B Cell Development under the Condition of Allelic Inclusion

Eiichiro Sonoda,*† Yael Pewzner-Jung,† Stephan Schwers,* Shinsuke Taki,§ Steffen Jung,† Dan Elat,¹ and Klaus Rajewsky*¹
*Institute for Genetics
University of Köln
Weyertal 121
D-50931 Köln Federal Republic of Germany
†Division of Medicine
Hadassah University Hospital
Jerusalem 91120 Israel
§Present address: Department of Immunology, Faculty of Medicine, University of Tokyo, 113 Tokyo, Japan.

Summary

Mice whose IgH alleles are engineered to encode two distinct antibody heavy (H) chains generate a normal-sized B cell compartment in which most cells stably express the two heavy chains. This demonstrates that “toxicity” of bi-allelic H chain expression and cell-autonomous mechanisms of silencing in-frame IgH gene rearrangements do not significantly contribute to allelic exclusion at the IgH locus. Notwithstanding, the stability of the various engineered IgH loci during B cell development in the bone marrow differed substantially from each other.

Introduction

B lymphocytes express an immunoglobulin (Ig) heavy (H) chain from only one of their IgH alleles. This phenomenon is called H chain allelic exclusion and reflects the fact that only one allele of a B cell carries an in-frame (“productive”) VHDHJH gene rearrangement. The analysis of IgH rearrangements in single B cell progenitors has shown that allelic exclusion is seen as soon as the cells begin to undergo such rearrangements (Löffert et al., 1996). This supports the prevailing view that allelic exclusion is due to a cell-autonomous developmental control mechanism by which gene rearrangements in the IgH locus are arrested once the cell expresses an H chain from one of its IgH alleles (reviewed by Rajewsky, 1996). It is believed that the initial signal for this arrest results from H chain expression at the cell membrane, mediated in the progenitor cells by the assembly of the pre-B cell receptor, a structure composed of membrane-bound H chains of class μ, the surrogate light (L) chain λ5/V pre-B, and the signaling units Igα and Igβ. In accord with this view, allelic exclusion was abolished in early progenitors by mutation of the λ5 gene (Kitamura et al., 1992; Löffert et al., 1996).

However, it is difficult to exclude the possibility that B cell progenitors expressing H chains from both IgH alleles are indeed generated, but rapidly counterselected so that they become undetectable. An extreme model of this kind postulated that H chain expression from both alleles at an early developmental stage is toxic for the cells, resulting in cell death (H chain “toxicity”; Wabl and Steinberg, 1982). Alternatively, cells having acquired productive VHDHJH joints on both IgH alleles may possess a mechanism to silence one of them. This latter possibility was advocated by Imanishi-Kari et al. (1993), who found that hybridomas from IgH transgenic mice could switch from the expression of an endogenous to that of the transgenic H chain upon loss of the endogenous IgH rearrangement in vitro. While such a mechanism cannot account for the underrepresentation of cells bearing two in-frame VHDHJH joints in the compartment of early progenitor cells, it could act as an additional safeguard ensuring allelic exclusion beyond pre-B cell receptor-mediated arrest of IgH gene rearrangement. The paradoxical finding that in λ5-deficient mice allelically included cells are present in the compartment of early B cell progenitors but absent from that of surface immunoglobulin-positive B cells (Kitamura et al., 1992; Löffert et al., 1996) could be explained in this way.

In an attempt to investigate these matters further, we have generated, by gene targeting, mice that carry distinct in-frame VVDHIH joints in their IgH alleles at the appropriate position. We now use these animals to explore whether H chain toxicity or silencing of in-frame VVDHIH joints plays a significant role in the establishment of allelic exclusion at the IgH locus.

Results

Replacement of the JH Locus by Distinct, In-Frame VVDHIH Rearrangements in the Mouse Germline

