Development of B-1 Cells: Segregation of Phosphatidyl Choline-specific B Cells to the B-1 Population Occurs After Immunoglobulin Gene Expression

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Summary

Adult mice have two easily recognizable subsets of B cells: the predominant resting population of the spleen, called B-2, and those called B-1, which predominate in coelomic cavities and can express CD5. Some antibody specificities appear to be unique to the B-1 population. Cells expressing antibody specific for phosphatidyl choline (PtC) are the most frequent, comprising 2-10% of peritoneal B cells in normal mice. To understand the basis for the segregation of the anti-PtC specificity to this population, we have produced transgenic (Tg) mice expressing the rearranged V,12 and V,4 genes of a PtC-specific B-1 cell lymphoma. We find that V,12-Tg and V,12/V,4 double-Tg mice develop very high numbers of PtC-specific peritoneal and splenic B cells. These cells have the characteristics of B-1 cells; most are CD5+, and are all IgM+, B220−, and CD23−.

In the peritoneum these cells are also CD11b+. In addition, adult mice have many splenic B cells (up to one third of Tg− cells) that express the V,12 Tg but do not bind PtC, presumably because they express a V, gene other than V,4. These cells appear to be B-2 cells; they are CD23+, CD11b−, IgM+, B220+, and CD5+. Thus, mice given either the V,12 Tg alone or together with the V,4 Tg develop a large population of PtC-specific B cells which belong exclusively to the B-1 population. Since B-2 cells can express the V,12 and V,4 gene separately, we interpret these data to indicate that the events leading to the segregation of PtC-specific B cells to the B-1 population in normal mice are initiated after Ig gene rearrangement and expression. These data are discussed with regard to hypotheses of the origin of B-1 cells. We also find that V,12-Tg mice have a marked decrease in the generation of Tg-expressing B cells in adult bone marrow, but not newborn liver. We speculate that this may be related to positive selection of V,12-expressing B cells during differentiation.

Adult mice contain, at least, two stable, distinct populations of B lymphocytes (for reviews see references 1-3); these have been designated B-1 (formerly CD5+, Ly-1 B) and B-2 (formerly conventional) (4). B-1 cells are the predominant population in the peritoneal cavity, and B-2 cells are the predominant population in spleen and lymph nodes. One of the notable differences between B-1 and B-2 cells are their expressed Vn repertoires and Ig specificities (5). Antigens recognized predominantly or exclusively by the mlgM of B-1 cells include phosphatidyl choline (PtC), 1 DNA, Ig (rheumatoid factor), and common bacterial carbohydrate antigens (e.g., phosphorylcholine [6-10]). Probably the most frequent Ig specificity expressed by B cells is that for the ubiquitous self-membrane phospholipid, PtC (6, 11). In normal mice, this specificity is expressed by 2-10% of peritoneal B cells and ~0.3% of normal splenic B cells (6) and appears to be expressed exclusively by B-1 cells (6, 8, 12, 13). IgM specific for PtC is encoded predominantly by either of two combinations of Vn and Vλ: Vn11 and a V,9 gene, or Vn12 and a V,4 gene (13-18). Each Vn gene pair is represented about equally in the adult B-1 population of B10.H-2H-4b (24b) mice (14). These findings and the restriction of the Vn CDR3 sequences of anti-PtC B-1 cells argue that these cells in the adult are antigen-selected (17, 19).

The origins of B-1 and B-2 cells are controversial. Two views have been offered. The more long-standing view is that B-1 and B-2 cells arise from distinct progenitors committed to their respective lineages before expression of Ig (20, 21;
for reviews see references 22 and 23). A more recent hypothesis (24, 25), stimulated by the findings of Ying-zi et al. (26), proposes that the different types of B cells are the result of distinct antigen-induced differentiative pathways. According to this hypothesis, commitment to one or the other pathway occurs after mIg expression. The signal determining which pathway is followed is the result of the first encounter with antigen; interaction with T1-2 antigens and no T1 interaction leads to the B-1 phenotype. The majority of IgM+ , IgD− B cells, which have not encountered antigen, are uncommitted, and these we term B-0 cells (25). B-0 cells are equivalent to resting B-2 cells as defined by the former hypothesis (4).

To understand the development of B-1 cells and, in particular, the basis for the segregation of anti-PtC B cells to the B-1 subset, we have produced transgenic (Tg) mice expressing a V12 heavy chain or V4 light chain derived from the PtC-specific lymphoma CH27. We have examined the ability of these mice to produce PtC-specific B-1 and B-2 (B-0) cells. We find that there is an exclusive segregation of the PtC specificity to the B-1 phenotype. In addition, we find that adult V12-Tg mice have a severely limited ability to generate B cells in the bone marrow. These results are discussed with regard to the origin of B-1 and B-2 (B-0) cells.

