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Peritoneal B cell engraftment in RAG and SCID immunodeficient mice

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**PERITONEAL B CELL ENGRAFTMENT IN RAG AND SCID IMMUNODEFICIENT
MICE**

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Marcia M. Stickler

December 1998

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Abstract

PERITONEAL B CELL ENGRAFTMENT IN RAG AND SCID IMMUNODEFICIENT MICE

by Marcia M. Stickler

Controversy exists over the origin of the three B cell subsets in mice: B-1a, B-1b, and B-2. The predominant theory contends that B-1a cells arise from a distinct lineage primarily because transfers of fetal tissue reconstitute B-1a cells while adult bone marrow transfers do not. The data reported in this study reveal that the amount of adult bone marrow progenitors transferred has little effect on the proportion of B-1a cells that engraft, however, allowing more time for engraftment significantly increases the proportion of B-1a cells that engraft. Transfers into non-irradiated SCID and RAG hosts demonstrate that B-1a cells engraft in the peritoneal cavity in similar proportions to syngeneic donor strains. Analysis of V-D-J junctions reveals that adult derived B-1a cells have N region insertions similar in frequency to that found in B-2 cells. The data presented in this study are consistent with the hypothesis that B cell subsets are derived from a single precursor.

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Introduction

There are three distinct B cell subsets, B-1a and B-1b cells (also called B-1 cells when grouped together), and B-2 cells in mice. These populations can be distinguished by their surface markers, anatomical location, regulation and function. B-2 cells, also called conventional B cells, are characterized as $\text{IgM}^{+/-} \text{IgD}^{++} \text{B220}^{++} \text{MAC-1}^{-}$. B-1a and B-1b cells are $\text{IgM}^{++} \text{IgD}^{+} \text{B220}^{+/-} \text{MAC-1}^{+}$. B-1a cells express CD5 (Ly-1) while B-1b and B-2 cells do not (reviewed in Kantor and Herzenberg, 1993). Both B-1a and B-1b cells predominate in the peritoneal and pleural cavities with a minor fraction present in the spleen and are undetectable in lymph nodes (Hayakawa et al., 1983; Hayakawa et al., 1986a; Hayakawa et al., 1986b). B-2 cells are ubiquitous.

B-1 cells are functionally unique from B-2 cells. B-1 cells are the major contributor to free IgA in the gut (Kroese et al., 1989), and a large percent of serum IgM (Ishida et al., 1992; Forster and Rajewsky, 1987; Riggs et al., 1990). B-1 cells are responsible for secretion of polyreactive IgM antibodies with low affinity specificity for autoantigen and bacterial cell wall proteins (Hardy and Hayakawa, 1986; Hayakawa and Hardy, 1988; Kantor and Herzenberg, 1993; Stall et al., 1996). In addition to secreting autoreactive IgM in normal mice, B-1a cells are enriched in certain inbred autoimmune mouse strains and are responsible for spontaneous IgM secretion in these mice (Hayakawa et al., 1983; Hayakawa et al., 1984).

In addition to phenotype, anatomical localization, and cell function, the regulation of cell populations is distinctly different between B-1 and B-2 cells. While B-2 cells are replenished from precursors in the bone marrow throughout adult life, B-1a (Hayakawa et al., 1985; Hayakawa et al., 1986a, Forster and Rajewsky, 1987) and B-1b (Kantor et al., 1995) cells are distinguished by their capacity for self renewal from mature immunoglobulin bearing cells. Along with self-replenishment, B-1a and B-1b cells exhibit a form of negative feedback regulation not seen with B-2 cells, that prevents continued production of B-1 cells from precursors in the bone marrow from about 6 weeks of age (Lalor et al., 1989a; Lalor et al., 1989b; Thomas-Vaslin et al., 1992).

There is considerable disagreement over the question of the origin of murine B cell subsets. The generally accepted theory holds that these cells are derived from distinct lineages because they have been demonstrated to arise at different times during development: B-1a cells arise primarily from fetal precursors, and B-1b and B-2 cells arise from both fetal and adult precursors (Hardy and Herzenberg, 1986; Herzenberg et al., 1986; Hayakawa and Hardy, 1988; Kantor and Herzenberg, 1993; Herzenberg and Kantor, 1993; Hardy et al., 1994). Such lineages are defined as phenotypically and functionally distinct sets of cells derived from unrearranged progenitors that have some capacity for self-renewal. B-1a and B-1b cells develop primarily from fetally-derived precursors that produce a long lived self-renewing population of mature cells that prevent further development from precursors in the adult animal by feedback inhibition. B-2 cells on the other hand, are relatively short lived and repopulate themselves from progenitor

cells throughout the life of the animal. Evidence used to support this theory was derived from transplantation studies. Previous studies have shown that transfers of fetal liver cells (Hayakawa et al., 1985; Hardy and Hayakawa, 1991; Hardy and Hayakawa, 1992a; Kantor et al., 1992) and fetal spleen cells (Hayakawa et al., 1985) readily replenish peritoneal B-1a cells in recipient mice; however, transfers of adult bone marrow into irradiated normal or irradiated immunodeficient adult hosts have consistently failed to reconstitute B-1a cells (Hayakawa et al., 1985; Hardy and Hayakawa, 1991; Hardy and Hayakawa, 1992a; Kantor et al., 1992; Kantor et al., 1995) but readily produce B-1b (Kantor et al., 1992; Kantor et al., 1995) and B-2 cells (Hayakawa et al., 1985; Hardy and Hayakawa, 1991; Hardy and Hayakawa, 1992a; Kantor et al., 1992; Kantor et al., 1995). Additionally, transfers of fetal omentum cells into adult mice has produced B-1a and B-1b cells but no B-2 cells (Solvason et al., 1991). In contrast to the accepted theory are studies that have reported B-1a cell engraftment from adult bone marrow. Thomas-Vaslin et al. (1992) transferred adult bone marrow into irradiated normal adult hosts and found significant B-1a cell engraftment in the peritoneal cavity. Huang et al. (1996) transferred adult bone marrow into newborn non-irradiated SCID mice and found B-1a cell engraftment in the spleen.

Adult bone marrow transfers that result in B-1a cell engraftment are consistent with a second theory about the development of B cells which contends that the B-1a phenotype is the product of a differentiative pathway available to any B cell (Haughton et al., 1993, Wortis, 1992). Subpopulations of B cells develop from a common pool of precursors that

are selected for by antigen crosslinking of surface Ig. Cells bearing Ig rearrangements that are polyreactive or monoreactive with polymeric (self or bacterial cell wall) antigen allows extensive crosslinking to occur. This crosslinking occurs in the absence of T cell interaction. Entry into the CD5⁻ B cell (B-1b and B-2) pathway involves interaction with a T helper cell without extensive crosslinking. Ying-zi et al. (1991) and Huang et al. (1996) have found that *in vitro* treatment of CD5⁻ B cells with anti-Ig induces the CD5⁺ phenotype. A theory that combines concepts from both of the contending theories was presented by Stall et al. (1996): fetal-type B cells, as defined by delayed MHC (Major Histocompatibility Complex) class II expression, are derived from unique precursors with the capacity to become B-1a cells, but they require appropriate signaling to mature into CD5⁺ B-1a cells, without which they mature into B-2 cells.

While evidence is very compelling for both theories, neither one has been definitively proven. CD5 induction by IgM crosslinking has only been demonstrated *in vitro* and evidence for separate lineages is conflicting. Studies that have not found B-1a cell reconstitution from adult bone marrow transfers were all done on irradiated adult hosts, with either limited numbers of cells transferred or were allowed only a limited time for reconstitution. One study has shown B-1a cell reconstitution from adult bone marrow in irradiated hosts (Thomas-Vaslin et al., 1992). This study transferred a higher number of cells, and allowed a longer time for reconstitution than the studies that showed little or no B-1a cell reconstitution. Reconstitution of peritoneal B-1a cells from adult bone marrow has not been demonstrated in adult non-irradiated hosts.

In this study adult bone marrow transfers were made into two different non-irradiated adult immunodeficient mouse models. Severe combined immunodeficient mice (SCID) are deficient in DNA double strand break repair, impairing V-D-J rearrangement resulting in a lack of B and T lymphocytes (Kirchgessner et al 1995). A mutation in the catalytic subunit of DNA-dependent protein kinase (p350) has been implicated as cause of the double strand break repair defect in laboratory SCID mice. The mutation greatly reduces p350 protein levels in these mice. The phenotype does not eliminate all B cell development however. The SCID defect appears in one of the final steps in V-D-J rearrangement: the joining of the free DNA ends of the coding strands. The recombination activating protein still binds and double stranded breaks still occur. Other DNA repair enzymes can repair the double stranded breaks. The SCID phenotype is described as leaky because on rare occasions coding junctions are formed during rearrangement. $RAG^{-/-}$ deficient mice lack either of the recombination activation genes (RAG) 1 or 2. These knockouts block recombination at an early stage when immunoglobulin gene rearrangement normally initiates and no double stranded DNA breaks occur. Both T and B lymphocytes require gene rearrangement to construct functional receptors. In $RAG^{-/-}$ mice, the mutation results in a complete lack of mature T and B cells (Mombaerts et al., 1992, Shinkai et al., 1992).