We had previously used classical gene targeting to generate a mouse strain (designated T15i) whose JH locus was replaced by the VVDHIH segment of the phosphorylcholine-binding antibody T15 (Taki et al., 1993). In this mutant, a neomycin resistance gene (neo’s) sits upstream of the V1(T15 gene. For the production of the additional “knock in” mutants required for the present study, we used the Cre-loxP recombination system to delete the neo’s gene after the replacement of JH by other VVDHIH segments (Gu et al., 1993). These other VVDHIH gene segments are derived from the (4-hydroxy-3-nitrophe-nyl) acetyl (NP)-binding antibody B1-8 (Reth et al., 1978; Bothwell et al., 1981) and antibody giD42, a mutant of the DNA-binding antibody D42 (Elat et al., 1988; Pewzner-Jung et al., 1996) with a reduced affinity for DNA. V1B1-8 was modified by a silent point mutation in codon 92, inactivating the “internal” heptameric recombination signal sequence (RSS) known to mediate the so-called V1 replacement reaction (Reth et al., 1986; Kleinfield et al., 1986), in an attempt to increase the stability of this gene segment in B cell development (see Discussion). The targeting strategy for the generation of the B1-8i
strain is depicted in Figure 1A, and the characterization of the B1-8i mutation by Southern blotting before and after neo deletion is shown in Figure 1B. The generation of the glD42i strain followed the same principles and will be detailed elsewhere (Y. P.-J., S. J., E. S., K. R., and D. E., unpublished data).

We thus had three mutant mouse strains at our disposal that carried distinct \( \text{V}_{\text{HD}}\text{DH}_{\text{JH}} \) gene segments in their IgH loci. Since the T15i and B1-8i strains had been generated by gene targeting in embryonal stem (ES) cells from strain 129 (IgH \( a \)), T15i and B1-8i mice expressed the transgenic H chains with constant (C) regions of the \( a \) allotype (Taki et al., 1993; see below). In contrast, glD42i mice were derived from targeted ES cells of C57BL/6 (IgH \( b \)) origin. Consequently, they expressed transgenic antibodies of the \( b \) allotype (see below). This allotypic difference facilitated the analysis of transgene expression in subsequent crosses.

Almost All B Cells in Heterozygous B1-8i and glD42i Mice Express H Chains from the Targeted Allele Only

In mice heterozygous for the B1-8i and glD42i mutations, the wild-type IgH alleles appear to be almost perfectly allelically excluded: when the IgH loci in these animals are chosen to carry different C region allotypes and the B cells in the peripheral blood (and also other organs; see below) are stained accordingly, it turns out that most B cells express the targeted allele only, even more so in the case of B1-8i than with glD42i (Figure 2A, middle diagrams; see also Table 1). Data in the next section and Table 1 show that in the case of B1-8i this corresponds to the expression of \( \text{V}_{\text{H}}\text{B1-8} \) itself. This also holds for glD42i (Y. P.-J., and D. E., unpublished data). This situation contrasts with that in heterozygous T15i mice, where approximately 60% of the cells express the wild-type allele and have inactivated the T15i allele by \( \text{D}_{\text{H}} \rightarrow \text{V}_{\text{H}}\text{D}_{\text{J}} \) or \( \text{D}_{\text{H}} \rightarrow \text{(D}_{\text{H}} \rightarrow \text{V}_{\text{H}}\text{D}_{\text{J}}) \) rearrangements (Taki et al., 1995).

Mice Carrying Two Distinct \( \text{V}_{\text{HD}}\text{DH}_{\text{JH}} \) Transgenes in Their IgH Alleles Produce B Cells, Most of Which Express Both Genes

Two types of crosses were performed to assess B cell development in mice carrying appropriately positioned distinct \( \text{V}_{\text{HD}}\text{DH}_{\text{JH}} \) gene segments in their IgH alleles. The first was a cross generating B1-8i/glD42i animals. Here
the analysis of H chain expression from the two IgH alleles was straightforward, because the two alleles differed by C region allotype (B1-8i, a; glD42i, b). The second cross involved B1-8i and T15i mice. In this case, the two mutant IgH alleles were allotypically indistinguishable, so V region markers were required for the analysis. For the T15 VH region, a monoclonal anti-idiotypic antibody is available that recognizes this structure specifically in association with most L chain V regions (Desaymard et al., 1984). However, in the case of VH1-8i-B1-8, the only monoclonal anti-idiotypic recognizing this structure by itself (antibody Ac146; Reth et al., 1979) fails to bind its target in association with certain L chains (unpublished data). This problem was circumvented by making B1-8i/T15i mice homozygous for the CkT mutation (Zou et al., 1993), such that they expressed L chains of type λ only. Since Ac146 is an anti-idiotypic raised against the λ1 chain–bearing antibody B1-8 and λ1 chains are the major α subtype, this provided an efficient way to quantitate VH1-8 expression in the B cell population of the mutant animals. This can be seen from the analysis of peripheral blood B cells from B1-8i, a/b; CkT/CkT, and various control mice (Figure 2). In the B1-8i/glD42i mice, the vast majority (>95%) of the IgM+ cells in the blood stained for both the a and the b allotype, demonstrating expression of both IgH alleles (Figure 2A).