**Materials and Methods**

**V12.CH27-Cμ Construct.** The V12.CH27-Cμ construct contains a V12-DSP2.9-Jμ1 segment joined to the 5′ end of a fragment containing the μ constant region gene (Cμ; IgM*) (see Fig. 1). The Cμ−μM fragment was isolated as a 12.8-kb segment from an EcoRI digest of HYHEL10-μ-δ (27), a kind gift of Dr. Christopher Goodnow (Howard Hughes Medical Institute and Department of Microbiology and Immunology, Stanford University, Stanford, CA). The V12-DSP2-Jμ1 segment was isolated as a 3.3-kb EcoRI fragment from CH27.LX, a B-1 (CD5+) cell lymphoma of B10.H-2H-4p/Wts origin (24, 24; IgM*) (28). Genomic DNA from CH27.LX was digested to completion with EcoRI and size fractionated on a 0.6% agarose gel. The fraction containing the 8.5-kb−productive V-J rearrangement was identified with the Jμ-Cμ probe pEC (33) and cloned into the BamHI site of EMBL3 (Promega Corp., Madison, WI). Positive clones were identified by hybridization to a Jμ-specific probe (a 2.9-kb HindIII fragment isolated from pECμ) and plaque purified. The V4-Jμ4-Cμ fragment was then cloned into the BamHI site in a modified pSV2-Neo vector (this vector has the polylinker described above except that the vector SfiI site was retained). The identity of the V4-Jμ4 segment in the clone used for transfection and microinjection was confirmed by dyeodeoxy sequence analysis (28).

The ability of the V4-Jμ4-Cμ fragment to encode a functional κ light chain was tested by transfection into P3-×63-Ag8.653, a nonsecreting myeloma line (34). The plasmid was linearized with NotI and electroporated into cells. Stable transfectants were selected in the presence of 0.4 μg/ml G418. Transfectants were selected for expression of secreted κ light chain (analyzed by ELISA). The V4-Jμ4-Cμ fragment was shown to pair with the V12.CH27-Cμ-encoded heavy chain by cotransfecting myeloma cells with both constructs. Stable transfectants secreting IgM,κ Ig were identified by ELISA.

**Microinjection and Founder Tg Mice.** The V12.CH27-Cμ and V4-Jμ4-Cμ fragments were isolated from vector DNA by digestion with Sall and BamHI, respectively, and purified by agarose gel electrophoresis. The DNA was microinjected separately into C57BL/6 × SJL)F1 (IgM*) fertilized eggs by the National Transgenic Facility of DNX, Inc. (Princeton, NJ). Mouse lines carrying either the V12.CH27-Cμ or the V4-Jμ4-Cμ Tg were identified by Southern blot analysis of tail DNA by use of the pJll or pECμ probes, or by PCR with, for V12, framework 1 (CTTCCTTAC-CCTGCTCTTACTTACTGTTCG; bp 54-82 of V12) and Jμ-specific (CTATCCTTACGAAAAGCTTCTGGCAGC; 95 bp 3′ of Jμ1) amplifiers and, for V4, V4 (5′ AGCTCAAGTATAAGTTC-CATTACTTGCAT 3′) and Jμ4 (5′ GTTACCCAAAACAGAACCC-AAAAGTTCACA 3′) amplifiers.

**Mice.** Lines of Tg mice have been maintained by backcrossing male Tg+ mice with female 244mice. Tg+ offspring were identified by PCR analysis or serology. Mice are bred and maintained in our own pathogen-free mouse colony.

**Hybridomas.** Hybridomas from V12-Tg mice were prepared as described previously (14). Briefly, spleen cells were cultured overnight in RPMI 1640 medium containing 50 μg/ml LPS and 20 μg/ml dextran sulfate. Cells were harvested and fused by use of polyethylene glycol with the Ag8.653 myeloma. Supernatant from wells containing growing cells was screened for IgM by ELISA.
Positive clones were expanded and then subcloned to ensure monoclonality.

**Genomic Blot Hybridizations.** To prepare hybridoma DNA, ~5 x 10⁷ cells were washed in PBS and resuspended in 0.5 ml digestion buffer consisting of 100 mM NaCl, 10 mM Tris-Cl, pH 8, 25 mM EDTA, pH 8, 0.5% SDS, and 0.1 mg/ml proteinase K. After an overnight incubation at 50°C, the DNA was extracted once with phenol, once with phenol/chloroform (1:1), and once with chloroform. The DNA was then precipitated by adding a half volume of 7.5 M ammonium acetate and 2 vol ethanol. The precipitated DNA was pooled out of the ethanol with a glass rod, rinsed with 70% ethanol, and resuspended in 100 µl Tris-EDTA (TE).

Analysis was by the method of Southern (35). DNA was digested with BamHI and electrophoresed through 0.7% agarose gels. The DNA was transferred to nitrocellulose and hybridized with the Jκ-specific probe pECκ (33). The final wash conditions were 30 mM NaCl/3 mM sodium citrate/0.1% SDS at 68°C.

