B Cell Antigen Receptor Specificity and Surface Density Together Determine B-1 versus B-2 Cell Development

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Summary

Mice expressing the immunoglobulin (Ig) heavy (H) chain variable (V) region from a rearanged $V_H^{12}$ gene inserted into the IgH locus generate predominantly B-1 cells, whereas expression of two other $V_H$ region transgenes ($V_H^{B1-8}$ and $V_H^{glD42}$) leads to the almost exclusive generation of conventional, or B-2, cells. To determine the developmental potential of B cells bearing two distinct B cell antigen receptors (BCRs), one favoring B-1 and the other favoring B-2 cell development, we crossed $V_H^{12}$ insertion mice with mice bearing either $V_H^{B1-8}$ or $V_H^{glD42}$. B cells coexpressing $V_H^{12}$ and one of the other $V_H$ genes are readily detected in the double IgH insertion mice, and are of the B-2 phenotype. In mice coexpressing $V_H^{12}$, $V_H^{B1-8}$ and a transgenic $\kappa$ chain able to pair with both H chains, double H chain–expressing B-2 cells, and B-1 cells that have lost $V_H^{B1-8}$ are generated, whereas $V_H^{B1-8}$ single producers are undetectable. These data suggest that B-1 but not B-2 cells are selected by antigenic stimuli in whose delivery BCR specificity and surface density are of critical importance.

Key words: B-1 cells • B-2 cells • immunoglobulin heavy chain • allelic inclusion • gene targeting

Two major B cell subsets, designated B-1 and B-2, exist in humans and mice. B-1 and B-2 cells can be distinguished by their cell surface phenotypes and anatomical localization. B-1 cells, found mainly in the pleural and peritoneal cavities, express high levels of surface IgM, low levels of B220 and IgD, and moderate levels of CD5. In addition, they do not express CD23. In contrast, conventional, or B-2, cells are the predominant B cells found in the spleen and lymph nodes; they express high levels of B220, IgD, and CD23 and moderate levels of IgM, and lack surface CD5 expression (for review see references 1, 2).

Different $V_H$ genes are preferentially expressed in B-1 and B-2 cells (3, 4). The distinct $V_H$ repertoire that is found in B-1 cells has led to the hypothesis that the specificity of the B cell antigen receptor (BCR) may in fact determine the differentiation of B cells into this subset (5, 6). It is known that antibodies that react with phosphatidyl choline (PTC) are produced largely by B-1 cells (7) and are mostly encoded by either of two $H$ and $L$ chain combinations, namely $V_H^{12}$ and $V_K^4$ or $V_H^{11}$ and $V_K^9$. Consistent with the view that BCR specificity plays a role in the development of B-1 cells, transgenic mice expressing $V_H^{12}$ alone, or in combination with $V_K^4$, generate mostly B-1 cells in all of the peripheral lymphoid organs, including spleens and lymph nodes (8).

We have generated by gene targeting various strains of Ig H chain insertion mice carrying different H chain variable (V) region genes targeted into their physiological position in the IgH locus. The inserted $V_H^{H_J_{12}}$ elements include the segments designated $V_H^{12}$ (8), $V_H^{B1-8}$ (9), and $V_H^{glD42}$ (10). Similar to conventional $V_H^{12}$ transgenic mice, $V_H^{12}$ insertion mice develop mainly B-1 cells. In contrast, mice whose IgH alleles were engineered to express $V_H^{B1-8}$ or $V_H^{glD42}$ develop mainly B-2 cells. This again suggests that BCR specificity may play a determining role in the differentiation of B-1 cells. If indeed signals transmitted through a BCR of a certain specificity lead to the generation of B-1 cells, then interference with the cell surface expression of that specific BCR may alter the differentiation process of these cells. Here, we generated IgH double ($V_H^{12}$ and $V_H^{B1-8}$ or $V_H^{12}$ and $V_H^{glD42}$) insertion mice to test whether the expression of a second H chain in $V_H^{12}$-expressing B cells may act in a dominant negative manner to perturb the generation of B-1 cells.
Materials and Methods

Mice. The B1-8f (11) and glD42i (10) IgH insertion mice were generated in the laboratory in Cologne and in collaboration with D. Eilat’s group at Hebrew University (Jerusalem, Israel), and have been described previously (references 10 and 11, as indicated behind each mouse strain). The generation of the V_{H}12f mice will be described elsewhere (Lam, K.-P., and K. Rajewsky, manuscript in preparation). The conventional V_{K}4 transgenic mice (8) were obtained from Stephen Clarke (University of North Carolina, Chapel Hill, NC). Mice used were 2–4 mo old and maintained in a conventional animal facility.