In the case of the B1-8i/T15i combination the situation was similar, but there was clearly a fraction of cells (approximately one third of all B cells) that was negative for the T15 idiotype (Figure 2B). The presence of the VH[T15] cells in the mutant mice was not surprising in light of our earlier data showing the frequent inactivation of the T15i allele through “secondary” rearrangements of upstream Dα or Dγ plus Vα elements into the transgenic VαDαJα gene segment in early B cell progenitors (Taki

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Ac146</th>
<th>Ac146&lt;sup&gt;ext&lt;/sup&gt;</th>
<th>Ac146&lt;sup&gt;ext&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells analyzed</td>
<td>21</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>VH[DαJα] rearramgements detected by PCR</td>
<td>19 (90%)</td>
<td>13 (93%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Sequenced</td>
<td>15</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>VH[DαJα] rearramgements identical to VH, B1-8</td>
<td>13</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>VH[DαJα] rearramgements different from VH, B1-8 heavy chain</td>
<td>2*</td>
<td>1*</td>
<td>—</td>
</tr>
</tbody>
</table>

*VαDαJα rearramgements use Jα elements other than Jα2 and therefore likely originate from the nontargeted allele.
et al., 1995). That this indeed explains the presence of the $V_{\gamma}B1-8$-only cells in the double mutant mice was demonstrated by Southern blot analysis of genomic DNA of single and double producing splenic B cells isolated from the animals by fluorescent cell sorting (Figure 3): in the $V_{\gamma}B1-8$-only cells, the 2.5 kb EcoRI fragment corresponding to the inserted $V_{\gamma}T15$ gene is essentially absent, and a new 5.1 kb band appears, characteristic of the $D_i \rightarrow V_{\gamma}D_{\gamma}J_{\gamma}$ rearrangement by which $V_{\gamma}T15$ is most frequently inactivated during early development (Taki et al., 1995). In accord with the interpretation that the $B1-8$-only cells in these animals are generated early in B cell development are flow cytometric data showing their abundance already in the population of newly generated B cells in the bone marrow (45% of these cells in one particular experiment; data not shown).

To make sure that the high frequency of double producing cells in the blood of the double transgenic animals was not peculiar to this particular cellular compartment, we selected the $B1-8i/giD42i$ combination and analyzed B cells in spleen and bone marrow for allotype expression. As depicted in Figure 4, the results were indistinguishable from those obtained for peripheral blood B cells. Finally, to confirm that the analysis of allotype expression by surface staining truly reflects endogenous expression of the corresponding IgH alleles and is not obscured by passively adsorbed serum antibodies, spleen cells from $B1-8i/giD42i$ mice were cultured in vitro in the presence of lipopolysaccharide (LPS) and interleukin-4 (IL-4) for 5 days and subsequently tested for cytoplasmic expression of IgM of a and b allotype. The results of this analysis (Figure 5) clearly demonstrate cytoplasmic expression of both allotypes in the vast majority of the cells.

**The Size of the Compartments of B Cells and Their Progenitors Does Not Depend on H Chain Allelic Exclusion**

In the bone marrow of the mouse, B lineage cells can be subdivided into developmentally ordered subpopulations on the basis of cell surface markers. In the classification of Hardy et al. (1991), early B cell progenitors express the CD43 antigen and distribute into fractions A, B, C, and C’ on the basis of other surface markers (Hardy et al., 1991; Ehlich et al., 1993). Upon further differentiation, the cells develop into CD43+ pre-B cells (expressing cytoplasmic μ chains; fraction D) from which IgM+ immature B cells (fraction E) and, finally, mature IgM+, IgD+ B cells emerge (fraction F). We have measured the sizes of these cellular compartments in the various single and double transgenic mice in two experiments (Table 2). In the first (Table 2, top), the numbers of CD43+ progenitors and CD43+ pre-B, immature B, and mature B cells were determined. In the second (Table 2, bottom), we measured the various CD43+ progenitor subsets. Despite substantial variations in the cell numbers, there is no indication from the results that the various cellular compartments differ in any systematic way between the single and double transgenic mice. The only consistent difference in cell numbers in Table 2 is seen between the wild type and all transgenics at the level of CD43+ progenitors (Table 2). Except fraction A (which is known to consist of a mixture of B and natural killer cell progenitors; Rolink et al., 1996), the size of this compartment is significantly smaller in the transgenic than the wild-type animals (Table 2, bottom). This is consistent with earlier data indicating rapid progression of B cell progenitors equipped with a transgenic H chain through early phases of development (Era et al., 1991). In the present data this effect is smallest in heterozygous $T15i$ mice. This may reflect the fact that in these animals the $T15$ transgene is frequently inactivated by secondary gene rearrangements (Taki et al., 1995), making the corresponding cells dependent on productive $V_{\gamma}D_{\gamma}J_{\gamma}$ joints in the wild-type IgH locus as in normal B cell development.