**Antibodies and Liposomes.** mAbs against the following molecules were used in these studies: IgM (AF6-78), IgM (DS-1), B220 (RA3-6B2), CD5 (53-7.3), and CD8 (53-6.7) were obtained from Pharmingen (San Diego, CA), either fluoresceinated, biotinylated, or conjugated to PE. Anti-CD23 (B3B4) (biotinylated) was the kind gift of Dr. Tom Waldschmidt (University of Iowa, Iowa City, IA) and anti-CD11b (Mac-1; M1/70) (biotinylated) was generously provided by Dr. Elizabeth Reap (University of North Carolina, Chapel Hill, NC). In two-color analyses, the biotinylated reagent was developed with streptavidin-PE (Jackson Immunoresearch Laboratories, West Grove, PA). In three-color experiments, directly fluoresceinated and PE-conjugated antibodies were combined with a biotinylated antibody revealed with streptavidin-RED670 (GIBCO BRL, Gaithersburg, MD). A monoclonal anti-Vκ12 idiotype (5CS, rat IgG) was produced in one of our labs (L. W. Arnold and G. Haughton). This antibody recognizes the product of the Vκ12 gene independently of light- and heavy-chain CDR3 sequences. The use of fluorochrome-encapsulating liposomes to detect membrane-bound PtC-specific Ig has been described previously (6). In these experiments, the liposomes encapsulated either carboxylfluorescein or sulforhodamine B.

**Immunofluorescence and Flow Cytometry.** To detect membrane molecules, single-cell suspensions were prepared in HBSS (without Ca²⁺, Mg²⁺, and phenol red) containing 0.1% sodium azide and 0.5% FCS (buffer). Cells were incubated with previously determined saturating amounts of antibody in 25–50 µl buffer for 20 min, after which they were washed three times with buffer and incubated with second-step reagents. After washing as before, the cells were analyzed with a FACScan® (Becton Dickinson, Mountain View, CA) with acquisition computer and software from Cytometry (Fort Collins, CO), unless otherwise noted. All data represent cells falling within the lymphocyte gate determined by forward and 90° light scatter; 10,000–50,000 cells were analyzed.

**ELISA.** Detection of Vκ12 Id, IgM κ allotype, κ light chain, and total IgM were performed by ELISA as previously described (6). Assays were quantified with mAb standards of known IgM concentration. Trapping reagents for each analysis were anti-Vκ12 Id, anti-IgMκ (DS-1; Pharmingen), goat anti–mouse κ, and goat anti–mouse IgM (Southern Biotechnology Associates, Birmingham, AL). Assays were developed with alkaline phosphatase–conjugated goat anti–mouse IgM (Southern Biotechnology Associates).

**Results**

**Tg Mice**

The expressed Vκ and Vκ rearrangements of the PtC-specific B cell lymphoma CH27 (19) were used to generate Tg mice to examine the development of Vκ12-expressing and PtC-specific B cells. These genes were cloned into Cκ (IgH-κ) or Cλ expression vectors (Fig. 1), and separate heavy- and light-chain Tg mice were produced. Four Vκ12 Cκ founder lines were established, and two (7-2 and 6-1) were selected for extensive study. Line 7-2 contains 1–2 copies of the Tg, and 6-1 contains 15–20 copies. Mice have been backcrossed to 24b (IghH) and are currently in their sixth backcross generation. Two Vκ4 founder lines have been established; one (1-2) having 5–10 copies has been characterized and used in these experiments. Mice transgenic for both Vκ12 and Vκ4 (double Tg) were made by mating 6-1 Vκ12-Tg females with 1-2 Vκ4-Tg males.

**Characterization of Tg Expression and B Cell Subpopulations in Vκ12-Tg Mice**

**Allelic Exclusion.** We have analyzed adult peripheral lymphoid tissues from lines 7-2 and 6-1 for cell surface expression of κ chains encoded by the Tg (IgMκ) and an endogenous allele (IgMκ). As shown in Figs. 2 and 3, 6-1 mice exclude nearly all expression of endogenous Cκ. Tg Cκ is expressed by >95% of splenic and peritoneal B cells, and ≤5% of cells express an endogenous Cκ (70% of these appear not to express the Tg). In contrast, 7-2 mice appear to express endogenous Cκ genes much more frequently. The majority (70–90%) of 7-2 peritoneal cells (PerC) express only the Tg, and 10–30% (variable between individual mice) express both the Tg and an endogenous rearrangement. On the other hand, the majority of 7-2 splenic B cells express an endogenous rearrangement, and about half of these also express low levels of Tg Cκ. Only ~10% of splenic B cells appear to express only the Tg.

Allelic exclusion in 1-2 Vκ-Tg mice was assessed by

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Figure 1. Vκ12 and Vκ4 constructs used to produce Tg mice. The rearranged genes were cloned from genomic DNA of CH27, a B-1 cell lymphoma expressing IgM specific for PtC. Restriction enzymes are as follows: S, SalI; N, NotI; E, EcoRI; B, BamHI; K, KpnI; Sf, SgrI; X, XbaI; H, HindIII.
Southern blot analysis of endogenous Jκ rearrangements in splenic LPS hybridomas. Hybridomas with hybridizing fragments in addition to the endogenous germine and Tg fragments were scored as having failed to undergo Tg-mediated allelic exclusion. 12 of 23 (~50%) hybridomas showed no evidence of endogenous Vκ gene rearrangements (data not shown). Allelic exclusion in (6-1 x 1-2)FI mice that express both Tgs appears to be predicted by the phenotype of the parents; they express no endogenous heavy chain genes, and most splenic B cells appear to express the Tg light chain, as judged by specificity for PtC (see below).

