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VpreB1/VpreB2/A5 Triple-Deficient Mice Show Impaired B Cell Development but Functional Allelic Exclusion of the IgH Locus

Takeyuki Shimizu, 2* Cornelia Mundt, † Steve Licence, † Fritz Melchers, 3* and Inga-Lil Mårtensson †

At the precursor B cell stage during bone marrow B cell development, Ig μH chain associates with surrogate L (SL) chain, which is encoded by the three genes VpreB1, VpreB2, and A5, to form the pre-B cell receptor (pre-BCR). Surface expression of the pre-BCR is believed to signal both proliferation and allelic exclusion of the IgH locus. Mice which lack either VpreB1/VpreB2 or A5 show a lack of precursor B cell expansion but normal IgH allelic exclusion. This would suggest that one of either A5 or VpreB can make a pre-BCR-like complex which is still able to signal allelic exclusion but not proliferation. To investigate this, we established mice lacking all components of the SL chain. These mice showed severely impaired B cell development which was similar to that previously found in mice lacking either A5 or VpreB1/VpreB2. Surprisingly, the IgH locus was still allelically excluded and thus the SL chain appears not to be involved in allelic exclusion. The Journal of Immunology, 2002, 168: 6286–6293.

Development of B lymphocytes from early progenitors is ordered by the stepwise rearrangements of the V(D)J segments of the IgH and L chain gene loci (1–4). In mice and humans, the IgH locus is rearranged before the κ and λ L chain loci (5, 6). It has been proposed that the product of a successful rearrangement at one IgH allele signals the developing B cell to turn off rearrangements at the other allele of the same locus, thereby ensuring that one B cell produces only one type of IgH (7–9). Precursor (pre-) B lymphocytes first rearrange D to J segments on both IgH alleles before they enter V to DJ rearrangements (3, 10). If the μH chain resulting from a productive VDJ rearrangement can pair with the surrogate L (SL) chain, a pre-B cell receptor (pre-BCR) is formed, in combination with SL chain, on the cell surface. The SL chain is formed by the noncovalent association of the VpreB and A5 proteins, and is assumed to form a λ L chain-like structure capable of disulfide-bound association with a μH chain (11–15). Approximately half of allproductively rearranged IgH loci have been found to produce μH chains capable of combining with the SL chain (16). Nonproductive VDJ rearrangements and productive rearrangements which result in nonpairing μH chains appear to change neither the phenotype nor the functional stage of the cell and they allow rearrangement at the second IgH allele. In contrast, pairing μH chains cause allelic exclusion by preventing the rearrangement of the second allele.

Surface deposition of the pre-BCR allows the cell to be stimulated to differentiate into two and five rounds of cell division (17). Such proliferating large pre-BII cells thereby expand the number of cells which produce SL chain-pairing μH chains. All other cells, either nonproductively VDJ rearranged on both IgH alleles or producing only nonpairing μH chains, do not expand, and may even be induced to apoptosis due to their lack of surface expression of the pre-BCR (18). A quantification by single-cell PCR analysis of the two μH chains in VDJ rearranged cells in the pre-BII and mature B cell compartments and the repertoire suppression of the SL chain nonpairing VH domains of μH chains produced from the productively rearranged alleles (and not seen in the nonproductively rearranged alleles) support such a scenario (16, 19).

Experimentally induced, as well as naturally occurring, mutations in the genes encoding the SL chain support the view that the pre-BCR signals proliferative expansion of pre-BII cells. Targeted disruption of the A5 gene in mice as well as a naturally occurring deletion mutation in humans both abolish proliferative expansion of pre-BII cells and reduce the rate of B cell generation compared with that expected from a normal number of DJH/rearranged pre-BI cells (20–22). In bone marrow without selective expansion of SL chain-pairing μH chain-producing precursors, subsequent sites of L chain rearrangements and production finally lead to selectable surface IgM+ immature B cells. These results also clearly indicate that an incomplete VpreB/μH chain pre-BCR-like complex (23) is not sufficient to signal proliferative expansion. Furthermore, targeted disruption of one of the two VpreB genes (in mice two genes encode the VpreB1 and VpreB2 proteins which are 98% structurally identical (12, 24)), i.e., VpreB1, is not sufficient to induce a marked defect in the generation of B cells, demonstrating that VpreB2 alone, together with A5, is sufficient to generate a functional SL chain and pre-BCR (25). However, the targeted disruption of VpreB1 and VpreB2 on the same chromosome gave rise to a B cell generation-defective phenotype which is apparently indistinguishable from that obtained following disruption of the A5 gene (26). This result suggests that there are no other proteins which can substitute for VpreB1 and VpreB2.