Antibodies. The following mAbs used in this study were produced and conjugated to fluorochromes in our laboratory: anti-B220 (RA3-6B2); anti-IgM (R33-24.12); anti-IgD (1.3-5); anti-CD43 (S7); anti-$\mu_{a}$ (RS3.1); anti-$\mu_{b}$ (M B86); anti-V_{H}B1-8 (Ac146); and anti-V_{H}12 (5C5). The anti-CD5 and anti-CD23 mAbs were purchased from PharMingen.

FACS® Analyses and Cell Sorting. Tissues and cell preparations for flow cytometric analyses and cell sorting were prepared as previously described (12). In brief, spleen cells were prepared by dissociation between frosted glass slides. Peritoneal cavity and bone marrow cells were obtained by injecting staining medium (PBS containing 3% FCS and 0.1% NaN$_{3}$) into the peritoneal cavity and femurs and tibia, respectively, using a 1-ml syringe with a 26-gauge needle. All cells were treated with RBC lysing solution (0.15 M NH$_{4}$Cl, 1 mM KHCO$_{3}$, and 0.1 mM Na$_{2}$EDTA) to eliminate erythrocytes. For FACS® analyses, cells were stained with optimal amounts of FITC-, PE-, and biotin-conjugated mAbs for 10 min on ice and washed three times with staining medium. Biotin-conjugated mAbs were revealed with streptavidin-Cyochrome. Flow cytometry analyses were performed on a FACScan™ (Becton Dickinson) and cell sorting was done on a FACStar®PLUS®.

Southern Blot Analysis. Genomic DNA was prepared from mouse livers and sorted splenic B cells (13), digested with BamHI and fractionated on a 1% agarose gel. After capillary transfer, the membrane was hybridized with a random-primed $\alpha$-P$_{32}$-labeled specific probe, as shown in Fig. 5.

Results

Different B Cell Populations Are Generated in glD42i, B1-8f, and V_{H}12f Mice. We used gene targeting to generate a series of IgH insertion mice in which the J_{H} locus was replaced by distinct V_{H}D_{H}J_{H} segments (14). These segments were taken from the 4-hydroxy-3-nitrophenyl acetyl-binding antibody, B1-8 (9); the antibody glD42, which is a reduced affinity mutant of the DNA-binding antibody, D42 (10); and the anti-Ptc antibody, V_{H}12 (8). The corresponding mice were designated B1-8f, glD42i, and V_{H}12f respectively. The B1-8f (11) and glD42i (10) mice have been described previously, whereas the generation of the V_{H}12f mice will be described elsewhere (Lam, K.-P., and K. Rajewsky, manuscript in preparation).

Flow cytometric characterizations of the B cell populations in the spleens of wild-type, glD42i, B1-8f, and V_{H}12f mice are depicted in Fig. 1. The majority of the B cells

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Figure 1. Phenotype of splenic B cells in (A) wild-type, glD42i/+ , V_{H}12f/+ , and V_{H}12f/glD42i; and (B) B1-8f/+ , V_{H}12f/+ , and V_{H}12f/B1-8f IgH insertion mice. Spleen cells obtained from wild-type and various Ig H chain insertion mice were stained with fluorochrome-conjugated allotypic (A, top) and idioypic (B, top) antibodies as well as antibodies that recognize various cell surface markers used to define B-1 and B-2 cells. Numbers indicate percentage of total lymphocytes in the top panels and percentage of total B220$^{+}$ cells in the others.
present in gld42i (Fig. 1 A) and B1-8f (Fig. 1 B) mice are B-2 cells in that they express high levels of CD23 (shown for gld42i mice), IgD (shown for B1-8f mice), and B220, the pan-B cell marker. In addition, they do not express CD5, a marker found on T and B-1 cells. In contrast, V_{H}12f mice generate cells predominantly of the B-1 phenotype in that they express intermediate levels of CD5, low levels of B220 and IgD, and no detectable CD23. These data are consistent with a previous report that showed the preferential generation of B-1 cells in the lymphoid organs of conventional V_{H}12-transgenic mice (8). Thus, different V_{H}12f gene specificity seems to bias the generation and/or selection of different B cell subsets in the mouse.