**Analysis of PerC.** The majority of B-1 cells in normal young adult mice are found in the peritoneal cavity. Mice of all three Vκ12-Tg lines (7-2, 6-1, and double) have high numbers of PerC B cells expressing the Tg (see Fig. 2). In line 6-1 and the double-Tg mice, nearly all PerC B cells express the Tg. In line 7-2 mice, 70-90% of PerC B cells express the Tg without endogenous heavy chain gene expression. Like PerC B-1 cells of normal mice, Tg+ B cells in these mice have high levels of IgM and low levels of B220 and CD23 (36) (compare with Tg- mouse PerC in Fig. 2). Moreover, most of these cells are CD5- and CD11b-. Thus, PerC Tg+ B cells have the cell surface characteristics of B-1 cells. About half of the PerC B cells in the double-Tg mouse shown in Fig. 2 are CD5+, but are CD23- and CD11b+ (i.e., B-1b cells), although the number of these cells varies among individual mice. Thus, a significant proportion of PerC cells in these mice appear to be B-1b (37). Nearly all the Tg+ B cells from mice of all three Vκ12-Tg lines are PtC-specific as shown by the binding of fluorescent liposomes (Fig. 2).
Analysis of Spleen Cells. In line 6-1 and double-Tg mice, essentially all B cells express the Tg without endogenous C<sub>m</sub>. However, in mice of both these lines, two clearly distinguishable B cell populations are present. The predominant one is mlgM<sup>hi</sup>, which expresses CD5<sup>+</sup>, binds liposomes, is B220<sup>-</sup>, CD23<sup>-</sup>, and CD11b<sup>-</sup> (Figs. 3 and 4). The other population (larger in 6-1 mice than in double-Tg mice) does not bind liposomes, is mlgM<sup>lo</sup>, CDS<sup>-</sup>, CD23<sup>+</sup>, and CD11b<sup>-</sup>. Three-color flow cytometric analysis of 6-1 splenic cells (Fig. 4) directly demonstrates that the majority of Tg<sup>+</sup> liposome-binding B cells are CD5<sup>+</sup>, B220<sup>-</sup>, CD23<sup>-</sup>, and that the majority of Tg<sup>+</sup> cells that do not bind liposomes are CD5<sup>-</sup>, B220<sup>hi</sup>, and CD23<sup>+</sup>. Data shown in Fig. 4 also show that the liposome-binding cells are larger and more granular than those B cells that do not bind liposomes. Therefore, the characteristics of the predominant liposome-binding population are those of B-1 cells, whereas those of the liposome-negative population are those of B-2 (B-0) cells. Thus, all PrtC-specific cells appear to have the B-1 phenotype.

The majority (80-90%) of splenic B cells in 7-2 mice have mostly endogenous heavy chains (Fig. 3). The number of CDS<sup>+</sup> B cells is slightly higher than in normal mice (~1.5-fold), and the number of cells specific for PrtC (binding liposomes) are increased ~15-40-fold above normal (Fig. 3). All of the cells specific for PrtC express the Tg. However, since the percentage of Tg<sup>+</sup> cells that bind liposomes and the percentage of Tg<sup>+</sup> cells that are CDS<sup>+</sup> and B220<sup>lo</sup> are similar (11 vs. 15 and 21%, respectively), it is likely that the PrtC-specific cells have the cell surface characteristics of B-1 cells (Fig. 3). Three-color analysis confirms that Tg<sup>+</sup> liposome-binding B cells are CD5<sup>+</sup>, CD23<sup>-</sup>, and B220<sup>lo</sup>, and Tg<sup>+</sup> liposome-negative cells are CD5<sup>-</sup>, CD23<sup>+</sup>, and B220<sup>hi</sup> (data not shown).

Expression of V<sub>a</sub>12-Tg Early in Ontogeny. Liver and spleen cells of newborn V<sub>a</sub>12-Tg mice were examined to determine the expression of the Tg and B cell development early in ontogeny (Figs. 5 and 6). Newborn livers of both 7-2 and 6-1 mice contain normal numbers of IgM<sup>+</sup> B cells. In liver and spleen of 7-2 mice, B cells expressing the Tg predominate, the reciprocal of that seen in the adult spleen (Fig. 3) and bone marrow (see below). In newborn 6-1 mice, nearly all Ig<sup>+</sup> B cells express the Tg, as seen in the adult. However, the IgM<sup>+</sup> B cells present in newborn Tg mice (both 7-2 and 6-1) are not predominantly CD5<sup>+</sup> (<5%) (Fig. 6; data not shown for 7-2 mice). In newborn 6-1 mice, 3-5% ofIgM<sup>+</sup> cells bind liposome (Fig. 6). In 6-1 mice, liposome-binding IgM<sup>+</sup> cells increase rapidly, so that by day 3, they represent 15-20% of IgM<sup>+</sup> cells and, by day 6 >80% of IgM<sup>+</sup> cells.
Figure 6. Analysis of spleen cells from newborn, 1-d-old, and 6-d-old V_{a12}-Tg mice. Spleen cells from young mice were analyzed for expression of mIgM, mlgM, CD5, and liposome binding. All analyses used three-color immunofluorescence: carboxyfluorescein-liposome versus PE.