Most studies examining engraftment of B-1a cells have used irradiated normal or irradiated SCID mice. Presumably SCID mice are irradiated because of their leaky rearrangement phenotype. Existing endogenous cells could interfere with the analysis of

engrafted cells. However, it is possible that the irradiation of mice used in these studies alters the microenvironment in which B-1a cells develop. Using RAG mice for transfers has an advantage over the use of SCID mice because they are non-leaky. This feature allows for the study of lymphocyte progenitor transfers in non-irradiated mice without the confounding effect of endogenous lymphocytes. Also, differentiation of even a few lymphocytes in SCID mice alters the hematopoietic microenvironment, causing maturation and differentiation of support cells (Shores et al., 1991; Hollander et al., 1995). RAG mice may have a more fetal-like hematopoietic environment due to a total lack of lymphocytes.

Another unique feature of B-1a cells examined in this study is found at the rearrangement junctions of immunoglobulin heavy chain genes. B-1a cells exhibit characteristic variable heavy chain rearrangements that distinguish them from B-1b and B-2 cells. This includes over-utilization of certain V_H segments and reduced utilization of N region insertions. IgH (immunoglobulin heavy) chain genes are constructed of four segments: variable (V), diversity (D), joining (J) and constant (C). As B cells mature, heavy chain genes are assembled by combining one of 13 D segments with one of four J segments, which then joins with one of the numerous V segments. The diversity of immunoglobulin specificity is derived from the multitude of combinations available from the 300-1000 (estimated) V segments, the 13 D segments and the four J segments. Additional diversity is obtained through imprecise joining. Further increases in diversity are obtained during joining where terminal deoxynucleotidyl transferase (TdT) inserts

non-template nucleotides termed N-region insertions between each gene segment (Landau et al., 1987; Giffillan et al., 1993). Finally association with a light chain can create an estimated antibody diversity upwards of 10^{11} potentially unique specificities.

All three subsets of B cells show considerable diversity in their V_H usage (Hardy, 1992b; Kantor, 1996; Kantor et al., 1997). However, V_{H11} and V_{H12} genes have been shown to be over-utilized in populations of peritoneal B-1a cells specific for autoantigens (Hardy et al., 1989; Carmack et al., 1990; Arnold et al., 1994). In addition, B-1a cells use N region insertions less frequently than B-2 cells or B-1b cells (Gu et al., 1990; Tornberg and Holmberg, 1995; Kantor et al., 1997), though insertions are more frequent in older mice (Gu et al., 1990). The lack of N region insertions in a substantial proportion of B-1a cells reveals that rearrangements in these cells takes place in the absence of TdT. TdT has been shown to be absent in B cells rearranging during fetal and neonatal development up until the first week of life (Feeney, 1990; Gu et al., 1990). This indicates that a substantial population of B-1a cells may develop early in ontogeny, and maintain themselves into adult life. Further support for early development of B-1a cells comes from the preferential use of J_{H1} rearrangements in B-1a cells. The use of J_{H1} in rearrangements is proportionally higher in B-1a cells, especially in V_{H11} rearrangements, than in B-1b and B-2 cells (Hardy et al., 1989; Carmack et al., 1990; Tornberg and Holmberg, 1995; Kantor et al., 1997). Similarly, the proportion of fetal pre-B cells utilizing J_{H1} segments is much higher than the proportion of adult pre-B cells that utilize J_{H1} (Gu et al., 1990). Whether these over-representations in gene segment usage in B-1a

cells is a result of antigen selection or directed utilization during joining is an area of controversy. This feature of B-1a cells has been used as further evidence that B-1a cells have unique origins from B-1b and B-2 cells.

To help elucidate the question of the existence of B-1a precursors in adult murine bone marrow, B cell reconstitution was examined in the peritoneal cavity of both syngeneic and allogeneic non-irradiated RAG and SCID recipients of adult bone marrow. Because there has been varying degrees of success with B-1a cell engraftment in irradiated hosts with regard to the amount of bone marrow transferred and the time allowed for engraftment, both of these variables were examined in non-irradiated syngeneic RAG recipients of adult bone marrow. In addition to examining the variables that effect B-1a cell engraftment, the variable heavy chain gene rearrangements of V_H11 and J558, a commonly occurring V_H segment in all three B cell subsets, were examined from FACS sorted peritoneal B-1a cells of C57Bl/6 mice and RAG recipients of C57Bl/6 adult bone marrow. It is hypothesized that B-1a cells of adult origin in reconstituted mice will contain N region insertions similar to that found in adult B-2 cells if these cells are following a pattern of development that suggests that they are not derived from unique fetal precursors which are lost after one week of life.

Materials and Methods

Mice

Adult BALB/c, C57Bl/6, and CB.17 SCID mice were purchased from Taconic (Germantown, NY). RAG-2^{-/-} mice were bred in house. All mice were kept under pathogen free conditions. C57Bl/6 mice were used for syngeneic bone marrow transfers into RAG-2^{-/-} (RAG) mice and allogeneic transfers into CB.17 SCID (SCID) mice. BALB/c mice were used for syngeneic transfers into SCID mice and allogeneic transfers into RAG mice. Two to four month-old donor and recipient mice were used for bone marrow transfers.

Bone marrow transfers and cell preparation

Bone marrow was obtained from BALB/c and C57Bl/6 donor mice. Femurs and tibias were flushed with Dulbecco's phosphate buffered saline (D-PBS) and a single cell suspension was created. T cells were depleted by complement lysis using anti-CD90.2 (Thy 1.2, Pharmingen, San Diego, CA) and rabbit and guinea pig complement. Anti-CD90.2 was added to extracted bone marrow at a concentration of 1 μ g/10⁷ cells. Cells were incubated on ice for 30 minutes, washed with D-PBS and added to 2.5 to 5 ml of MAR18.5 supernatant (mouse anti rat IgG) plus a 1 in 20 dilution of rabbit and guinea pig complement (Cedarlane, Ontario, Canada). Cells were incubated for one hour at 37°C. T

depleted bone marrow cells were isolated using Histopaque (density = 1.119; Sigma, St. Louis, MO). For the bone marrow titration experiment 10^6 , 5×10^6 , 10^7 or 3×10^7 T cell depleted C57Bl/6 adult bone marrow cells in a volume of 100 μ l D-PBS were injected into the tail vein of RAG recipients. Mice were sacrificed and their peritoneal cavity (PerC) cells were analyzed at eight weeks after transfer. B cell subset and T cell engraftment was compared between the groups receiving different numbers of bone marrow cells and donor mice which served as a positive control. For all other transfers, 10^7 cells were injected into the tail vein of SCID and RAG recipient mice, and cells were analyzed 12 weeks after transfer. Syngeneic and allogeneic transfers were made with both immunodeficient strains of mice.

PerC cells were harvested at the time of sacrifice. PerC cells were also collected from C57Bl/6 and BALB/c donor control mice as well as SCID and RAG control mice that were not engrafted. PerC cells were obtained by flushing the peritoneal cavity with 3 ml D-PBS. This method allowed for a recovery of approximately 90% of the injected volume. Any red cells present were lysed with ammonium chloride (Sigma). Cells were washed and counted using trypan blue exclusion.