V_{H}12-expressing B cells bearing a second I region gene develop into conventional B cells. B cell development under the condition of H chain allelic inclusion had previously been analyzed in mice bearing V_{H}B1-8 and V_{H}glD42 (15). In these double I expression insertion mice, B cells expressing two functional V_{H}12f alleles are readily generated in the bone marrow and not counter-selected in the peripheral lymphoid organs. Here, we cross V_{H}12f mice with gld42i and B1-8f mice to examine the developmental potential of B cells bearing two distinct BCRs, one that is preferentially expressed in B-1 and the other in B-2 cells.

Expression of the V_{H}12 and gld42i alleles in B cells can be identified by the expression of their constant regions as the former is of the α and the latter, of the β allototype. Flow cytometric analyses of the B cells in double V_{H}12f/gld42i mice revealed that the majority of the cells in the spleen (Fig. 1 A), bone marrow, and lymph nodes (data not shown) of these mice coexpress both V_{H}12f genes. Similar results were also obtained when V_{H}12f mice were crossed with B1-8f mice. Expression of V_{H}12 and V_{H}B1-8 can be distinguished by staining with the anti-idiotype (Id) mAb 5C5 (8) and Ac146 (9) respectively. The 5C5 mAb recognizes V_{H}12 independent of the L chains whereas the Ac146 mAb recognizes V_{H}B1-8 in association with λ and the majority (~80%) of the κ L chains (9). As shown in Fig. 1 B, the majority of the splenic B cells in V_{H}12f/B1-8f mice coexpress both Ids, indicating that they are double-Id expressors. This is also true for the B cells in the bone marrow and lymph nodes and of these data (not shown). Taken together, these data indicate that V_{H}12-expressing B cells can coexpress another V_{H}12f gene.

Surprisingly, phenotypic characterization of the IgH “double-producers” in V_{H}12f/gld42i and V_{H}12f/B1-8f mice revealed that these B cells express high levels of B220 and IgD (shown for V_{H}12f/B1-8f mice; Fig. 1 B); and the majority of them are also CD23-positive (shown for V_{H}12f/gld42i mice; Fig. 1 A). In addition, these double-producers do not express CD5 on their cell surfaces. Thus, in contrast to B cells that express V_{H}12 only, B cells that coexpress V_{H}12f/V_{H}glD42, or V_{H}12f/V_{H}B1-8 assumed a phenotype that is characteristic of B-2 cells.