Overall, the data in Table 2 indicate efficient generation of B cells in the bone marrow of both single and double transgenic mice. As shown in Table 3, both types of mice also possess normal-sized splenic B cell compartments. Together, these results indicate that neither the generation nor the maintenance of B cells differs dramatically between these animals.

**Discussion**

**Replacing $J_{\gamma}$ by $V_{\alpha}D_{\gamma}J_{\gamma}$ Gene Segments: Variations in Locus Stability and Allelic Exclusion, and Limitation as a Model for Receptor Editing**

Taking our previous work (Taki et al., 1993, 1995) together with the present results, it appears that different mutant IgH loci, in which $J_{\gamma}$ is replaced by distinct $V_{\alpha}D_{\gamma}J_{\gamma}$ gene segments, can dramatically differ in their stability during B cell development. The stability of the mutant loci is probably determined by their ability to serve as a substrate for recombination with upstream $D_i$ elements.
Development of Allelically Included B Cells

Figure 4. IgM Allotype Expression on B Cells in Bone Marrow and Spleen of 8-Week-Old IgH Insertion and Control Mice

Single cell suspensions were prepared from each organ and stained as in Figure 2. Only cells falling into the lymphocyte gate were analyzed.

early in development, namely at the stage of \( D_\lambda \rightarrow J_\lambda \) joining in the wild type. As we have previously shown (Taki et al., 1995), \( D_\lambda \) elements are frequently rearranged at that stage into the T15i locus, via two "hotspots" of recombination characterized by heptameric RSS in the inserted gene. Since these hotspots are localized in the leader intron of \( V_\lambda T15 \) and its first framework region, respectively, these secondary rearrangements (in which upstream \( V_\lambda \) genes can also be involved, presumably through "tertiary" rearrangement later in development) usually inactivate the inserted \( V_\lambda T15 \) gene, allowing \( V_\lambda \rightarrow D_\lambda J_\lambda \) joining on the other (wild-type) IgH allele. In contrast, as shown by the present data, the B1-8i locus exhibits a high degree of stability during B cell development. In the case of gld42i, the situation is less clear-cut, but significantly better exclusion of the wild-type allele by gld42i than by T15i points to the significantly higher stability of the former.

The stability of the engineered loci may well be affected by the presence or absence of hotspots of recombination. Thus, the major recombination breakpoint in the case of \( V_\lambda T15 \) lies in the leader intron (Taki et al., 1995) and is characterized by a RSS heptamer and adjacent CCAG motif (Chou and Morrison, 1993). V\( _\lambda \)B1-8 lacks both of these structures, and V\( \lambda \)gld42 with its intermediate stability possesses the heptamer but lacks the CCAG motif in this position. However, when other mutant IgH loci are also taken into consideration (Chen et al., 1995; Cascalho et al., 1996), a more complex picture seems to emerge. Thus, a large fraction (~20%) of the peripheral B cells of the animals of Cascalho et al. (1996) express \( V_\lambda D_\lambda J_\lambda \) joints modified by recombination with upstream \( D_\kappa \) and \( V_\kappa \) segments, whereas we did not see a single such event in B cells from animals carrying the B1-8i mutation (Table 1). In both cases the engineered \( V_\lambda D_\lambda J_\lambda \) joints encode the same antigenic specificity in combination with \( \lambda \) chains, the analysis being done in both cases in mice in which the \( \kappa \) locus had been inactivated. Therefore, the different behavior of the two mutant loci most likely relates to differences in their molecular structures. However, none of these differences appears to provide a satisfactory explanation for the different phenotypes. Thus, a neo' and the DQ52 element are present in the mutant locus of Cascalho et al., while both are missing in B1-8i. DQ52 is also missing, however, in T15i (which is unstable), and in the case of D42i locus stability appears independent of the presence or absence of the neo' gene in a first approximation (Y. P.-J. and D. E., unpublished data). Further, we engineered the B1-8i allele not to contain the internal RSS heptamer in framework 3, classically involved in the V\( \lambda \) replacement reaction (Reth et al., 1986; Kleinfield et al.,

