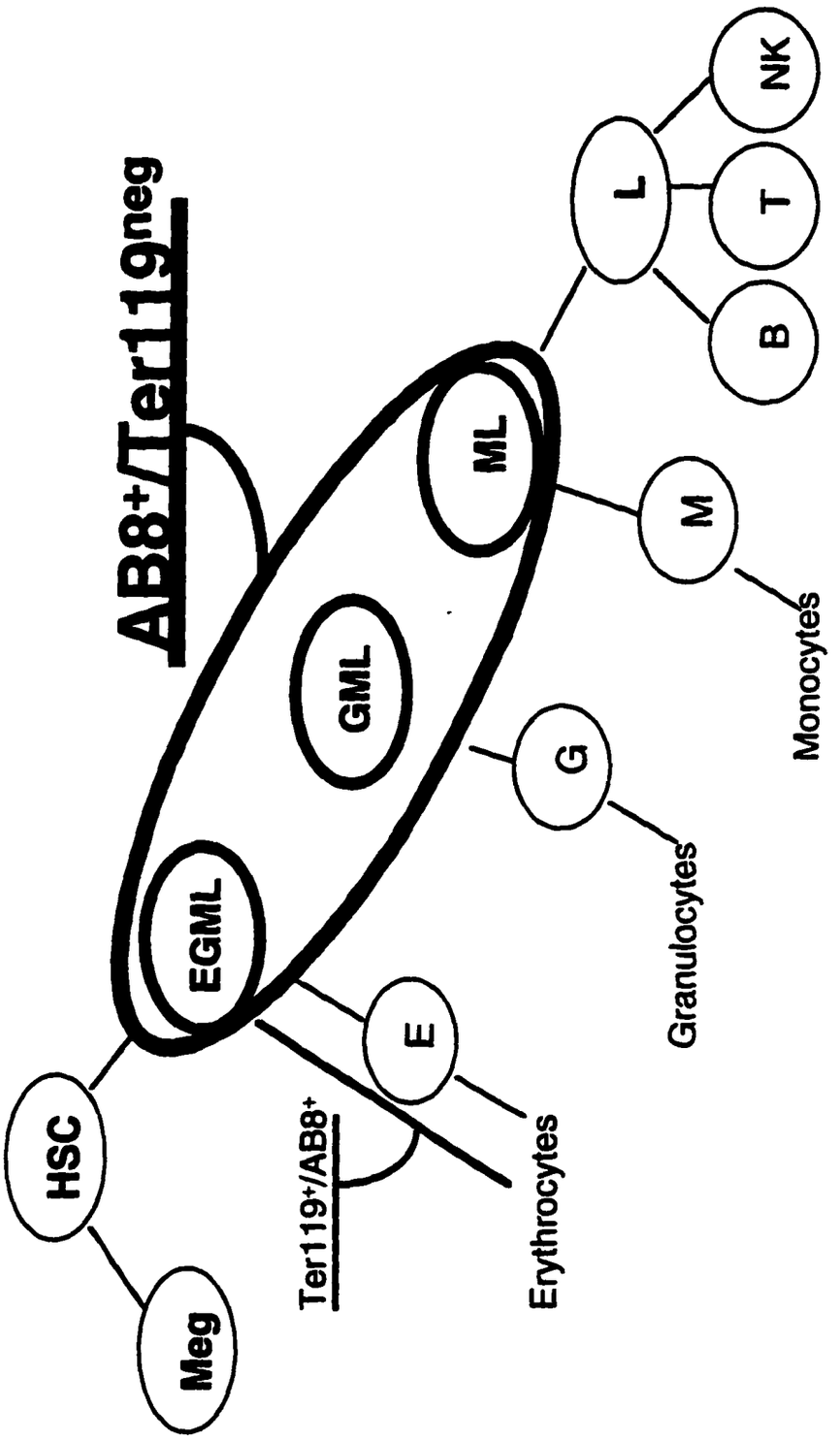
In fetal liver, the population does not contain stem cell potential even at 11 dpc, indicating that this marker is absent from hematopoietic stem cells that seed the fetal liver, which originate in the paraaortic splanchnopleura. This population does give rise to myeloid-lymphoid colonies in the limiting dilution assays at 14, 16, and 18 dpc, but not at 11 dpc. In these cultures, unlike the bone marrow cultures, the AB8+/Ter119neg cells give rise to mixed myeloid-lymphoid colonies as well as lymphoid colonies independent of myeloid-type cells. This suggests that the AB8+/Ter119neg population in fetal sites is more heterogeneous than in adult bone marrow or that the cells in transition to the B lineage from fetal sites are longer lived than those in the bone marrow; thus they can survive and form colonies which would then be accounted for in the long-term cultures. In the T220-29 stromal cultures, the AB8+/Ter119neg population does generate B lineage cells at each stage of B cell differentiation in culture. Of the cells in the cultures that do progress to express surface immunoglobulin, some do coexpress CD5, indicating this progenitor population has B1 lineage potential, which was not as definitive in the cultures with the adult bone marrow fraction. The fraction of cells AB8 marks in fetal hematopoietic tissues will enable more precise delineation of these transitional progenitor populations. The identification and isolation of transitional porgenitors can be used to ask questions of lineage commitment and differentiation.