FACS staining and analysis

Approximately 10^6 PerC cells in 50 μ l were stained with 5 μ l of each of the following antibodies: a) anti-IgM FITC (clone R6-60.2; Pharmingen, San Diego, CA), b) anti-B220

PE (clone RA3-6B2; Caltag, Burlingame, CA), c) anti-CD5 CyChrome (clone 53-7.3; Pharmingen), and d) anti-MAC-1 biotin (clone M1/70.15; Caltag), incubated for 15 minutes at 4°C, followed by a 15 minute, 4°C staining with 3 µl streptavidin APC (Caltag). For the bone marrow titration and FACS sorting experiments the cells were incubated with anti-CD16/32, to block the binding of Ab to Fc receptors (clone 2.4G2; Pharmingen), for five minutes at room temperature before staining with anti-IgM FITC, anti-CD5 PE (clone 53-7.3; Pharmingen), anti-B220 PerCP (clone RA3-6B2; Pharmingen) and with anti-MAC-1 biotin and streptavidin APC as above. Staining with the second protocol yielded the same results as the first protocol when the cells are analysed as shown in Figure 1. Cells were washed and resuspended in FACSFlow buffer (Becton Dickinson, San Jose, CA). Class II MHC (major histocompatibility complex) stainings were performed to determine the origin of B-1a cells in allogeneic transfers. PerC cells were stained with anti I-A^d PE (BALB/c and SCID specific) and anti-I-A^b FITC (C57Bl/6 and RAG specific). Four color analysis was performed on the FACSCalibur (Becton Dickinson). Analyses were assessed with CellQuest software (Becton Dickinson). B and T cells were quantified as described (Figure 1). B-1a cells were identified by positive expression of IgM, CD5, and MAC-1. B-1b cells were identified by positive expression of MAC-1 and IgM, and negative expression of CD5. B-2 cells were identified by a broad positive expression of IgM and bright expression of B220 and a negative expression of MAC-1 and CD5. Regions for all lymphocyte subpopulations were drawn after gating on lymphocytes in forward and side scatter. B-1a

Figure 1. B cell populations in the PerC of a Balb/c mouse. B-1a cells are identified by the presence of bright IgM and dim CD5 levels (R1) and by the presence of MAC-1 (R3). B-1b cells are CD5- IgM bright and MAC-1 positive (R3). B-2 cells are B220 bright, IgM+, MAC 1- and CD5-. T cells are identified by the presence of bright CD5 levels, and the absence of surface immunoglobulin (R4). All plots were gated on low forward and side scatter lymphocytes. B-1a cells (magenta) were enumerated by gating on IgM bright, MAC 1+ (R3) cells to achieve a cleaner population. B-1b cells (magenta) were enumerated by subtracting the B-1a cells (R1) from R3. B-2 (green) cells were quantified by subtracting IgM bright, MAC 1+ cells (R3) from the total B cell population (R5). To calculate the total number of cells in a population, the percentage of cells for that population is determined as a percentage of total cells acquired, and is multiplied by the total number of cells obtained from the PerC.

cells from C57Bl/6 and syngeneic RAG engrafted mice were sorted for PCR of V_H genes using the same gate that was set for B-1a analysis.

RNA isolation and cDNA synthesis

Total RNA was isolated from individually sorted B-1a cell populations from four C57Bl/6 mice and four RAG mice engrafted with C57Bl/6 bone marrow using RNeasy Blood Mini Kit (Qiagen, Valencia, CA). Cell numbers for each sample ranged from 5×10^3 to 3×10^4 . cDNA was constructed using 0.5 µg oligo (dT) 12-18mer primers and 10 µl RNA from a 30 µl preparation. The sample was incubated at 70°C for 10 minutes and 0°C for one minute. Tris (30mM), 5mM MgCl₂, and 30mM KCl, and 1 µl of 10 mM each dATP, dGTP, dCTP, and dTTP was added to the reaction mix and incubated for five minutes at 42°C in a 20 µl reaction. Two hundred units SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD) was added to the reaction. The contents were incubated at 42°C for 50 minutes. cDNA production was followed by an RNase digestion using 2 units *E. coli* RNase.

PCR

Two rounds of nested PCR were used to amplify V_H genes from the cDNA produced from FACS sorted B-1a cell populations. For the first round of PCR, DNA was amplified

from the Framework 1 (FR-1) region of the V_H segment to a 5' region of the C_μ segment. Five μl of the extracted DNA (approximately 1500 to 8000 genome copies) was used for the first round of amplification (Figure 2). In the second round of PCR, specific V_H genes were amplified from 5 μl of the first round reaction (Table 1). Primers for the second round of amplification were designed using known V_H11 and J558 sequences, and the four J_H sequences, using regions in the gene segments that have the least homology with one another to ensure that only the specific gene segments are amplified. V_H11 and J558 variable gene segments were amplified with each of the four J_H genes for each of eight mice. Both rounds of PCR were amplified in 30mM Tris, 1.65 mM MgCl₂, and 30mM KCl. Both primers (0.3 μM of each), 1 μl of 10mM each dATP, dGTP, dCTP, and dTTP and 5 units of Taq polymerase (Boeringer Mannheim, Indianapolis, IN) were used in the 100 μl reaction volume. For both rounds of amplification the following program was used: 96°C for 3 minutes for denaturation of DNA; 35 cycles of the following: 97°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds (Kantor et al., 1997); followed by an extension at 72°C for five minutes. PCR reactions were performed using PCR Minicycler (MJ Research, Watertown, MA).

Figure 2. Rearranged heavy chain gene segments. External primers in the first round of PCR were specific to the conserved Framework 1 (FR-1) V_H region (V_HExt) and the 5' end of the Cμ region (CμExt). The second round of primers was specific to the V_H families J558 or V_H11 and the J_H regions.

Table 1. Primers for PCR. V_HE and CμE were used for the first round of amplification (Kantor et al., 1997). Specific V_H and J_H primers were used in the second round of amplification. V_H11 and J558 were each amplified with each of the J_H primers. The V_H primers were designed with a *Not*I restriction site, and the J_H primers include an *Xho*I restriction site (underlined).

Primer	Sequence
V _H Ext	TGGAGGTGCAGCTGCAGGAGTCTGG
CμExt	ATGGCCACCTTATCAGA
V _H 11	<u>CGCGCGGCCG</u> CACATTAATTCTGATGGCAG
J558	<u>CGCGCGGCCG</u> CAAGGCTTTTGGCTACACTT
J _H 1	<u>GCGCTCGAG</u> AGGAGACGGTGACCGT
J _H 2	<u>GCGCTCGAG</u> TGAGGAGACTGTGAGA
J _H 3	<u>GCGCTCGAG</u> CAGAGACAGTGACCAG
J _H 4	<u>GCGCTCGAG</u> AGGAGACGGTGACTGA

Ligation

PCR products were purified with QiaQuick PCR Purification Kit (Qiagen). Products were digested with 10 units each *NotI* and *XhoI* restriction endonucleases in 50 mM Tris, 10 mM MgCl₂, 100 mM NaCl, and 1 mM dithioerythritol (DTE) overnight at 37°C. pBluescript KS- phagemid (Stratagene, La Jolla, CA) was also digested with *NotI* and *XhoI*, followed by treatment with 2.5 units of calf intestinal phosphatase to prevent reannealing of the vector. Digests were electrophoresed through a 1.0% GTG agarose gel to eliminate small digested ends. Gels were viewed on a long wave UV transilluminator to excise bands. *V_{H11}* derived PCR products were approximately 225 base pairs and J558 products were approximately 300 base pairs in length. DNA was extracted from the agarose with Qiaquick Gel Extraction Kit (Qiagen). Ligations were performed for 5 minutes at room temperature with DNA Rapid Ligation Kit (Boeringer Mannheim).

Transformation

Five µl of the ligation product was added to 200 µl of competent JM101 *Escherichia coli* cells. Cells were placed on ice for 30 minutes, then heat shocked for 2 minutes at 42°C. Cells were incubated for one hour in 1 mL Luria Broth (LB) in a 37°C shaker at 250 RPM to allow expression of the ampicillin resistance gene. Cells were plated on X-gal/IPTG selection (white colonies contain inserts, blue colonies do not) LB agar plates with 50 µg/ml carbenicillin, and grown over night at 37°C. Two or three colonies per

PCR product were selected and grown overnight in 5 ml LB with 250 µg carbenicillin in a 37°C shaker at 250 RPM. Plasmids were isolated with Qiaprep 8 Turbo Mini Prep Kit (Qiagen).

Sequencing and analysis

Plasmid DNA was sequenced using the specific V_H primers that were used in the PCR reaction. Approximately 500 ng DNA from the plasmid prep was added to 6 pmoles of primer. Sequencing was done in house (Genencor International Inc., Palo Alto, CA) with ABI Prism BigDye Terminator cycle sequencing kit using Amplitaq DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequencing was done with Applied Biosystems Model 373 DNA Sequencer.

V_H and J_H sequences were identified by sequence homology with known sequences retrieved from GenBank or EMBL databases. Sequences were aligned using DNA Star MegAlign software (Madison, WI). Diversity (D) segments were identified with the longest matching sequence to the four known families: SP2, FL16, D_{Q52} , (Ichihara et al., 1989) and D_{ST4} (Feeney and Riblet, 1993). Second D segments were assigned only if the match was five nucleotides or greater (Kantor et al., 1997). P elements were identified by their complementary sequence (forming a palindrome) to V_H , D, or J_H sequences that are present in their full coding sequence (Lafaille et al., 1989). The remaining nucleotides were then assigned to the N region segments.

Statistical analyses

Unpaired T test analyses were performed on the engraftment data to determine if there are significant differences in the levels of B-1a cells present in the peritoneal cavities of mice engrafted with adult bone marrow and the donor strains of mice. *Chi* square analyses were performed on sequence data to determine if there are significant differences in the frequency of N region insertions in the B-1a cells of donor mice and engrafted mice.