Figure 5. Cytoplasmic IgM Allotype Expression in Splenic B Cells Stimulated with LPS

Spleen cells were cultured with LPS and IL-4 for 5 days. Recovered viable cells were fixed with 2% formaldehyde, permeabilized with saponin, and stained with biotin-RS3.1 (anti-\( \mu^\alpha \)) and FITC-MB86 (anti-\( \mu^\beta \)). Biotin-RS3.1 was counterstained with PE-streptavidin. The numbers indicate the percentage of cells within the lymphocyte gate.
Thus be complex, it is important to point out that the in pre-B and immature B cells (Li et al., 1993) where of Chen et al. (1995), it is involved only in a minority of et al., 1996). The insertion of N sequences is known

\[ \text{Strain N} \]

loci largely represent an artifact of this experimental initiated secondary rearrangements of V HDHJH loci as

an earlier stage than that of an immature B cell reacting rearrangements of V HDHJH joints engineered into the J H

While the mechanism(s) controlling the stability of “knocked in” IgH loci during B cell development may thus be complex, it is important to point out that the secondary rearrangements occurring in some of these loci largely represent an artifact of this experimental system, in which D\( _i \) elements are still present upstream of the V\( _i \)D\( _{HJH} \) joint, in contrast with the physiological situation. These rearrangements differ from “receptor editing” as originally proposed by Tieg et al. (1993) both in terms of developmental timing (as they are initiated at an earlier stage than that of an immature B cell reacting to self-antigen, namely that of D\( _i \) \( \rightarrow \) J\( _H \) joining; Taki et al., 1995) and mechanistically (as they are mostly, if not always, initiated by recombination of an upstream D\( _i \) instead of a V\( _i \) element as in the classical V gene replacement reaction [Reth et al., 1986; Kleinfeld et al., 1986] into a V\( _i \)D\( _{HJH} \) joint). That the former is indeed the case is indicated not only by the direct demonstration of Taki et al. (1995), but also by the insertion of N sequences into most of the newly formed V\( _i \)D\( _{HJH} \) joints isolated from the various mutants (Chen et al., 1995; Cascalho et al., 1996). The insertion of N sequences is known to be due to the activity of terminal deoxynucleotidyl transferase (Alt and Baltimore, 1982; Gilfillan et al., 1993; Komori et al., 1993), an enzyme which is down-regulatedºknocked inº IgH loci during B cell development may

Bone marrow cells of two femora from 7- to 10-week-old mice were counted, and the size of the various fractions was calculated as described previously (Ethlich et al., 1993). (Top) The number of cells in fractions A–C, D, E, and F was determined by three-color staining using FITC-R33-24-12 (anti-\( \mu \)), PE-RA3-6B2 (anti-B220), biotin-S7 (anti-CD43), and Cy-Chrome-streptavidin. (Bottom) The number of cells in fractions A, B, and C–C was determined by four-color staining using FITC-30F1 (anti-HSA), PE-BP-1, biotin-S7 (anti-CD43), APC-RA3-6B2 (anti-B220), and Texas red-streptavidin.

Values represent the mean ± SD. Values in top and bottom sections are from two independent experiments. N, number of mice analyzed.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total Cells (x 10^6)</th>
<th>Lymphocytes (x 10^6)</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A–C x 10^6</td>
<td>D x 10^6</td>
<td>E x 10^6</td>
</tr>
<tr>
<td>Wild type/wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1-8i/wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glD42i/wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T15i/wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1-8i/glD42i</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1-8i/T15i</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Cell Numbers of Bone Marrow Fractions A–F of IgH Insertion Mice