Table 1. Analysis of Splenic B Cells in 6-d-old V_{a12}-Tg* Mice

| Mice  | Number of mice tested | Spleen weight (mg) \( \times 10^7 \) | Total recovered cells \( \times 10^6 \) | IgM* cells \( \% \) | Liposome-positive cells \( \% \) | B220* , IgM* cells \( \% \) |
|-------|-----------------------|---------------------------------|-------------------------------|----------------|-----------------|----------------|
|       |                       |                                 |                               |                |                 |                 |
| Tg+   | Experiment 1          | 2                               | 25.65 ± 3.35*                | 5.99 ± 0.53    | 4.60 ± 0.60    | 2.73 ± 0.11    | 4.05 ± 1.05    | 2.43 ± 0.42    | 1.70 ± 0.10    | 1.02 ± 0.15    |
|       | Experiment 2          | 5                               | ND                            | ND             | 4.28 ± 0.45    | -               | 3.42 ± 0.45    | -               | 4.20 ± 0.77    | -               |
| Tg-   | Experiment 1          | 4                               | 29.08 ± 4.03                 | 5.36 ± 0.87    | 3.60 ± 0.36    | 1.93 ± 0.29    | <0.01          | NA             | 1.88 ± 0.25    | 1.01 ± 0.25    |
|       | Experiment 2          | 2                               | ND                            | ND             | 4.95 ± 0.75    | -               | <0.01          | -               | 5.75 ± 0.75    | -               |

* Standard deviation.
Table 2. Frequency of B Cell Subpopulations in Adult Bone Marrow of V_{12}-Tg Mice

| Mice | Total IgM<sup>+</sup> | Total IgM<sup>−</sup> | Total IgM<sup>+</sup> | B220<sup>+</sup>, IgM<sup>+</sup> | B220<sup>+</sup>, IgM<sup>−</sup> | B220<sup>−</sup>, IgM<sup>+</sup> | B220<sup>−</sup>, IgM<sup>−</sup> | B220<sup>+</sup>, IgM<sup>−</sup> | B220<sup>−</sup>, IgM<sup>−</sup> | PtC specific |
|------|----------------------|----------------------|----------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|----------------------|
| 7-2 Tg | 4 | 12.5 | 3.8 | 8.6 | 55.3 | 40.8 | 2.3 | 5.1 | 2.4 | 5.8 | 1.4 |
| | | (11.5-13.3) | (49.5-60) | (32.3-49) | (1.5-3.0) | (4.0-7.5) | (2.0-2.7) | (4.0-7.5) | (0.8-1.6) |
| | | [22.4] | [6.9] | [15.6] | [74] | [4.2] | [9.6] | [4.3] | [10.5] | [2.5] |
| 6-1 Tg | 4 | 6.1 | 5.9 | 0.2 | 30 | 27 | 3.8 | <0.1 | 1.9 | 0.1 | 2.8 |
| | | (5.3-7.2) | (24-38) | (18-37) | (3.1-5.0) | (1.4-2.2) | (1.6-5.0) | (1.6-5.0) |
| | | [20.3] | [19.7] | [0.6] | [90] | [12.7] | [6.3] | [9.3] |
| Tg<sup>−</sup> | 6 | 26.4 | 26.4 | 55.1 | 30.5 | - | 10.6 | - | 17.5 | 0.4 |
| | | (20-34) | (50.0-60.5) | (25-36) | (7.5-13.0) | (9.0-28.5) | (0.2-1.0) | (0.2-1.0) |
| | | [47.9] | [47.9] | [55] | [19.2] | [31.8] | [0.7] |

The bone marrow B cell subpopulation distribution from several mice was determined as described in Figs. 6 and 7 and summarized here. The Tg<sup>−</sup> control mice include Tg<sup>−</sup> mice of the 7-2 line (two mice), 6-1 line (two mice), and 2q<sup>b</sup> Tg<sup>−</sup> (two mice). No differences were observed among these mice. For 7-2 mice, percent of cells in columns denoting IgM<sup>−</sup> cells contain only those that are IgM<sup>−</sup>, IgM<sup>b</sup>-<sup>−</sup>. Cells in the IgM<sup>b</sup> columns are IgM<sup>b</sup>+ or IgM<sup>−</sup>, IgM<sup>b</sup>-<sup>−</sup>. Also, in 7-2 mice, the sum of the B220<sup>+</sup>, IgM<sup>+</sup> columns exceeds the total IgM<sup>+</sup> cells because of the inability to completely resolve IgM<sup>+</sup> cells from IgM<sup>−</sup> cells. Numbers in parentheses are ranges; those in brackets are percentage of B220<sup>+</sup> cells. All PtC-specific B cells are IgM<sup>−</sup>, IgM<sup>b</sup>-<sup>−</sup>.