Development of B lymphocytes that coexpress V_{H}12 and V_{H}B1-8 into conventional B-2 cells is not due to restricted Light chain usage. The specificity of the BCR is determined by the variable regions of the H and L chains. Thus, the loss of the B-1 phenotype in cells coexpressing V_{H}12 and V_{H}B1-8 or V_{H}12 and V_{H}gld42 may be due to altered Ig L chain usage. It is conceivable that the L chains that associate with both V_{H}12 and V_{H}B1-8 or V_{H}12 and V_{H}gld42 under the condition of H chain allelic inclusion are different from those that normally associate with V_{H}12 alone. This altered L chain usage could affect the specificity of the V_{H}12 receptor and thus could influence the generation and/or selection of B-1 cells. To examine this possibility, we crossed V_{κ}4 L chain transgenic (tg) mice (8) with V_{H}12f/+, B1-8f/+, and V_{H}12f/B1-8f mice. The V_{κ}4 gene used in the generation of the transgenic mice was initially isolated from a CD5+ B lymphoma cell line that together with V_{H}12 recognizes PtC (8). In addition, this V_{κ}4 L chain can also associate with the B-1 H chain to form a BCR of innocuous specificity. Association of the V_{κ}4 L chain with either or both V_{H}12 and V_{H}B1-8 is demonstrated in Fig. 2. We had previously shown that the bone marrow pre-B cell compartment is absent in Ig transgenic mice whose H and L chains pair to form a BCR of an innocent specificity (16). This probably reflects the fact that precursor cells carrying functional Ig H and L chain transgenes rapidly differentiate into IgM+ B cells. We have used this phenomenon to examine the association of V_{κ}4 with both V_{H}12 and V_{H}B1-8. As shown in Fig. 2, B220+...
pre-B cells are present in wild-type and in the various single and double IgH tg mice and represent ~8% of the cells present. However, this population is fivefold reduced in the B1-8f/+, Vκ4tg; and Vκ4tg mice. The cells were stained with antiidiotypic Ac146 and 5C5 mAbs, and with anti-B220, anti-IgD, and anti-CD5 mAbs. Numbers indicate percentage of total splenic lymphocytes.

Interestingly, two populations of B cells are present in the spleens of Vκ4tg mice (Fig. 3, middle). The Ac146−5C5− double producers that coexpress Vκ4, and Vκ4, B1-8, Vκ4 receptors. The Ac146−5C5− population (fraction b) seems to have lost the surface expression of the B-1 H chain and appears to express only Vκ4. In contrast, Ac146+ 5C5− cells (fraction c) that express only Vκ4, B1-8, Vκ4 are not seen in these mice.

FACS® analyses of the Ac146+ 5C5+ double producers indicate that these cells are conventional B cells in phenotype, as they express high levels of B220 and IgD and lack CD5 expression. They are indistinguishable from the B cells found in B1-8f/+; B1-8f/+; Vκ4tg; or Vκ4tg; and Vκ4tg; glD42i mice. Thus, the development of allelically included Vκ4-expressing B lymphocytes into conventional B cells is not likely to be due to altered L chain usage, as it occurs also in the presence of the Vκ4 transgene. B Cells Expressed Vκ4 only in Vκ4tg mice have lost the B-1 phenotype. The Ac146−5C5− B cells present in the spleens of Vκ4tg mice appear to have lost the surface expression of the B-1 H chain. Thus, they are de facto single Vκ4, Vκ4 expressors and, not surprisingly, have a B-1 phenotype (Fig. 3, middle).

Further analyses revealed that B cells expressing Vκ4 only can also be found in the peritoneal cavities of Vκ4tg mice (Fig. 4). As shown in Fig. 4, the Ac146−5C5− cells comprise a large fraction (>90%) of the B cells present in the peritoneal cavity of
these mice and possess, as expected, a B-1 cell phenotype (data not shown). Although we cannot exclude the possibility that cells bearing V<sub>H</sub>1-8 but not expressing the Ac146<sub>i</sub> due to pairing with certain L chains are also included in this population, such cells should represent a minor fraction. This is supported by FACS<sup>a</sup> analysis of control B-1-8f/+ mice (Fig. 4), which suggests that the majority of the B cells (>80%) in the peritoneal cavity express the Ac146<sub>i</sub>.