### Table 3. Numbers of Splenic Lymphocytes in IgH Insertion Mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total Cells (x 10^6)</th>
<th>T Cells (%)</th>
<th>B Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type/wild type</td>
<td>5</td>
<td>7.6 ± 1.8</td>
<td>44.8 ± 2.8</td>
</tr>
<tr>
<td>B1-8i/wild type</td>
<td>3</td>
<td>9.0 ± 0.8</td>
<td>36.0 ± 5.7</td>
</tr>
<tr>
<td>glD42i/wild type</td>
<td>3</td>
<td>4.8 ± 0.9</td>
<td>60.6 ± 4.2</td>
</tr>
<tr>
<td>T15i/wild type</td>
<td>2</td>
<td>7.2 ± 1.8</td>
<td>44.5 ± 4.5</td>
</tr>
<tr>
<td>B1-8i/glD42i</td>
<td>5</td>
<td>8.8 ± 0.7</td>
<td>38.2 ± 6.0</td>
</tr>
<tr>
<td>B1-8i/T15i</td>
<td>2</td>
<td>9.4 ± 0.2</td>
<td>43.0 ± 1.0</td>
</tr>
</tbody>
</table>

T and B cell ratios from spleens of 7- to 10-week-old mice were determined by fluorescence-activated cell sorter analysis using FITC-145-2C11 (anti-CD3) and PE-RA3-6B2 (anti-B220). Values represent the mean ± SD. N, number of mice analyzed.

* 7-month-old mice.
Once Generated, B Cells Expressing H Chains from Both IgH Alleles Are Not Counterselected by B Cell-Intrinsic Mechanisms

The present data establish for two different combinations of V_H region genes that mice engineered to express distinct H chains from their two IgH alleles generate double expressing B cells in normal numbers in the bone marrow and a normal-size compartment of such cells in the periphery. As far as we can see, this excludes the possibility that B cell-intrinsic mechanisms such as H chain toxicology (Wabl and Steinberg, 1982) or monoallelic silencing of H chain expression (Imanishi-Kari et al., 1993) contribute to the establishment of allelic exclusion at the IgH locus to any significant extent. It also further supports the view (Loffert et al., 1996) that the virtual absence of double producing B cells in X5-deficient mice (in which allelic exclusion at IgH does not operate) reflects a peculiarity of B cell development in this mutant strain. It is particularly striking in this context that there is no indication in the present experiments of a selection of single producers in the generation of the peripheral pool of “naive,” IgM^+ IgD^− B cells: the fraction of such cells is not larger in this compartment than that of the newly generated B cells in the bone marrow of B1-8i/T15i mice.

However, we consider it likely that double producing B cells would lose in competition with single producers under selection by antigen. Indeed, as shown by Lozano et al. (1993), cells expressing transgenic light chain V regions from several transgene copies are counterselected in the germinal center reaction in which somatic antibody mutants binding antigen with high affinity are generated and selected. Using the genetically engineered animals described in this study, we plan to identify the phases of B cell development at which such competition (and, therefore, antigenic selection) occurs.

Experimental Procedures

Construction of the B1-BVDJ Targeting Vector

The targeting vector pVHl2neo was constructed as described (Taki et al., 1993) with slight modifications. A 0.8 kb C_m intron fragment was polymerase chain reaction (PCR) amplified from genomic DNA from the 129/Cla-derived E14.1 cell line (Kühn et al., 1991) using the 5’ primer, 5’-TGCTTACGATTTGACGCC-3’ (containing a ClaI restriction site) and the 3’ primer, 5’-GAACTGGAGTACTCAAG CTA-3’. This short arm of homology together with a 9 kb BamHI-XhoI fragment 5’ of DQ52 (Taki et al., 1993) and containing the HSV-tk gene (Thomas and Capecchi, 1987), were cloned into the ClaI-PstI restriction site of the circular Cre-encoding plasmid pIC-Cre (Gu et al., 1993) was transfected by electroporation into 2 × 10^7 targeted ES cells. DNA from G418-sensitive clones was digested with HindIII and hybridized with probe A (Figure 1Ac). Of 21 G418-sensitive clones, 12 revealed a successful neo deletion.

Generation of B1-Bi Mice

Two ES cell clones bearing the rearranged V_M gene and without the neo gene were injected into blastocysts of C57BL/6 mice and transplanted into uteri of F1 (BALB/c × C57BL/6) foster mothers. Chimeric mice were mated to C57BL/6 mice. Tail DNA from chinchilla offspring, indicating germine transmission of the ES cell genome, was analyzed by Southern blot. Tail DNA was digested with EcoRI and hybridized with probe A (Figure 1Bb). Mice heterozygous for B1-Bi carry a 6.5 kb wild-type fragment and an additional 3.2 kb band, derived from the targeted allele. Heterozygous mice from one of the ES cell clones were bred to homozygosity and further analyzed.