Figure 8. Analysis of subpopulations of B cells in the bone marrow of V<sub>12</sub>-Tg mice. Bone marrow cells were stained with FITC-anti-IgM<sup>+</sup> or -IgM<sup>−</sup> and biotinylated anti-B220 (6B2) (revealed with PE-streptavidin). A lymphocyte gate was established based on forward and 90° light scatter between Tg<sup>+</sup> mice and Tg<sup>−</sup> littermates. The increase in the frequency of CD5<sup>+</sup> B cells directly parallels the increase in liposome-binding B cells. As shown in Fig. 6 B, >90% of liposome-binding IgM<sup>+</sup> cells are CD5<sup>+</sup>, whereas <10% of IgM<sup>−</sup>, PtC-negative cells express CD5.

Expression of V<sub>12</sub>Tg in Adult Bone Marrow. To examine the early stages of B cell differentiation in adult mice, bone marrow cells from 2-4-mo-old V<sub>12</sub>-Tg mice were analyzed for expression of mIgM allotypes and B cell markers. The data are shown for representative individual mice in Figs. 7 and 8 and summarized in Table 2. 6-1 mice have about two-fold fewer total B220<sup>+</sup> cells and about fourfold fewer Ig<sup>+</sup> bone marrow B cells than Tg<sup>−</sup> controls. This is particularly evident in the B220<sup>+</sup>, IgM<sup>+</sup> population, where the decrease from normal is ninefold, but is also apparent (about three-fold) in the less mature B220<sup>+</sup>, IgM<sup>+</sup> population (Fig. 8 and Table 2).

Line 7-2 mice have normal numbers of total bone marrow B220<sup>+</sup> cells, but there are about twofold fewer IgM<sup>+</sup> B cells than in Tg<sup>−</sup> controls. Surprisingly, in sharp contrast to the as shown. Cells within the gate were analyzed for B cell subpopulations. The distribution of total B220<sup>+</sup> cells (d) was divided into three regions (as diagrammed at the bottom of the figure): a, B220<sup>+</sup>, IgM<sup>−</sup> (pre- and pre-B cells); b, B220<sup>−</sup>, IgM<sup>+</sup> (mature B cells); c, B220<sup>−</sup>, IgM<sup>+</sup> (immature B cells). Percentages denote percent of total cells in lymphocyte gate. Note that in 7-2 mice the number of B220<sup>+</sup> cells expressing IgM<sup>+</sup> includes those expressing IgM<sup>−</sup> only and those expressing both heavy chain allotypes. The same is true of those expressing IgM<sup>b</sup>−<sup>−</sup>.
newborn, where 80% of IgM+ B cells express the Tg, the majority (70%) of bone marrow IgM+ B cells are producing only an endogenous heavy chain.

**Serum Ig in V\textsubscript{H}12-Tg Mice.** In normal mice, a large proportion of serum IgM is derived from cells of the B-1 population (22, 38). We have analyzed sera from Tg mice for expression of V\textsubscript{H}12 idiotypic and PtC specificity. As shown in Fig. 9, V\textsubscript{H}12-Tg mice have high levels of V\textsubscript{H}12 idiotype in their serum. Line 7-2 mice have ~50-fold more V\textsubscript{H}12 Id than Tg- mice; 6-1 and double-Tg mice have 100–200-fold more. All mice have about the same levels of total serum IgM (800–1,100 μg/ml; data not shown). V\textsubscript{H}12-Tg mice also have high levels of serum IgM specific for PtC (anti-BrMRBC), as detected by their ability to bind BrMRBC (Fig. 10). Thus, the PtC-specific B-1 cells in these mice are secreting substantial amounts of IgM.

**Discussion**

To examine the selection of PtC-specific B cells and the basis for their segregation to the B-1 population, we have produced Tg mice expressing either the V\textsubscript{H}12 µ heavy chain or the V\textsubscript{\lambda} light chain used to encode anti-PtC antibodies. We find that V\textsubscript{H}12-Tg mice have large numbers of B-1 cells in spleen and PErC, almost all of which are specific for PtC. In 6-1 and double-Tg mice, these cells are the predominant splenic B cell population. These mice do not have PtC-specific B-2 (B-0) cells, although they do have substantial numbers (up to 30%) of splenic B-2 (B-0) cells that express the V\textsubscript{H}12 Tg. These cells do not bind PtC, presumably because they express a light chain V gene other than V\textsubscript{\lambda}. Thus, PtC-specific B cells segregate to the B-1 population.