Notably, both the A5-deficient as well as the VpreB1/VpreB2 double-deficient mice showed allelic exclusion of the IgH locus in the immature/mature B cell compartments (19, 26, 27), questioning the idea that the pre-BCR-like complex, composed of μH chain...
and one of the SL chain components, signals allelic exclusion in pre-B cells.

To delete all genes encoding the SL chain, we have generated a triple-deficient mouse strain in which both VpreB genes as well as the $\lambda^5$ gene have been disrupted by targeted integration of deleting elements on the same chromosome. Interestingly, the triple-mutant mice showed the same B cell generation defect as reported for the $\lambda^5$- and the VpreB1/VpreB2-deficient mice. Surprisingly, however, immature and mature B cells in these mice still showed IgH allelic exclusion.

Materials and Methods

Targeting vectors

A targeting vector for the VpreB1-$\lambda^5$ locus was made as follows. A 5.5-kb XhoI-HindIII fragment which contains the second and third exons and the 3' region of the $\lambda^5$ gene was isolated from phage clone 7pB12-2 (28). This fragment was cloned between the XhoI-HindIII sites of pBlueScript II (Stratagene, CA). The thymidine kinase gene cassette was inserted downstream of the $\lambda^5$ gene. In a separate vector, the neomycin (neo) resistance gene cassette was inserted between the SalI-XhoI sites of pBlueScript II. A 1.6-kb SacII-BglII fragment, which contains the region 5' of the VpreB1 gene, was isolated from pUC5E6.5X (24) and inserted between the SacII-BamHI sites. The resulting 5' VpreB1-neo gene hybrid was cut out using SacII and inserted into the blunt-ended XhoI site of the vector which contained the 3' end of the $\lambda^5$ gene and the thymidine kinase gene. As a result, the entire region of the VpreB1 gene and the first exon of the $\lambda^5$ gene were deleted in the targeting construct. The neo gene was cloned in the opposite transcriptional orientation to VpreB1 and $\lambda^5$. The targeting vector for VpreB2 has been described previously and uses hygromycin as a selection marker (26). In addition, the neo cassette has a loxP site on either side which thus the hygromycin gene has a loxP site on only one side (see Fig. 1).

Gene targeting in ES cells and establishing SL chain-deficient mice

E14 embryonic stem (ES) cells (strain 129) were cultured on irradiated embryonic fibroblasts (Incyte Genomics, St. Louis, MO) in DMEM (Life Technologies, Grand Island, NY) with 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO), 1000 U/ml ESGRO (Life Technologies), gentamicin, and 15% FCS. The VpreB1-$\lambda^5$ targeting vector was linearized with N执法人员. The ES cells were transfected and cultured with 300 μg/ml G418 (Life Technologies) and 2 μM gancyclovir (Roche, Basel, Switzerland). Single colonies were screened for VpreB1-$\lambda^5$ homologous recombination by Southern blotting. Two homologously targeted ES cell clones (522 and 515) were transfected with the linearized VpreB2 targeting vector and selected in 300 μg/ml hygromycin (Roche Molecular Biologicals, Mannheim, Germany). Single colonies were screened for VpreB2 homologous recombination by PCR (26). Positive clones were confirmed by Southern blotting.