Flow Cytometric Analysis of Cells

Single cell suspensions from bone marrow, spleen, and peripheral blood were stained with monoclonal antibodies (MAbs) and analyzed by FACSScan (Becton Dickinson) for three-color analysis or FACSStar (Becton Dickinson) for four-color analysis. The following MAbs were used in the flow cytometric analysis as fluorescence isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), Cy-Chrome, or biotin conjugates: MB86 (anti-B1-8; Reth et al., 1979); Tc54 (anti-T15; Desaymard et al., 1984); 30F1 (anti-HSA; Hardy et al., 1991); Ac146 (anti-B1-8; Reth et al., 1979); Tc54 (anti-T15; Desaymard et al., 1994); and 10C5 (anti-V_A; Sanchez et al., 1991). Biotin conjugates were revealed by PE-streptavidin (Southern Biotechnology), Cy-Chrome-streptavidin (PharMingen), or Texas red-streptavidin (Boehringer Mannheim). Idiotype stainings using Ac146 and Tc54 were performed after washing the cells with isotonic saline buffer (pH 4) in order to strip Fc receptor-bound serum IgG class antibody (Kumagai et al., 1975). Cells in the lymphocyte gate, as defined by forward and side light scatter (Forster et al., 1989), were analyzed. Dead cells were gated out by propidium iodide staining.

Splenocytes from the B1-Bi/T15i double insertion mice carrying the C_c mutant mutation (Zou et al., 1993) were washed with isotonic saline buffer (pH 4) and stained with FITC-Tc54 (anti-T15) and biotin-Ac146 (anti-B1-8), followed by streptavidin-PE and Cy-Chrome-RAM-A68B2 (anti-B20). B1-8 single and B1-Bi/T15i double positive B cells were sorted by FACSStar (Becton Dickinson). The purity of sorted B1-8 single and B1-Bi/T15i double positive B cells was 82% and 79%, respectively.

Generation of B1-BVDJ Targeted ES Cells

Not-linearized targeting construct (30 µg) was transfected by electroporation into 2 × 10^7 E14.1 ES cells (Kühn et al., 1991). The transfected cells were selected with G418 (350 µg/ml) and Gancyclovir (2 µM). Double resistant colonies were identified by PCR using a 5’ primer (5’-ACAGTTACTAGCGTGAGTACT-3’) located at the DFL16.2 element in V_d and a 3’ endogenous primer (5’-GGAACTAGTACTCAAGGTA-3’) located 61 bp 3’ of the EcoRI site and 5’ of the E9 enhancer (Taki et al., 1993). PCR amplification was performed for 40 cycles using a thermal cycler (Biometra). Each cycle consisted of 1 min at 95°C, 1 min at 61°C, and 2 min at 72°C. Putative targeted transfectants, positive for a 1.3 kb PCR fragment, were further analyzed by Southern blotting. Genomic DNA from PCR-positive colonies was EcoRI digested and hybridized with probe B (Figure 1Ac). Homologous recombinants were identified by a 6.5 kb band, corresponding to the wild-type allele, and an additional 3.2 kb band, corresponding to the B1-BVDJ targeted allele. Out of 60 G418 and Gancyclovir double resistant clones, 2 appeared to carry the targeted allele.

To delete the neo gene, 40 µg of the circular Cre-encoding plasmid pIC-Cre (Gu et al., 1993) was transfected by electroporation into 2 × 10^7 targeted ES cells. DNA from G418-sensitive clones was digested with HindIII and hybridized with probe B. Targeted clones with a successful neo deletion were identified by a 2.3 kb wild-type band and an additional 1.5 kb band (Figure 1Ad). Targeted clones that retained the neo gene were identified by a 2.6 kb band instead of the 1.5 kb band (Figure 1Ac). Of 21 G418-sensitive clones, 12 revealed a successful neo deletion.
Genomic DNA from sorted cells was prepared as described previously (Laird et al., 1991) and hybridized with probe A after EcoRI digestion (Fig. 1).

LPS Culture and Cytoplasmic Immunoglobulin Staining
Spleen cells were cultured in RPMI medium (GIBCO BRL) supplemented with 10% fetal calf serum, 40 μg/ml LPS, and 10% IL-4-containing supernatant (J ung et al., 1993). Cells were recovered, subjected to ficoll gradient centrifugation, and fixed with 2% formaldehyde for 20 min. Fixed cells were permeabilized with saponin (Sander et al., 1991) and stained with biotin-43.5 (anti-μ), biotin-22.9 (anti-κ), biotin-22.8 (anti-λ), and FITC-10.9 (anti-γ). Biotinylated mAbs were revealed by PE-streptavidin (Southern Biotechnology). Flow cytometric analysis was performed using a FACScan (Beckton Dickinson).