To determine the nature of the lack of B-1-8 gene expression in these V<sub>H</sub>12-only cells, we first sorted 5C<sup>5</sup>·Ac146<sup>-</sup> cells from the spleen of V<sub>H</sub>12f/B-1-8f, V<sub>k</sub>4tg mice and analyzed the targeted IgH loci by Southern blotting using a probe located 5' of the DQ<sub>52</sub> region. The wild-type, targeted V<sub>H</sub>12f and B-1-8f alleles should yield fragment sizes of 2.3, 3.4, and 13.5 kb (Fig. 5, A and B, lanes 1–3), respectively. In the 5C<sup>5</sup>·Ac146<sup>-</sup> B cells sorted from the spleen of V<sub>H</sub>12f/B-1-8f, V<sub>k</sub>4tg mice, the band corresponding to the targeted B-1-8f allele is missing (Fig. 5 B, lane 4), suggesting that the gene has been replaced. Similar results were also obtained from 5C<sup>5</sup>·Ac146<sup>-</sup> B cells isolated from the peritoneal cavities of V<sub>H</sub>12f/B-1-8f mice (data not shown). Thus, 5C<sup>5</sup>·Ac146<sup>-</sup> B cells in the spleens and peritoneal cavity of V<sub>H</sub>12f/B-1-8f, V<sub>k</sub>4tg or in the peritoneal cavity of V<sub>H</sub>12f/B-1-8f have lost V<sub>H</sub>1-8 and consequently express only V<sub>H</sub>12. Loss of the B-1-8f allele could occur by rearrangement of upstream V or D gene segments into the B-1-8 VDJ and resulting in a nonfunctional allele (17–19). It is not known presently at which developmental stage the replacement of the B-1-8f allele occurs. FACS<sup>a</sup> analyses of the bone marrow of V<sub>H</sub>12f/B-1-8f or V<sub>H</sub>12f/B-1-8f, V<sub>k</sub>4tg mice suggest that the immature and mature B lymphocytes in this compartment are predominantly double producers (data not shown).

Reduced Expansion of B Lymphocytes in Mice Coexpressing V<sub>H</sub>12 and Another H Chain. Assessment of the number of B cells in wild-type and various IgH insertion mice revealed that V<sub>H</sub>12-expressing B lymphocytes undergo pronounced cellular expansion. As shown in Table I, V<sub>H</sub>12f/+ mice kept in a conventional animal facility generally have 2- and 20-fold more B cells in the spleen and peritoneal cavity respectively, compared with wild-type, B-1-8f/+ or glD42i/+ mice. Interestingly, the number of splenic B cells in mice coexpressing V<sub>H</sub>12 and either V<sub>H</sub>1-8 or V<sub>H</sub>4 is similar to that in wild-type, B-1-8f/+ or glD42i/+ mice, suggesting that the expansion of V<sub>H</sub>12-expressing B cells is curtailed in these mice. However, the number of peritoneal B cells in V<sub>H</sub>12f/B-1-8f or V<sub>H</sub>12f/glD42i mice suggests that the immature B cells that accumulate in the peritoneal cavities of the double IgH insertion mice are mainly V<sub>H</sub>12 expressors (see Fig. 4) that have lost expression of the other H chain (see Fig. 5).

The presence of a V<sub>k</sub>4 L chain transgene leads to an even greater increase in the number of splenic B cells as V<sub>H</sub>12f/+ or V<sub>k</sub>4tg mice have threefold more cells than do V<sub>H</sub>12f/B-1-8f mice and eightfold more cells than do either V<sub>k</sub>4tg or B-1-8f/+ mice. This is probably due to

| Genotype          | Spleen (× 10<sup>7</sup>) | Peritoneal cavity (× 10<sup>6</sup>) |
|-------------------|---------------------------|-------------------------------------|
| +/-               | 2.45 ± 0.83               | 0.85 ± 0.28                         |
| V<sub>H</sub>12f/ +| 5.76 ± 0.26               | 15.40 ± 6.72                        |
| B-1-8f/+          | 2.47 ± 0.42               | 0.24 ± 0.11                         |
| glD42i/+          | 1.08 ± 0.47               | 0.76 ± 0.26                         |
| V<sub>H</sub>12f/B-1-8f | 2.00 ± 0.13               | 11.63 ± 0.38*                       |
| V<sub>H</sub>12f/glD42i | 1.60 ± 0.21               | 10.58 ± 0.32*                       |
| +/- + V<sub>k</sub>4tg | 2.20 ± 0.69               | 0.95 ± 0.33                         |
| B-1-8f/+ V<sub>k</sub>4tg | 1.60 ± 0.40               | 0.31 ± 0.10                         |
| V<sub>H</sub>12f/V<sub>k</sub>4tg | 15.19 ± 1.71              | 13.20 ± 3.70                        |
| V<sub>H</sub>12f/B-1-8f V<sub>k</sub>4tg | 6.96 ± 1.90               | 11.20 ± 2.53*                      |

The number of B lymphocytes is estimated by anti-B220 and anti-IgM staining of splenic and peritoneal cavity cells in flow cytometry analyses. Mice analyzed were kept in a conventional animal facility and were 2–4 mo old. Groups of more than three mice were analyzed for each genotype.