Four ES clones targeted at both the VpreB1-$\lambda^5$ and VpreB2 loci were used to establish clonogenic mice. Two of these (515-3H11 and 522-H2A3) had the two mutations integrated on the same chromosome, whereas the other two ES clones (522-N3C4 and 515-4F4) had integrated the two mutations on separate chromosomes. The agouti mice (129 × C57BL6 background) with targeted mutations from 515-3H11 and 522-H2A3 ES clones were backcrossed to establish homozygous SL chain-deficient mice. The genotypes of the mice were determined by PCR using previously described primers (26) and/or Southern blotting with probes A and B shown in Fig. 1. Homozygous SL chain-deficient mice were also crossed with mice expressing cre recombinase ubiquitously. These were then intercrossed to obtain homozygous SL chain-deficient mice with a deletion of the neo cassette on both alleles (SL$\lambda$neo-deficient) but with the hygromycin cassette intact. These mice were screened by several PCRs to confirm that the genotype was correct using a combination of primers detecting VpreB1, VpreB2, $\lambda^5$, neo, and hygromycin. In addition, they were also screened for the presence of an ~700-bp PCR product indicative of the neo deletion using one primer located just upstream of the recombinant loxP site (5'-TTGTCTCATTGATGCGACCAAGGCCC-3') and another primer downstream of the XhoI site in $\lambda^5$ intron 1 (5'-CAAGCTTCTTTGACTGCG-3'). Conditions were as follows: 30 cycles of 94°C for 30 s, 55°C for 20 s, and 72°C for 20 s. Mice were established and analyzed under PPL 80/1143 and PPL 80/1501.

SL chain expression

Total RNA was prepared from two million bone marrow cells from 3-week-old mice using RNAzol (Tel-Test, Friendswood, TX). cDNA was synthesized using SuperScript II reverse transcriptase-polymerase and random primers (Life Technologies). PCR primers and reaction conditions for the VpreB genes were described previously (24). Primer sequences for $\lambda^5$ exon 2 were 5'-TTGTCTCATTGATGCGACCAAGGCCC-3' and 5'-GGTTCTTCTTTGACTGCG-3'. Conditions were as follows: 30 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s.

One million bone marrow cells from 3-week-old mice were cultured on irradiated stromal cells (S17) in RPMI 1640 medium (Life Technologies) supplemented with 50 μM 2-ME, antibiotics, 10% FCS, and 10% IL-7. One week later, the cultured cells were collected and expression of the SL chain on the cell surface and in the cytoplasm was analyzed by FACS.

FACS analysis

Cell suspensions were prepared by conventional methods and stained in HBSS (Life Technologies) supplemented with 10 mM HEPES and 3% FCS. After staining, the cells were suspended in the same medium containing propidium iodide (0.25 μg/ml) to exclude dead cells. To analyze the allele of IgM, bone marrow cells were stained with PE-labeled anti-IgM, biotin-labeled anti-IgD, and allophycocyanin-labeled anti-B220 Abs in the presence of 5% rat serum. After washing, the cells were stained with FITC-labeled anti-IgM Abs and tricolor-labeled streptavidin. Cytoplasmic staining (for detection of SL chain) was performed according to the manufacturer’s instructions (Caltag Laboratories, Burlingame, CA). The Abs used were as described previously (26). All data were acquired on a FACS-Calibur and analyzed using the CellQuest program (BD Biosciences, Mountain View, CA).

Immunization and ELISA

Control and homozygous mutant littermates were immunized by i.p. injection of either 100 μg of NP-chicken γ-globulin in alum or 5 μg of 4-hydroxy-3-nitrophenlyacetyl (NP)–Ficoll. Two weeks after immunization, the mice were bled and sera were prepared and analyzed by ELISA as previously described (26). The sera were analyzed as 5-fold serial dilutions. Immunizations were performed under PPL 80/1263.

Results

Establishment of SL chain-deficient mice

The three genes encoding the SL chain (VpreB1, VpreB2, and $\lambda^5$) are all located on mouse chromosome 16 (29, 30); therefore, targeting of these three genes in ES cells was performed in two steps. In the first step, the VpreB1 gene and the first exon of the $\lambda^5$ gene were replaced with the gene encoding neomycin resistance (Fig. 1). Two correctly targeted ES cell clones (clones 522 and 515, data not shown) were used for the second step in which the VpreB2 gene was substituted by the hygromycin resistance gene (Fig. 1). The double-targeted ES cell clones were injected into blastocysts and four clones gave rise to germline transmission. In two of these (522-H2A3, 515-3H11), the two targeting events were found to segregate together, demonstrating that they were on the same chromosome. The heterozygous mutant mice were bred for homozygosity which was confirmed by Southern blotting as shown in Fig. 1. Both strains showed the same phenotype and the homozygous VpreB1$^{-/-}$VpreB2$^{-/-}$,$\lambda^5^{-/-}$ mice are hereafter called SL chain-deficient mice.