The AB8+/Ter119 negative population does mark cells in transition from stem cells to lineage-committed cells. The extent of the multipotential ability of this population is not determined. If applied to a schematic representation of these transitional populations postulated by Shing (73) in a current opinions review, AB8 may

mark all the transitional populations or only the myeloid-lymphoid transitional populations, as illustrated in Fig. 1. This scheme of differentiation from the stem cell to lineage-committed cells is based on a collection of hematopoietic transcriptional-factor gene-deficient mouse models. None of these populations, with the exception of the B-myeloid lineage cells described in fetal liver cells (97, 102), have actually been proven, isolated, or characterized. AB8 represents a unique tool that could actually isolate these populations and address their potential as well as address the commitment processes these cells would be able to undergo.

These studies have (i) identified a monoclonal antibody and corresponding molecule that is associated with an early, probably noncommitted progenitor population in adult bone marrow and fetal hematopoietic sites, (ii) demonstrated that this population from either bone marrow or fetal liver does not have stem cell potential but can generate myeloid and lymphoid-type colonies in limiting dilution assays, (iii) demonstrated that this population in adult bone marrow and fetal hematopoietic sites can generate B lineage cells and clearly B lineage cell from fetal liver, and (iv) illustrated the potential use of this antibody to isolate a transitional progenitor to address questions of lineage commitment and differentiation.

Figure 1. A model of AB8 expression during hematopoietic differentiation. The differentiation scheme used is based on studies from transcriptional factor gene inactivation mice. The transitional multipotential populations depicted have not yet been identified or isolated, except for the ML population in fetal liver.



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APPENDIX

ANIMAL USE APPROVAL

AI14782-19

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Executive Vice President and Provost
Institutional Animal Care and Use Committee

MEMORANDUM

DATE: FEBRUARY 26, 1997

TO: KEARNEY, JOHN

FROM: Clinton J. Grubbs, PhD
Chairman, Institutional Animal Care and Use Committee

SUBJECT: Notice of Approval

Title: REGULATION OF B CELL CLONAL DIVERSITY

Agency: NIAID

Committee Action Date: February 26, 1997

IACUC Protocol Number: 9703481

Species: MICE
Stress Level: B

This application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC). Animal use is scheduled for review one year from the Committee Action Date noted above.

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. Its animal care and use program is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

As a condition of approval, the IACUC required the following modification(s) of this application:

__Change in agent, dosage, route of administration, or schedule of administration
__Change in method or schedule of sample collection
__Change in pre- or postoperative care
__Change in endpoint
__Additional training for personnel
__Monitoring by Animal Resources personnel or by IACUC members
__Additional information or clarification
__See attached
X None

Please forward this notice to the appropriate granting agency.

If you have any questions, call the IACUC office at 934-7692.

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Chantal Marie Moratz

Major Subject Microbiology

Title of Dissertation Characterization and Analysis of a Hematopoietic Progenitor Population Detected Using AB8

Dissertation Committee:

John F Kearney, **Chairman**

Max D Cooper

R Pat Bucy (97K)

Rick Dunn

Cary T. Uhler

Director of Graduate Program Susan Jacobs

Dean, UAB Graduate School John F. Modden

Date 9/10/97