Results

Bone marrow transfer titration

C57Bl/6 T cell depleted adult bone marrow was transferred into RAG mice to determine if decreasing the number of cells transferred has an effect on the relative proportions of B cells that engraft. The following groups of RAG mice were inoculated with the indicated number of T cell depleted bone marrow cells: a) 3×10^7 (three mice), b) 10^7 (five mice), c) 5×10^6 (two mice), and d) 10^6 (five mice). Five C57Bl/6 donor control mice were also analyzed. Two of the three mice in the 3×10^7 group were eliminated because of an animal housing error that consisted of a male mouse being placed with two female mice which resulted in a pregnancy of one of the females (both the male and the pregnant female were eliminated). Eight weeks after transfer, PerC cells were harvested as described in the Methods section. The cells were counted, stained, and analyzed by flow cytometry for B-1a, B-1b, B-2, and T cells as described in the Methods section. When decreasing amounts of T cell depleted bone marrow were transferred into RAG mice, the total number of B cells decreased for all three subset populations, almost to zero when 10^6 cells were transferred (Figure 3). T cell engraftment, although variable among recipients of the same number of bone marrow cells, remained similar to the levels in mice of the donor strain, regardless of the number of cells transferred.

The relative proportion of the B cell subset engraftment eight weeks after transfer was compared between the above groups of RAG mice receiving decreasing amounts of T cell

depleted bone marrow. The number of cells transferred had little effect on the proportion of B-1a cells that engrafted. There was a however, a decrease in the proportion of B-1b cells with a corresponding increase in the proportion of B-2 cells between the group receiving the highest and the group receiving the lowest amount of bone marrow cells (Figure 4). No conclusion can be made on the significance of this decrease in B-1b cells and increase in B-2 cells because only one mouse was analyzed for the 3×10^7 group. Additionally, cell numbers for all three B cell subpopulations were very low for the 10^6 group resulting in highly variable percentages within this group. The most dramatic effect of decreasing the amount of bone marrow cells for transfer was with the relative proportions of total B and T cells in the peritoneal cavity. The proportion of B cells dropped and the proportion of T cells rose as decreasing amounts of bone marrow were transferred (Figure 5).

Figure 3. Total number of B-1a, B-1b, B-2 and T cells in the peritoneal cavities of C57Bl/6 mice and RAG recipient mice with decreasing amounts of transferred T cell depleted adult C57Bl/6 bone marrow cells. The group of RAG mice that received 10^7 bone marrow cells was from a separate experiment.

Figure 4. B cell subset percentages in the peritoneal cavities of C57Bl/6 mice and RAG recipient mice with decreasing amounts of transferred T cell depleted adult C57Bl/6 bone marrow cells. Values are expressed as a percentage of total B cells in the peritoneal cavity. The group of RAG mice that received 10^7 bone marrow cells was from a separate experiment.

Figure 5. Percentages of B and T cells (expressed as a percentage of lymphocytes) in the peritoneal cavities of C57Bl/6 mice and RAG recipients with decreasing amounts of transferred T cell depleted adult C57Bl/6 bone marrow cells. The group of RAG mice that received 10^7 bone marrow cells was from a separate experiment.

Length of time allowed for engraftment

While the amount of bone marrow cells transferred had a small impact on the proportion of B-1a cells that engrafted, the time allowed for engraftment had a more pronounced effect on B-1a cell engraftment. T cell depleted bone marrow cells (10^7) were transferred into 16 RAG mice in two separate experiments. Five mice were examined at eight weeks and 11 were examined at 12 weeks. The same five C57Bl/6 mice were used as donor controls for both the titration and the engraftment time experiments. The length of time allowed for engraftment had a positive effect on the

proportion of B-1a cells that engrafted (Figure 6). There was no significant difference between the proportion of B-1a cells present in the peritoneal cavity of donor C57Bl/6 mice and the engrafted RAG mice analyzed at 12 weeks; however, the mice analyzed at eight weeks had a significantly smaller proportion of B-1a cells than both the donor mice and the RAG mice that were allowed 12 weeks to engraft (mean percentages, T test, $P = 0.012$ and $P = 0.013$ respectively).

Figure 6. Percentage of B-1a cells in the peritoneal cavities of C57Bl/6 mice and RAG mice eight and 12 weeks after receiving 10^7 T cell depleted adult C57Bl/6 bone marrow cells. The groups of RAG mice were from two separate inoculations.

Bone Marrow Transfers in allogeneic and syngeneic RAG and SCID mice

In this study, 42 RAG and SCID mice were inoculated with adult bone marrow and cells in the peritoneal cavity were analyzed for B and T cell engraftment. Twelve RAG mice were syngeneically inoculated and nine SCID mice were allogeneically inoculated with 10^7 C57Bl/6 derived T-cell-depleted bone marrow cells. Fourteen RAG mice were allogeneically inoculated and seven SCID mice were syngeneically inoculated with 10^7 BALB/c derived T- cell-depleted bone marrow cells. Five of the allogeneic RAG recipients did not engraft any detectable B or T cells and five possessed donor T cells but no detectable B cells. All 10 of these animals were excluded from analysis. All other animals (32 out of 42) had donor T and B cells.

Origin of engrafted peritoneal B cells

PerC lymphocyte cells were analyzed for MHC class II markers specific for donor cells. Analysis was done with allogeneic hosts to determine the origin of their B cells. Both BALB/c and SCID mice express I-A^d on their B cells. C57Bl/6 and RAG mice express I-A^b. Most of the B cells (97.3%) in the allogeneic SCID hosts were from donor origin (I-A^b) (Figure 7). Because none of the MHC class II positive I-A^b cells in the non-inoculated RAG mice contain surface IgM (data not shown), it can be concluded that 100% of the mature B cells in the inoculated RAG hosts are of donor origin (I-A^d).

Figure 7. MHC II staining in donor strain mice and allogeneic adult bone marrow recipients. Representative plots are shown. Cells were gated on lymphocytes in forward and side scatter. Balb/c and SCID mice express class II I-A^d, and C57Bl/6 and RAG mice express class II I-A^b. In the allogeneic SCID recipients 97.3% of the MHC class II B cells are of donor origin. In the non-engrafted RAG mice, because none of the MHC class II positive I-A^b cells have surface immunoglobulin (data not shown), it can be concluded that 100% of the B cells in engrafted RAG mice are of donor (I-A^d) origin.

Peritoneal lymphocyte engraftment in RAG hosts

Total numbers of B-1a, B-1b, B-2, and T cells in the peritoneal cavity were enumerated for donor and engrafted mice (Table 2a). Both the syngeneic and allogeneic RAG hosts engrafted poorly. In the syngeneic hosts, the total number of B cell engraftment was only 22% of the total number observed in donor mice. All three subsets of B cells were significantly lower in numbers than the donor mice. There was little difference in reconstitution levels between the subsets of B cells in the syngeneic RAG hosts with the exception of a slightly higher level of B-1b engraftment (Table 2b). The total number of B-1a cells engrafted in syngeneic RAG recipients was 22.3% of the level of these cells in the donor strain, while B-1b cell reconstitution was 30.6%, and B-2 cell reconstitution 20.2% (Table 2b). The reduction of these lymphocyte subpopulations in the host was due in part to a reduction in total lymphocyte cell numbers. There was also a proportionally greater reduction of B cells than T cells in the peritoneal cavity (Figure 8), which were present at 59% of levels found in donor mice (Table 2b). In the allogeneic RAG recipients, the total number of B-1a cells was 19.3%, the total number of B-1b was 7.6% and the total number of B-2 cells was 4.6% of the level of these populations in normal BALB/c donor mice. T cell reconstitution was 31% of normal BALB/c mice. Similar to the syngeneic RAG mice, there was an overall reduction in lymphocyte numbers compared to the donor. There also was a similar decrease in the proportion of B cell lymphocytes in the peritoneal cavity (Figure 8). B-1b and B-2 cell engraftment was particularly low (Table 2a and 2b). Allogeneic and syngeneic RAG hosts had roughly the

same level of B-1a cell engraftment when analyzed as a percentage of their respective donors, but the allogeneic hosts had much lower levels of B-1b and B-2 cells than syngeneic hosts (Table 2b).