There are several possible explanations for the segregation of PtC-specific B cells to the B-1 population in normal mice. One, cells committed to the B-1 lineage can rearrange the V\textsubscript{H}12 gene (and/or the V\textsubscript{\lambda} gene), but B-2 lineage cells cannot. Two, B-1 lineage cells can rearrange and express the V\textsubscript{H}12 gene (and the V\textsubscript{\lambda} gene), but B-2 lineage cells, although they can rearrange V\textsubscript{H}12 (and V\textsubscript{\lambda}), cannot express it. Three, V\textsubscript{H}12 may be rearranged and expressed by cells of either lineage, but PtC-specific B-2 cells are either unselected, deleted, or anergized, whereas B-1 cells are selected and clonally expanded. Four, PtC-specific B cells are induced to become B-1 cells because of the nature of their interaction with antigen. The first three possibilities are consistent with the lineage hypothesis, whereas the final possibility is consistent with the induced differentiation hypothesis. The V\textsubscript{H}12-Tg mice allow us to exclude some of these possibilities.

A mechanism for a process of directed Ig V\textsubscript{H} gene rearrangement that differs between pre-B cells of the two lineages is unlikely since this would have to include multiple genes, and there are V genes known to be used by B cells of both phenotypes (e.g., V\textsubscript{\alpha} and several genes of the J558 V186.2 and V3 subfamilies) (10, 39, 40). However, there is at least one report that B-1 and B-2 lineage cells may use different sets of V\textsubscript{H} genes (41), although selective processes cannot be excluded as an explanation for these data. The data from the V\textsubscript{H}12-Tg mice demonstrate that this prediction is not necessary. We have provided the appropriately rearranged V\textsubscript{H}12 gene, with its normally associated regulatory elements, to B cells of all lineages, yet both V\textsubscript{H}12-expressing B-1 and B-2 (B-0) cells are produced, and PtC-specific B cells appear to belong exclusively to the B-1 subset. Data from the V\textsubscript{\lambda} mice demonstrate that about half of splenic B cells express this Tg, whereas B-1 cells constitute only 5% of splenic B cells. Therefore, this V\textsubscript{\lambda} gene is expressed by B-2 cells, and yet PtC-specific B-2 cells are not detectable.

The second prediction, that only B-1 lineage cells can express the V\textsubscript{H}12 gene, requires that B-1 lineage cells have DNA binding proteins that recognize regulatory elements of a subset of V\textsubscript{\alpha} and/or V\textsubscript{\lambda} genes (e.g., V\textsubscript{H}12 and V\textsubscript{\lambda}). The
data from the $V_{H}12$-Tg mice (most easily demonstrated in the 6-1 line mice) prove that, at least, the $V_{H}12$ and $V_{H}4$ genes are not such genes, since they are expressed by both B-1 and B-2 (B-0) cells. Therefore, such a proposed mechanism for shaping the B-1 repertoire is unnecessary.

Antigen-driven selective mechanisms, therefore, remain the most likely basis for the restricted and exclusive repertoire of B-1 cells. The high frequency of PtC-specific B cells must be due largely to antigen-driven clonal expansion. Analysis of PtC-specific B cells in normal mice clearly establishes that B cells with this specificity are antigen driven (17, 19). $V_{H}$ use by B cells of 6-1 Tg mice will be diverse and, thus, the large number of cells specific for PtC in adult peritoneum and spleen must be due in part to antigen-driven clonal expansion of this subset of $V_{H}12$-expressing B cells. Indeed, selection is so powerful that $V_{H}12$-only-Tg mice are not sub-
population of B cells expressing receptors with diverse specificities. The latter explanation would require the elimination of the vast majority of cells expressing a receptor that is not PtC specific and, thus, the spleens of Tg + mice should contain low numbers of IgM + B cells compared with Tg- mice. As shown in Table 1, they contain the same number. The PtC specificity requires the pairing of a particular $V_{H}$ with the $V_{H}12$ expressed in the Tg mice and, assuming random $V_{H}4$ use, this would mean that only ~1 cell in 200 would express a receptor specific for PtC. Thus, for $V_{H}12$-Tg mice to acquire $2.43 \times 10^{6}$ PtC-specific B cells by day 6 (Table 1) without division of the PtC-specific B cells would require the production of $5 \times 10^{6}$ B cells ($2.43 \times 10^{6} \times 200$), or a production of $10^{8}$ cells per day. This would mean that the mouse would need to produce ~30-40 times more IgM + cells each day than are contained in the spleen of a 6-d-old mouse (normal or Tg +). Thus, it seems inescapable that PtC-specific B cells increase as a result of clonal selection and proliferation. It is also apparent, however, that the number of total splenic B cells that are not PtC specific in 6-d-old Tg + mice is only ~17% of the total number of such cells in the Tg - mice. Thus, it is possible that some PtC-negative cells are eliminated, although these data cannot establish this. Selection beginning on or about birth is consistent with our previous analysis of $V_{H}12$ rearrangements indicating considerable restriction of $V_{H}$ CDR3 sequences in the neonate (42). Selection of B cells in $V_{H}12$-Tg mice is substantially different from that studied in Tg mice expressing antibody specific for other self-antigens where the cells are deleted or

anergized (for reviews see references 43 and 44), suggesting that anti-PtC has an important normal function in mice.