Reverse Transcription-PCR and Sequence Analysis of IgH Gene Rearrangements from Single Cells
As a result of the breeding procedure, the 7-month-old B1-B1, a/b; C57/C57B1 mouse analyzed by reverse transcription-PCR was also transgenic for a chicken ovalbumin-specific mAb (Shinkai et al., 1993). However, we consider this fact irrelevant for the present analysis.

Single B cells were deposited into 0.5 μl microtubes containing 10 μl of lysis buffer (50 mM Tris-HCl [pH 8.3], 90 mM KCl, 3.6 mM MgCl2, 10 mM DTT, 0.5% NP-40, 0.2 U of PRIME RNase Inhibitor [5' to 3', Inc., Boulder, CO] and 6.2 U of RNase Guard [Pharmacia, Freiburg, Federal Republic of Germany]) using a FACStar plus (Beckton Dickinson). Cells were immediately frozen on dry ice and stored at −80°C until cDNA synthesis.

cDNA was prepared by a modification of the method of Kantor et al. (1997). Microtubes containing single cells were heated to 65°C for 1 min to denature the RNA and then chilled on ice for at least 3 min. After a brief centrifugation, 2.5 μl of the cDNA reaction mixture (60 mM Tris-HCl [pH 8.3], 90 mM KCl, 3.6 mM MgCl2, 10 mM DTT, 150 ng of random hexamers [Boehringer Mannheim], 5 mM dNTP) was added, and the tubes were placed at 22°C. After addition of 100 U of Superscript II Reverse Transcriptase (GIBCO BRL), tubes were incubated at 22°C for 10 min, and then cDNA synthesis proceeded for 15 min at 37°C and 15 min at 42°C, followed by a denaturation step for 6 min at 90°C.

Amplification of μ cDNAs was carried out in three rounds using a promiscuous 5' Vμ primer (MsVμE, 5'-GGGAAATTCAGGGTGCAGCT GCAGGGAGTCGG-3'), which was matched to test all known μV families (Kantor et al., 1997), and nested Cμ 3' primers (CμE, 5'-CACAGGGGCTCTCAGGACAGACG-3'; CμA, 5'-AGGGGGAG ACATTGGGACAGG-3'; CμL, 5'-ACATTGGGAGACGACTC-3'). In the first round of amplification, 2.5 μl of the cDNA reaction was transferred to a microtube containing PCR buffer (GIBCO BRL), 200 μM each dNTP, 2 mM MgCl2, 3 U of Taq DNA polymerase (from GIBCO BRL), and MsVμE and CμE primers (5 pmol of each). The final reaction volume was 50 μl. After an initial denaturation at 96°C for 2 min, amplification was performed for 35 cycles (30 s at 97°C, 30 s at 50°C, 30 s at 72°C), followed by a final elongation step at 72°C for 5 min. The second round of amplification was done accordingly, using 1.5 μl of the first round product as a template and MsVμE and CμA primers (10 pmol of each). In the third round of amplification, 1.5 μl of second round PCR product was amplified for another 15 cycles using MsVμE and CμL primers (10 pmol of each).

PCR products obtained after three round of amplification were cut from preparative 1.2% agarose gels, and DNA was isolated using Spin-X columns (Costar). Cycle sequencing was performed using the MsVμE primer and the Ready Reaction DyeDeoxyTerminator Cycle sequencing kit (Applied Biosystems) following the instructions of the manufacturer. Sequencing was done using an ABI 373A automatic sequencer (Applied Biosystems).

Acknowledgments
Correspondence should be addressed to K. R. We thank A. Eger and C. Göttlinger for technical help, P. Sanchez for the monoclonal anti-VαX antibody, F. Schwenk for critical discussion, U. Ringelstein for the artwork, and G. Schmall for help with the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft through SFB 243, the German-Israeli Foundation, the Human Frontier Science Programme, and the Land Nordrhein-Westfalen.

Received December 20, 1996.

References


Endogenous Ig production in μ transgenic mice. I. Allelic exclusion at the level of expression. J. Immunol. 150, 3311-3326.


GenBank Accession Numbers

The nucleotide sequence data for the IGH rearrangements reported in Table 1 will appear in the EMBL and GenBank nucleotide sequence databases under accession numbers Z84197-Z84200.