Peritoneal lymphocyte engraftment in SCID hosts

Bone marrow transfers in SCID mice revealed a different pattern of engraftment from that observed in RAG hosts. Engraftment was higher for all subsets of lymphocytes in the SCID hosts than in the RAG hosts. Differences in B cell subset reconstitution was evident in both syngeneic and allogeneic SCID hosts (Table 2a and 2b). Both SCID hosts showed similar patterns of engraftment, with B-1b cells engrafting at higher levels than B-1a and B-2 cells (Table 2b). Although the syngeneic hosts engrafted more of all three subsets of B cells than the allogeneic host (Table 2a), the allogeneic SCID hosts engrafted a higher level of the three B cell subsets compared to their C57Bl/6 donor mice than the syngeneic host did compared to their BALB/c donors (Table 2b). Total numbers of B-1a cells in the syngeneic SCID hosts were 52.2% of the total number in the BALB/c donor mice, B-2 cells were 46% of the levels found in the donor mice, while B-1b cell engraftment was greater than the donor mice (Table 2b). In the allogeneic engrafted SCID mice, both B-1a and B-1b cell engraftment levels were greater than the donor, while B-2 reconstitution was 79% of the level found in the donor mice. In the syngeneic SCID hosts the numbers of both B-1a and B-2 cells were significantly reduced from donor numbers (Table 2a). For the allogeneic engraftments into SCID mice, none of the

Table 2a. Total B-1a, B-1b, B-2, and T cells in the peritoneal cavities of control and engrafted mice. RAG mice were engrafted with 10^7 T cell depleted syngeneic C57Bl/6 or allogeneic Balb/c bone marrow. SCID mice were engrafted with 10^7 T cell depleted syngeneic Balb/c or allogeneic C57Bl/6 bone marrow. PerC cells were analyzed 12 weeks after transfers. Live cells were counted on a hemacytometer using trypan blue exclusion, and multiplied by the fraction of B-1a, B-1b, B-2, or T cells to the total population as determined by FACS analysis. Significant differences between donors and engrafted hosts as determined by a T test are indicated with asterisks.

	n	Number of cells recovered from PerC $\times 10^5$			
		B-1a	B-1b	B-2	T
C57Bl/6 donor:	8	1.66 $\pm$ 0.99	0.72 $\pm$ 0.69	3.37 $\pm$ 1.85	3.29 $\pm$ 1.67
Syngeneic RAG	11	0.37 $\pm$ 0.35***	0.22 $\pm$ 0.18*	0.68 $\pm$ 0.54***	1.96 $\pm$ 1.98
Allogeneic SCID	8	1.76 $\pm$ 1.30	1.84 $\pm$ 1.38	2.68 $\pm$ 1.29	1.99 $\pm$ 1.16
Balb/c donor:	8	5.23 $\pm$ 1.42	1.84 $\pm$ 1.01	8.55 $\pm$ 2.41	4.69 $\pm$ 1.32
Allogeneic RAG	4	1.01 $\pm$ 1.10***	0.14 $\pm$ 0.10**	0.39 $\pm$ 0.46***	1.44 $\pm$ 1.16**
Syngeneic SCID	7	2.73 $\pm$ 1.79**	2.17 $\pm$ 1.92	3.96 $\pm$ 3.22**	5.45 $\pm$ 2.46
SCID	5	0.15 $\pm$ 0.28	0.17 $\pm$ 0.34	0.18 $\pm$ 0.33	0.17 $\pm$ 0.14
RAG	4	0	0	0	0.10 $\pm$ 0.13

* P < 0.05

** P < 0.01

*** P < 0.001

Table 2b. Peritoneal B-1a, B-1b, B-2, and T cells in recipients of adult bone marrow. Numbers represent the mean number of lymphocyte subsets in engrafted mice reported as a percentage of the cells in the donor mice. Numbers are derived from Table 2a: $[(\text{Total number peritoneal lymphocyte subset in engrafted mice}) / (\text{Total number peritoneal lymphocyte subset in donor mice})] * 100$. RAG mice were engrafted with 10^7 T cell depleted syngeneic C57Bl/6 or allogeneic Balb/c bone marrow. SCID mice were engrafted with 10^7 T cell depleted syngeneic Balb/c or allogeneic C57Bl/6 bone marrow. PerC cells were analyzed 12 weeks after transfers.

	n	<u>Total number peritoneal lymphocyte subset in engrafted mice * 100</u>			
		B-1a	B-1b	B-2	T
Syngeneic RAG	11	22.3	30.6	20.2	59.6
Allogeneic RAG	4	19.3	7.6	4.6	30.7
Syngeneic SCID	7	52.2	117.9	46.3	116.2
Allogeneic SCID	8	106.0	255.6	79.5	60.5

differences were significant. The percentage of B and T cell engraftment showed opposite patterns in syngeneic and allogeneic SCID hosts, with the proportion of T cells going up in the syngeneic and down in the allogeneic engraftments, and the proportion of B cells going down in the syngeneic and up, though not significantly, in the allogeneic hosts compared to the respective donor mice (Figure 9).

Figure 8. B and T cell percentages in the peritoneal cavities of C57Bl/6 and BALB/c adult bone marrow donor mice and syngeneic and allogeneic engrafted RAG recipients. Mice were analyzed 12 weeks after transfers. Mean values are reported. Error bars represent one standard deviation. P values are given for lymphocyte populations that are significantly different from donor mice.

Figure 9. B and T cell percentages in the peritoneal cavities of C57Bl/6 and BALB/c adult bone marrow donor mice and allogeneic and syngeneic engrafted SCID recipients. Mice were analyzed 12 weeks after transfers. Error bars represent one standard deviation. P values are given for lymphocyte populations that are significantly different from donor mice.

Comparison of B subset proportions in engrafted immunodeficient mice

B-1a engraftment was examined as both a percentage of lymphocytes and as a percentage of B cells in the peritoneal cavity (Figure 10). While there are significant differences in the percentage of B-1a cells in the syngeneic engrafted hosts (for RAG, $P < 0.05$; for SCID, $P < 0.01$) when calculated as a percent of lymphocytes, this is largely due to a proportional increase of T cells (Figures 8 and 9). There is no significant difference in the proportion of the three subsets of B cells between syngeneic RAG hosts and C57Bl/6 donors (Figure 11) when they are calculated as a percentage of total B cells. For

Figure 10. Comparison of peritoneal B-1a populations in donor, immunodeficient and engrafted mice. Representative plots are shown. The number of experimental mice examined are reported for each figure. The top percentage value represents the average percentage of B-1a out of the total peritoneal lymphocyte population, and the bottom figure represents the average percentage of B-1a cells out of the total peritoneal B cell population. Errors are given in parentheses, and represent one standard deviation.

both the syngeneic and allogeneic engrafted SCID hosts there was no significant difference in the proportion of B-1a cells, but there was an increase in B-1b cells and a corresponding decrease in B-2 cells compared to their respective donors (Figure 12). The proportion of B-1a cells in allogeneic RAG hosts nearly doubled, while the proportion of B-2 cells was less than half that of BALB/c donors (Figure 11).

Figure 11. B cell subset populations in adult bone marrow donors and RAG hosts reported as a percentage of total B cells in the peritoneal cavity. Mean values are reported. Error bars represent one standard deviation. P values are given for B cell populations that are significantly different from donor mice.

Figure 12. B cell subset populations in adult bone marrow donors and SCID hosts reported as a percentage of total B cells in the peritoneal cavity. Mean values are reported. Error bars represent one standard deviation. P values are given for B cell populations that are significantly different from donor mice.

N region insertions in FACS sorted B-1a cells

B-1a cells were sorted from the peritoneal cavity of four C57Bl/6 mice and four RAG mice engrafted with C57Bl/6 bone marrow as described in the Methods section. The mean purity of the B-1a cell FACS sorts was $95.3\% \pm 3.1$ of the total B cell population (Figure 13). Because the purity was not 100% B-1a cells, some of the sequences could have been derived from B-1b or B-2 cells, but the comparisons made are relative and there is an equal chance for impurities in both groups.

Figure 13. B-1a FACS sort. Representative plots are shown. **A.** B cells in the peritoneal cavity of a C57Bl/6 mouse before sorting. **B.** B cells in the peritoneal cavity of a C57Bl/6 mouse after sorting for B-1a cells. The number of experimental animals is given. Percentages given represent the mean percentage of B-1a cells of the total population of B cells. In **B.** the numbers represent four C57Bl/6 mice and four RAG recipients of C57Bl/6 bone marrow.

Variable heavy chain transcripts were amplified using RT-PCR. Sequences of both J558 and V_H11 with each of the four J_H segments were examined and compared between the donor mice and the engrafted mice (Table 3a-d). All of the V-D junctions in the engrafted mice had N-region insertions (Table 3a), while 72% of the V-D junctions in the donor mice had N-region insertions (Table 3b). This difference is significant (*Chi square*: $P = 0.0197$). There were also significantly more J558 sequences with N-region insertions at both junctions in the engrafted mice (63% in the engrafted mice and 33% in the donor mice; $P = 0.0489$); however none of the J558 rearrangements from donor mice or engrafted mice had a lack of N-region insertions at both junctions. Both the donor mice and the engrafted mice showed low numbers of N region insertions at both junctions (Tables 3c and d). This was particularly true with the V_H11-J_H1 rearrangements. When these rearrangements are eliminated from analysis, approximately half of the rearrangements of the engrafted mice have N region insertions at either junction and one third of the rearrangements of the donor mice have N region insertions. These differences are not significant when analyzed using *Chi square*. There is no difference in the amount of nucleotides added at the junctions between the donor and the engrafted mice.