*Greater than 90% of the B cells express only V<sub>H</sub>12 (see Fig. 4).

†Approximately 50% of the B cells are single V<sub>H</sub>12 expressors (see Fig. 3).
ligand-mediated clonal expansion, as V_H12 together with V_k4 recognizes PtC (8). Again, this expansion is modulated in V_H12/B1-8f, V_k4tg mice (Table I). The three- to fivefold increase in the number of splenic B cells in this mouse strain compared with V_k4tg or B1-8f/+, V_k4tg mice is probably due to the fact that >50% of these cells in V_H12/B1-8f, V_k4tg mice are single V_H12 expressors (Fig. 3).

Discussion

V_H12 insertion mice, like conventional V_H12 transgenic mice (8), generate mostly B-1 cells, whereas V_H,B1-8 and V_H,glD42 insertion mice produce predominantly conventional, or B-2, cells. This is in line with the concept that BCR specificity is a major determinant in B-1 versus B-2 cell development. The novel findings in this study are that the coexpression of V_H12 with either of the two other V_H region genes in double IgH insertion mice (which express wild-type χ chains or a V_k4 transgene) results in the generation of a population of double-producing B-2 cells. In addition, in such animals a population of single-producing B cells appears, namely, B-1 cells expressing only V_H12.

Why do V_H12-expressing B cells that coexpress a second H chain not differentiate into B-1 cells? This can perhaps, best be explained by postulating that signaling via a BCR of a certain specificity, expressed at the cell surface at high density, is required to drive the differentiation of B cells into the B-1 subset. In our experiments, the provision of a second H chain of a different specificity presumably acts in a “dominant-negative” manner to dilute out the V_H12-containing BCR complexes on the cell surface. Assuming equivalent production of H chains from the various inserted VH,DKL segments and equivalent pairing of the H and L chains involved, only 25% of the Ig molecules on the cell surface of double-producing cells would carry V_H12 regions on both H chains. This reduced density of B-1–specific BCRs may not provide sufficient signal for the development of B-1 phenotype.

The hypothesis that BCR signaling is responsible for the development of the B-1 cell phenotype has been supported by experiments that show that under certain conditions the cross-linking of sIg on splenic B cells may lead to development of a B-1 cell phenotype on B-2 cells (20). The skewed development of B-1 and B-2 cell subsets in many gene-targeted mice with mutations in specific signaling molecules is also consistent with this hypothesis. For example, CD19-deficient mice (21, 22) and xid (23) mice that have a mutation in the blk gene have reduced numbers and a lack of B-1 cells, respectively. In contrast, lyn-deficient mice (24, 25) and motheaten mice that have a mutation in SHP-1 (26) have increased numbers of B-1 cells. Moreover, the introduction of the xid defect into V_H12 conventional transgenic mice leads to the predominance of V_H12-expressing B cells with a B-2 cell phenotype (27), compared with wild-type V_H12 transgenic mice that generate mostly B-1 cells.

The appearance of large numbers of V_H12-expressing B-1 cells in the double mutants that have lost expression of the second H chain is of particular interest. Such loss variants are rare in V_H,B1-8/V_H,glD42 mice (15), emphasizing the stability of these targeted IgH loci in B cell development. This suggests that the V_H12-only cells in the present system are strongly selected, in accord with the concept that B-1 cell development is driven by BCR signaling. It will be of interest to determine at which stage of development the loss of the second IgH allele occurs in these cells and whether its loss in mature (B-2) double producers will change their phenotype to that of B-1 cells.

It is apparent that B-2 cell development depends to a lesser extent than that of B-1 cells on density of BCRs of certain specificities at the cell surface (reference 15 and the data presented here). This might reflect a lesser dependence of B-2 cells on positive selection by (self)-antigens. The requirement of BCR expression for B-2 cell survival (11) would then largely be a cell-autonomous phenomenon.

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