However, these data do not formally establish whether these selective processes operate before or after lineage commitment. Under the concept of the lineage hypothesis, PtC-specific B-1 cells could be antigen selected and undergo clonal expansion, whereas PtC-specific B-2 cells could be unable to undergo clonal expansion or be functionally eliminated by clonal deletion or anergy. In the absence of clonal expansion, the

restrictions in V-(D)-J rearrangement and $V_{H}$ association would limit the frequency of these cells in the repertoire to an undetectable level in normal mice. For the same reasons, PtC-specific B-2 cells that are tolerated by anergy would be undetectable. However, unexpanded or anergized B-2 cells in $V_{H}12/V_{H}4$ double-Tg mice would be at a detectable frequency (~50% of B cells) since both the rearranged $V_{H}$ and $V_{H}$ genes are provided. That we do not detect PtC-specific B-2 cells in these mice indicates that neither of these mechanisms is responsible for the absence of B-2 cells with this specificity. Thus, by the lineage hypothesis, the only selective mechanism to exclude the PtC specificity from the B-2 lineage appears to be clonal deletion, either because of a failure to be positively selected in the bone marrow or to negative selection. These data are also consistent with the antigen-induced differentiation hypothesis (24, 25), which states that newly generated PtC-specific B cells would be selected into the B-1 population. Thus, since PtC is a ubiquitous self-antigen, all B cells with anti-PtC receptors would encounter antigen and be induced to become B-1 cells, and there would be no B-2 (B-0) cells with this specificity. This model is more conservative in that it requires only an additional consequence of mIg receptor signaling. In either case, we conclude that the events leading to the segregation of the PtC specificity to B-1 cells are initiated after Ig gene rearrangement and expression.

The most surprising observation from the analysis of $V_{H}12$-Tg mice is the decreased numbers of Ig + B cells in adult bone marrow, despite the apparent normal development of Ig + cells very early in ontogeny. This decrease is greater than is apparent from just the comparison of the number of Ig + cells (Table 1). Many of the Tg + B cells in 7-2 and 6-1 mice are CD5 + and bind liposomes (37 and 47% of Tg + cells, respectively [Table 1]), while only a small percentage of Tg- B cells in these mice express CD5. These cells were probably clonally selected mature B-1 cells that have migrated to the bone marrow, since newly generated B cells specific for PtC should be infrequent because of restriction in light chain association for this specificity. Excluding the PtC-specific B cells, the reduction in the number of newly generated B cells from normal is 10- and 8.5-fold in 7-2 and 6-1 mice, respectively.

This reduction in B cell development appears to affect Tg-expressing B cells preferentially. 7-2 mice produce B cells expressing the Tg and B cells expressing an endogenous $V_{H}$ gene (Fig. 6). In newborn 7-2 mice, 80-90% of B cells are Tg +. Thus, the Tg in these mice appears to exclude endogenous rearrangement well. Since the mechanism of allelic exclusion should not be different in the adult, the fact that the number of Tg + B cells in adult bone marrow is less than
half the number of endogenous IgM+ B cells suggests that the differentiation of Tg+ B cells in the bone marrow is selectively affected. B cells expressing endogenous IgM do not compensate entirely for the reduction in Tg+ B cell differentiation, probably because allelic exclusion by the Tg results in relatively few cells expressing endogenous IgM.

We speculate that the impairment of B cell differentiation in these Tg mice may be related to positive selection of developing Vn12-expressing B cells. We (25, 42, 45) and others (40, 46-48) have proposed that developing B cells, like developing T cells (for reviews see references 49 and 50), require rescue from an inevitable progression to apoptosis by ligand interaction (positive selection). We have recently demonstrated that positive selection of Vn12-expressing B cells in normal neonatal and adult mice is dependent on the sequence of Vn CDR3 (42 and Ye, J., L. W. Arnold, S. K. McCray, and S. H. Clarke, manuscript in preparation). This selection may occur at the pre-B cell stage and therefore be independent of the light-chain, or it may occur at the immature B cell stage after light chain expression. We have preliminary evidence for the formation from sorted IgM+ B lineage cells of adult bone marrow (Ye, J., and S. H. Clarke, unpublished observation). There may also be a requirement for positive selection after light-chain expression, and this could explain the loss of a significant fraction of B cells in the bone marrow of Vn12-Tg mice if only some Vn12/Vn combinations can be rescued. An alternative, but not necessarily mutually exclusive, explanation is that the very high levels of circulating PtC-specific IgM in Tg mice interfere with positive selection of pre-B or B cells by competing with their cell surface Ig receptors for ligand. This idea would also predict the observation that B cell development in neonatal mice would not be disrupted since there would be probably at most only low levels of circulating anti-PtC antibody because of the scarcity of cells with this specificity.

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