Another interesting observation with the V_H11 rearrangements is their relative lack of diversity with D segment usage, especially when arranged with J_H1. In both the donor and engrafted mice the same segments of D gene show up. The 5' 10 nucleotides of DSP, TGGTAACTAC, and a 10 nucleotide region of DFL, TAGCAGCTAC, were found both in the donor mice and in the engrafted mice for both independent sorting experiments and

a rearrangement with the 13 5' nucleotides of another member of the DSP family, CGGTAGTAGCTAC, and another member of the DSP family, TAGTAACTAC, were found in both the donor and recipient mice in the second sorting experiment. The latter was also found in donor mice of the first sorting experiment joined to a J_H1 and another joined to a J_H3. Not shown in Tables 3c or 3d is that these same V-D-J rearrangements were found in multiple mice within the same group of mice for each sorting experiment. Rearrangements with V_H11-J_H1 and TGGTAACTAC and CGGTAGTAGCTAC in multiple mice with a specificity for phosphatidylcholine has been reported by others (Seidl et al., 1997a). Additionally Tornberg and Holmberg (1995) reported V_H11 rearrangements with TGGTAACTAC, CGGTAGTAGCTAC, and TAGTAACTAC. Because careful precautions were taken to prevent cross-contamination, because the experiments were run several months apart, and because this was not seen with the J558 rearrangements, this is not believed to be an artifact of contamination.

Table 3a. J558 V-D-J junctional sequences from rearranged transcripts of FACS sorted B-1a cells from four C57Bl/6 mice. The variable segment is given beginning with TGT (Cys). P elements are shown for both the V-D junctions and the D-J junctions. N region insertions are also reported for both junctions. All of the D region is shown and the J region is reported to GAT for J_H1, GAC for J_H2 and J_H4, and GCT for J_H3 (ASP for J_H1, 2, and 4 and Ala for J_H3). J_H and D_H families are noted. D-D joined segments are separated with a vertical line. The proportion of junctions with N region insertions is indicated at the bottom of both N region columns.

Clone	V	P	N	P	D	P	N	P	J	JH	DH
JB1A	TGTACAAGA		GCC	G	CTTACTATGGTTAC	G		ACTGGTACTTCGAT		1	DSP
JB9A	CGTACAAGA		GCCGCT		TACTATGGTTACGAC			TGGTACTTCGAT		1	DSP
JB13A	TGTGCAAGA	T	TGGGGGG		TGACTGGTAC			TTCGAT		1	DSP
JB13B	TGTGCAAG		G	AG	CTACGGTAGTAGCT		TT	TACTGGTACTTCGAT		1	DFL
JB2B	TGTACAAGA		GCGCGG		TTACG		TG	ACTTTGAC		2	DSP
JB6A	TGTGCAAGA				TGGTTAC		TTTTTC	TTGAC		2	DSP
JB10B	TGTGCAAGA				AACGAT GG TAGCT		CCC	TTGAC		2	DSP+DFL
JB14A	TGTGCAAGA	T	CCCG		TGATTACGAC	G		ACTTTGAC		2	DSP
JB3A	TGTGCAAGA		GAGGGA		TATGGT			CCCTGGTTGCT		3	DSP
JB3B	TGTGCAAGA		AG		TGATTACGAC		TC	GTTTGCT		3	DSP
JB7A	TGTGCAAGA				TGGTTACT		TTTTC	TTGAC		2	DSP
JB7B	TGTGCAAGA		AGGGGGGT		ATACTATAG T TACGAC	G		GGTTTGCT		3	DSP+DSP
JB15A	TGTGCAAG				GGGA		G	GGTTTGCT		3	DQ
JB4A	TGTGCAAGA		AGGA		GTAA		GGGG	GCTATGGAC		4	DSP
JB8B	TGT			TT	AACTGGGAC		GGGG	GCTATGGAC		4	DQ
JB12A	TGT		GGGG		TGGTAAGTAC GAAGATTAT			TACTATGCTATGGAC		4	DSP+DSP
JB16A	TGTGCAAGA	T	TT		GGCT		ATATATCCT	CTATGGAC		4	DST
JB16B	TGTGCAAGA		AGGTATTGGCCC		TATGGTT			TGGAC		4	DSP
										13/18	10/18

Table 3b. J558 V-D-J junctional sequences from rearranged transcripts of FACS sorted B-1a cells from four RAG recipients of C57Bl/6 adult bone marrow. The variable segment is given beginning with TGT (Cys). P elements are shown for both the V-D junctions and the D-J junctions. N region insertions are also reported for both junctions. All of the D region is shown and the J region is reported to GAT for J_H1, GAC for J_H2 and J_H4, and GCT for J_H3 (ASP for J_H1, 2, and 4 and Ala for J_H3). J_H and D_H families are noted. D-D joined segments are separated with a vertical line. The proportion of junctions with N region insertions is indicated at the bottom of both N region columns.

Clone	V	P	N	P	D	P	N	P	J	JH	DH
JR1B	TGTGCCAGA		GAC		TGTAC		ATGGACCTT		TACTGCTNCTTCGAT	1	DSP
JR5A	TGTGCAAGA		AGCCGA		TACGGTAGTAGCTAC G TGGTA				TGGTACTTCGAT	1	DFL+DSP
JR2A	TGTACAAGA	T	GCC		TCTAC		GGGGGG		GACTACTTTGAC	2	DSP
JR6A	TGTGCAAGA		G	G	CTATGATGGT				TACCTTGAC	2	DSP
JR2B	TGTACAAGA		GAAGATG		ACTATGATTACGAC		GAAG		GGTTTGCT	3	DSP
JR4B	TGTGCAAGA	T	TTGGGG		AGATAC		C		CTATGCTATGGAC	4	DFL
JR9A	TGTACAAGA		GCCGCT		TACTATGGTTACNAC				TGGTACTTNGAT	1	DSP
JR13B	TGTGCAAG		GAG		CTACGGTAGTAG		CTTT		TACTGCTACTTCGAT	1	DFL
JR10A	TGTACAAGA	T	GCCT		CTAC		GGGGGG		GACTACTTTGAC	2	*
JR14A	TGTGCA		GCACG		GGTAA		GG		GACTACTTTGAC	2	DSP
JR14B	TGTGCAAGA		TGGGAGACAA		TAGCTAC		GAC		GACTACTTTGAC	2	DFL
JR11A	TGTGCAAG		GC		CAGCTCGGGCTAC	GT			CCTGGTTTGCT	3	DST
JR11B	TGTGCAAGA		GAAGATCCAAT		CTAT				*GG	3	DSP
JR15A	TGTGCAAGA	T	AT		TACGGTAGTAG		CCCT		TTTGCT	3	DLF
JR8A	TGTGCAAGA		G		GGG				ATGCTATGGAC	4	DQ/DST
JR12A	TGTGCAAGA	TCT	AAGGCA		GATGGTTACTAC		TATTC		CTATGCTATGGAC	4	DSP
JR12B	TGAGCAAGA		AG		TGATGGT				TACTATGCTATGGAC	4	DSP
JR16B	TGTGCAAGA	T	TTGGGG		AGGTAC		C		CTATGCTATGGAC	4	DSP
JR3C	TGTGCAAGA		TCGGC		CTACTATAGTAACT		CC		TTTGCT	3	DSP
										19/19	12/19

Table 3c. VH11 V-D-J junctional sequences from rearranged transcripts of FACS sorted B-1a cells from four C57Bl/6 mice. The variable segment is given beginning with TGT (Cys). Elements are shown for both the V-D junctions and the D-J junctions. N region insertions are also reported for both junctions. All of the D region is shown and the J region is reported to GAT for J_H1, GAC for J_H2 and J_H4, and GCT for J_H3 (ASP for J_H1, 2, and 4 and Ala for J_H3). J_H and D_H families are noted. D-D joined segments are separated with a vertical line. Two separate sorting experiments are separated with a solid horizontal line. The proportion of junctions with N region insertions is indicated at the bottom of both N region columns. Nucleotides that can be placed in either the D or J region are placed in the D region and are underlined.

Clone	V	P	N	P	D	P	N	P	J	JH	DH
VB1A	TGTATGAGATA				TGTA <u>ACTAC</u>				TGGTACTTCGAT	1	DSP
VB2B	TGTGCAAG		GGG		CTACTATGATTAC		GCGAACTC		TGAC	2	DSP
VB5B	TGTATGAGATA				TAGC <u>ANCTAC</u>				TGGTACTTCGAT	1	DFL
VB5C	TGTATGAGATA				TAGTA <u>ACTAC</u>				TGGTACTTCGAT	1	DSP
VB3C	TGTATGAGATA				TGGTA <u>ACTAC</u>				T	3	DSP
VB7A	TGTATGAGATA				TAGTA <u>ACTAC</u>				*T	3	DSP
VB9B	TGTATGAGATA				TAGCAGCT <u>AC</u>				TGGTACTTCGAT	1	DFL
VB11A	TGTATGAGATA				TGG				T	3	DSP
VB11B	TGTATGAGATA				CAG				T	3	DST
VB13A	TGTATGAGA				CC				TGGTTTGCT	3	DSP
VB13B	TGTATGAGATA				TAGCA				NCT	3	DFL
VB15A	TGTGCAAGA				CTGGG TA <u>ACTA</u> TGTAAC		CACG		GCT	3	DQ+DSP+DSP
VB16A	TGTATGAGATA				C				TATGCTATGGAC	4	**
VB4C	TGTGCAAGAGA				TGG				CTACTATGCTATGGAC	4	DSP
208H	TGTGCAAGA		CATAGCCC		ACGAC		GGGGG		TACTTTGAC	2	DSP
208G	TGTA		AAAGATCGTGCC		TATGG TAGCT <u>AC</u>				GAC	2	DSP
2	TGTATGAGAT		TGGC		CTATGGTA <u>ACTAC</u>		GGC		TACTTTGAC	2	DSP
21A	TGTATGAGATA				CGGTAGTAGCT <u>AC</u>				TGGTACTTCGAT	1	DSP
21F	TGTANGATATA				TAA <u>CNA</u>				CTACTGGTACTTCGAT	1	DSP
21G	TGTATGAGATA				TAGCAGCT <u>AC</u>				TGGTACTTCGAT	1	DFL
21J	TGTATGAGATA				TGGTA <u>ACTAC</u>				TGGTACTTCGAT	1	DSP
53E	TGTATGAGATA				CGGTAGTA		A		CTACTGGTACTTCGAT	1	DSP
53L	TGTATGAGATA				TAGTA <u>ACTAC</u>				TGGTACTTCGAT	1	DSP+DSP

Table 3c, continued

Clone	V	P	N	P	D	P	N	P	J	JH	DH
89G	TGTATGAGATA				CGGTAGTN			ACTG	TGGNACTTTCGAT	1	DFL
101A	TGTATGAGATA				TAGTAACCTAC				TGGTACTTTCGAT	1	DSP
101I	TGTAT		A		CGGTAGTACCTAC				TGGTACTTTCGAT	1	DFL
209	TGTATG				GGATACGGTAGTA				GCT	3	DQ
211A	TGTATGAGAT		TCTT		TACTACGGTAGTAGCTAC			GCT	GAC	2	DFL
211B	TGTATGAGATA	TA			ATGGT				TACTACTTTTGAC	2	DSP
212G	TGTATGAGA		CTCGT		TGAT			G	ACTATGCTATGGAC	4	DSP
214B	TGTATGAGATA	TA			ATGGT				TACTACTTTTGAC	2	DSP

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* Missing J region nucleotides extends beyond the GCT (Ala) sequence.

** This D region could belong to any family.

Table 3d. V_H1.1 V-D-J junctional sequences from rearranged transcripts of FACS sorted B-1a cells from four RAG recipients of C57Bl/6 adult bone marrow. The variable segment is given beginning with TGT (Cys). P elements are shown for both the V-D junctions and the D-J junctions. N region insertions are also reported for both junctions. All of the D region is shown and the J region is reported to GAT for J_H1, GAC for J_H2 and J_H4, and GCT for J_H3 (ASP for J_H1, 2, and 4 and Ala for J_H3). J_H and D_H families are noted. Two separate sorting experiments are separated with a solid horizontal line. The proportion of junctions with N region insertions is indicated at the bottom of both N region columns. Nucleotides that can be placed in either the D or J region are placed in the D region and are underlined.

Clone	V	P	N	P	D	P	N	P	J	JH	DH
VR5A	TGTATGAGATA				<u>TAGCAGCTAC</u>				TGTACTTCGAT	1	DFL
VR3A	TGTATGAGATA				TAGTA				*ACT	3	DFL/DSP
VR4C	TGTATGAGA		CTCGTT		GAT		GA		CTATGCTATGGAC	4	DSP
VR9A	TGTATGAGATA				<u>TAGCAGCTAC</u>				TGCTCACTGTC	1	DFL
VR9C	TGTATGAGATA				TAG		CA		GCT	3	DFL
VR10A	TGTGCAAG		GAGGGC		TAAC		CCC		TTTGAC	2	DSP
VR10B	TGTGCAAGA		CAAGAA		CTAC		AATGGCCG		TACTTTGAC	2	**
VR13A	TGTATGAGATA				<u>TGCTAACTAC</u>				TGTACTTCGAT	1	DSP
VR14A	TGTATGAGATA				<u>CGGTAGTAGCTAC</u>				TGTACTTCGAT	1	DFL
VR14B	TGTATGAGAT		TGGCC		TATGGTAACTAC		GGC		TACTTTGAC	2	DSP
VR10C	TGTGCAAG		GGG		CTACTATGATTAC		NCGAAC		TTTGAC	2	DSP
VR11B	TGTATGAGATA				CGGTAGTA				GCT	3	DFL
VR15B	TGTATGAGATA				TAGCA				GCT	3	DFL
VR12B	TGTATGAGATA				<u>CGGTAGTAGCTAC</u>				TGTACTTCGAT	1	DFL
VR12C	TGTATGAGATA				<u>TGCTAACTAC</u>				TGTACTTCGAT	1	DSP
<hr/>											
236A	TGTGCAAGA		CGC		TGG				GAC	2	SP2
219E	TGTGTAAGA		CAAGACGACTATA		GGTA		CGGGGGGGGG		CTATGCTATGGAC	4	SP2
29E	TGTATGANATA				<u>TAGTAACCTAC</u>				TGTACTTCGAT	1	DSP
29G	TGTATGAGATA				<u>TGNNAACTAC</u>				TGTACTTCGAT	1	DSP
29M	TGTATGAGATA				<u>TAGCAGCTAC</u>				TGTACTTCGAT	1	DFL

Table 3d, continued									
Clone	V	P	N	P	D	P	N	P	DH
173C	TGTATGAGATA				CGGTAGTAGCTAC			TGGTACTTCGAT	DFL
217A	TGTATGAGATA	TA			ATGGTTACTAC			TTTGAC	DSP
218B	TGTATGAGATA				TGGTAA			CT	DSP

	7/23	7/23
* Missing J region nucleotides extends beyond the GCT (Ala) sequence.		
** This D region could belong to any family except DQ.		

Discussion

The goal of this study is to demonstrate that precursors in adult bone marrow have the potential to give rise to B-1a cells, and in doing so lend support to the hypothesis that the three B cell subsets in mice, B-1a, B-1b, and B-2 are progeny of a single precursor. Presently, the predominant belief is that these three B cell populations arise from distinct progenitors that have the capacity to give rise to only one of the three types of B cells. The strongest evidence for this theory comes from transplantation studies in irradiated hosts. Fetal bone marrow and spleen cells have given rise to all three subsets of B cells, but adult bone marrow has lacked the ability to give rise to B-1a cells (Hayakawa et al., 1985; Hardy and Hayakawa, 1991; Hardy and Hayakawa, 1992a; Kantor et al., 1992; Kantor et al., 1995). Similarly, fetal omentum has failed to give rise to B-2 cells (Solvason et al., 1991). These data and other data that reveal the uniqueness of B-1a and B-1b cells such as phenotype, anatomical location, function, and regulation have been used to develop the separate lineage hypothesis that was first described by the Herzenbergs and coworkers (reviewed in Herzenberg et al., 1986; Herzenberg and Kantor 1993; Kantor and Herzenberg, 1993; Hardy et al., 1994).

There are three potential variables that can affect the ability of B-1a cells to engraft from adult bone marrow. These are the number of progenitors that are transferred, the length of time allowed for engraftment, and whether or not the recipients are irradiated. Hayakawa et al. (1985) transferred 10^7 adult bone marrow cells into irradiated recipients

and analyzed engrafted cells after only four weeks, and found almost no B-1a cells. Kantor et al. (1992; 1995) transferred 2×10^6 bone marrow cells into irradiated recipients and found a six-fold reduction in the proportion of B-1a cells compared to the donor mice after eight weeks of engraftment. Thomas-Vaslin et al. (1992) transferred 10-fold more bone marrow cells into irradiated recipients than Kantor et al. (1992; 1995) did and they allowed three months for engraftment. They observed B-1a cell engraftment to be approximately half of the level of B-1a cells found in the donor strain. Finally, Huang et al. (1996) transferred 5×10^6 adult bone marrow cells into non-irradiated newborn SCID mice and allowed 10 months for engraftment. They found that B-1a cells comprised 21% of T cell depleted spleen cells. This data is suspect however because the proper controls were not shown and 21% of B-1a cells in a spleen is about 10-fold higher than what has been reported in normal mice (reviewed in Hayakawa and Hardy, 1988; Kantor and Herzenberg, 1993). In contrast to the data shown in Figure 6 of this report, Kantor et al., (1997), without showing data, has stated that in irradiated recipients the level of B-1a cell engraftment does not change after eight weeks when 2×10^6 cells are transferred. The effect of both of these variables, the amount of bone marrow transferred and the time allowed for engraftment, using non-irradiated hosts, has not until now been systematically studied within a single report.

If the proportion of B-1a cells that engraft falls relative to the proportion of the other B cell subsets as a result of transferring a limited number of stem cells it can be argued that progenitors for these three populations of B cells are distinct and are present in

different proportions in the adult bone marrow. If the relative proportions remain constant, however, it is more likely that they are derived from a single precursor that has the potential to give rise to all three subsets. Allowing a longer time for engraftment to occur, is simply giving the cells enough time to populate the mouse. This is more likely an environmental factor that influences the rate that the three subsets develop rather than a factor influenced by the origins of the cells.

In this paper T cell depleted adult bone marrow was transferred into non-irradiated adult RAG and SCID mice. The number of transferred cells was varied as was the length of time allowed for engraftment. There was a small effect associated with the number of cells that were transferred, although the statistical significance of this effect is unknown because there was only one data point in the group receiving the largest number of bone marrow, and because very small numbers of cells B cells were analyzed in the group receiving the smallest number of cells. It is possible that the proportion of B-2 cells increases as fewer bone marrow cells are transferred because there is a dramatic increase in the ratio of T cells to B cells in the peritoneal cavity as fewer cells are transferred. This could skew B cell development towards B-2 cell production, though this remains to be tested. The most pronounced effect of decreasing the amount of bone marrow transferred is the steep decline in the numbers of all three subsets of B cells that engraft. Increasing the amount of time allowed for engraftment has a notable effect on the proportion of B-1a cells that develop. After 12 weeks of engraftment the proportion of B-1a cells in the peritoneal cavity mirrors that of the donor mice, but at eight weeks engraftment is only

2/3 the level of the donor. This difference is statistically significant. Of these two variables, time more than the number of cells transferred has an impact on the proportion of B-1a cells that engraft.

To compare engraftment in non-irradiated RAG and SCID immunodeficient mouse models, syngeneic and allogeneic adult bone marrow transfers were performed. Allogeneic transfers allowed for the tracking of the source of B cells in engrafted mice by analyzing their MHC class II markers. In the RAG mice this was not an issue because no endogenous mature B cells are present. In the SCID mice, however, B cells are present in very low numbers (about 3% of that found in BALB/c mice). The vast majority of the B cells in the engrafted SCID mice are of donor origin. This indicates that the majority of B cells in the syngeneic transfers are also of donor origin.

In the syngeneic transfers of both models, B-1a cells engrafted to similar proportions as the levels found in their bone marrow donors. The RAG hosts engrafted nearly the same proportions of all three B cell subsets as found in the donor mice, while the SCID hosts had a higher proportion of B-1b and a lower proportion of B-2 cells than the donor mice. These results are very different from what has been seen with transfers into irradiated hosts where relative percentages of B-1 cells fall and B-2 cells rise. These differences are likely due to a combination of allowing more time to engraft and not irradiating the hosts.

To demonstrate that B-1a cells, when derived from adult hematopoietic stem cells, contain heavy chain junctional sequences that resemble any B cell which differentiates in an adult microenvironment, junctional sequences of J558 and V_H11 rearrangements from FACS sorted B-1a cells derived from donor mice and engrafted RAG mice were analyzed. Within the large J558 family there is a wide diversity of junctional sequences that are utilized in each of the three B cell subsets (Kantor et al., 1997). The data shown in this report reflect that diversity. N region insertions were frequent in both the donor mice and the engrafted mice. The frequency of N region insertions in the J558 transcripts reported in this study for the donor mice is similar, though somewhat higher at both junctions, than the frequency reported elsewhere for B-1a cells (Kantor et al., 1997). The frequency of N region insertions for B-1a cells in the engrafted mice in this study, however, is very similar to that reported for B-2 cells (Kantor et al., 1997). There was significantly more N region insertions at the V-D junction of B-1a cells of the engrafted mice than in the donor mice. This lends support to the notion that the general lack of N region insertions in B-1a cells is a product of when the cells developed rather than being an innate feature of this lineage. B-1a cells can develop from precursors in the adult bone marrow and their junctional sequences are very similar to other B cells derived from adult bone marrow.

Unlike J558 rearrangements, the V_H11 rearrangements reported in this study showed limited diversity with respect to D segment usage and limited N region insertions, particularly when joined with J_H1, for both the donor and engrafted mice. This lack of

junctional diversity has also been reported elsewhere for V_H11-J_H1 rearrangements (Carmack et al., 1990; Hardy et al., 1994; Kantor, 1996). Repeated joinings of V_H11-DSP-J_H1 and V_H11-DFL-J_H1 in multiple mice have been reported in B-1a cell fractions specific for phosphatidylcholine (Seidl et al., 1997a; Seidl et al., 1997b). In fact the rearrangements have been shown to be derived multiple times independently (from different precursors) within the same mouse. This restricted repertoire of V_H11 rearrangements is most likely due to selection for specificity of autoantigen (phosphatidylcholine) that is highly enriched in peritoneal B-1a cell fractions (Hayakawa et al., 1984; Hardy et al., 1989; Carmack et al., 1990). The fact that there was not a significant increase in N region insertions in V_H11 rearrangements as was demonstrated for J558 rearrangements is likely due to autoantigen selection for V_H11 junctions without insertions. V_H11 expressing B cells could be selected into the population of B-1a cells because of their autoantigen or bacterial cell wall antigen specificity that results in excessive IgM crosslinking due to polyreactive or polymeric interactions with this type of antigen. This type of selection into a population is consistent with the theory that the B-1a phenotype is a product of a differentiative pathway available to any B cell precursor, and that the subpopulations of B cells are derived from a common pool of precursors.

It is also possible that the rearrangements containing V_H11-J_H1 in the engrafted mice are the result of a carryover of rearranged mature circulating B-1a cells in the donor mice during the bone marrow transfer. A recent report by Wasserman et al., (1998) demonstrates that the immunoglobulin heavy chain association with a surrogate light

chain that forms the pre-B cell receptor inhibits B cell proliferation in fetal liver. The V_H11 gene allows continued pre-B cell proliferation in fetal liver. It does not form an efficient association with a surrogate light chain, something that is critical for B cell differentiation in adult pre-B cells. This suggests that the V_H11 rearrangements in the engrafted mice may not be of adult origin but rather, are carryovers of B-1a cells that rearranged early in development in the donor mice. This hypothesis can be confirmed by transferring Ig⁻ FACS sorted bone marrow cells and analyzing for the presence of adult derived V_H11 rearrangements in the B-1a cells of engrafted mice.

The body of evidence that supports the hypothesis that B cells are derived from separate lineages is very compelling. Transfers into irradiated hosts have shown that precursors in fetal tissue can reconstitute B-1a cells but precursors in adult bone marrow cannot. This report, however, presents data that support the hypothesis that the three B subsets are not born of unique progenitor cells. These data show definitively that precursors for B-1a cells are present in adult bone marrow, and can reconstitute the peritoneal B-1a cell population to the same extent that B-1b and B-2 cells can be reconstituted. In a normal adult mouse B-1a cells are suppressed from development from progenitor cells because of feedback inhibition; however, when adult cells are transferred into a mouse without mature lymphocytes stem cells are released from negative feedback and B-1a cells develop normally.

The difference in engraftment found in this study compared to studies that have not found B-1a cell engraftment from adult bone marrow transfers is partly due to the length of time allowed for engraftment. This is probably not the only reason, however, because studies using irradiated hosts find almost no B-1a cells in the peritoneal cavity. Irradiation of host mice causes a series of events that may effect the ability of B-1a cells to develop, including release of inflammatory cytokines and gut damage (Xun et al., 1994; Hill et al., 1997). However, if the radiation is a major factor for preventing B-1a reconstitution, it remains unclear as to why B-1a cells can be generated from fetal tissues but not from adult bone marrow in irradiated recipients. Future studies will test the effect of radiation of host mice on B cell engraftment.

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IMAGE EVALUATION TEST TARGET (QA-3)

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