Lack of VpreB and $\lambda^5$ expression in SL chain-deficient mice

Total RNA from bone marrow cells of SL chain-deficient as well as control mice was prepared and reverse transcribed. The existence of the SL chain gene mRNA was first analyzed by using primers specific for VpreB1 and VpreB2 (Fig. 2A). In wild-type and heterozygous mutant mice, expression of both genes was detected, while in the homozygous mutant mice, no message was found. Since mRNA encoding a ubiquitously expressed gene (hypoxanthine guanine phosphoribosyl transferase) was detected, we concluded that the VpreB genes are not expressed in the SL chain-deficient mice.
Thereafter, we analyzed for \( \lambda_5 \) message using primers specific for exon 2 and exon 3, since these are still present after the targeting event. As shown in Fig. 2B, there is a properly spliced message, as judged by the size of the band, present in the homozygous mutant mice, with levels around 10-fold decreased as compared with that of the control. Northern blot analysis on total RNA prepared from in vitro-cultured pre-B1 cells (the same cultures as used for protein analysis, see below) was then performed. A \( \lambda_5 \) cDNA probe gave rise to a signal in RNA from control but not homozygous mutant cells, whereas an actin probe gave a signal of the same strength in both RNA preparations (data not shown). Thus, the truncated \( \lambda_5 \) mRNA levels were too low to be detected by Northern blotting.

The \( \lambda_5 \) exon 2/3 mRNA in the homozygous mutant mice is probably the result of cryptic transcription initiation sites just upstream of exon 2 in intron 1: we did not detect any transcripts starting further upstream in the intron (data not shown). The level of exon 2/3 expression might be due to sequences in the neo cassette. Especially since this was cloned in the opposite direction, positioning the enhancer in the cassette just upstream of the potential cryptic initiation sites in the \( \lambda_5 \) intron. To test whether the enhancer in the neo cassette had any effect on the \( \lambda_5 \) exon 2/3 transcripts, we deleted the neo cassette by crossing SL chain-deficient mice with transgenic mice expressing cre recombinase ubiquitously. The offspring were intercrossed resulting in SL chain-deficient mice with the neo cassette deleted on both alleles. These mice are hereafter termed SL\( \Delta \)neo-deficient mice. We analyzed bone marrow RNA from these mice by RT-PCR, again using primers for \( \lambda_5 \) exon 2/3. As shown in Fig. 2B (\( \Delta \)neo), by deleting the enhancer in the neo cassette the transcripts are no longer detectable in the homozygous mutant mice. Thus, the enhancer in the neo cassette was the cause of the truncated \( \lambda_5 \) transcripts.

We also searched for possible translation starts sites and open reading frames to determine whether a truncated \( \lambda_5 \) protein could be translated from the exon 2/3 transcripts. Even if the transcript starts in intron 1, it would have to be closer than 159 bp from the splice site as determined by RT-PCR assays (data not shown). There are no translation start sites with an open reading frame that could encode a protein in this region. The few ATG present all probably the result of cryptic transcription initiation sites just upstream of exon 2 in intron 1: we did not detect any transcripts starting further upstream in the intron (data not shown). The level of exon 2/3 expression might be due to sequences in the neo cassette. Especially since this was cloned in the opposite direction, positioning the enhancer in the cassette just upstream of the potential cryptic initiation sites in the \( \lambda_5 \) intron. To test whether the enhancer in the neo cassette had any effect on the \( \lambda_5 \) exon 2/3 transcripts, we deleted the neo cassette by crossing SL chain-deficient mice with transgenic mice expressing cre recombinase ubiquitously. The offspring were intercrossed resulting in SL chain-deficient mice with the neo cassette deleted on both alleles. These mice are hereafter termed SL\( \Delta \)neo-deficient mice. We analyzed bone marrow RNA from these mice by RT-PCR, again using primers for \( \lambda_5 \) exon 2/3. As shown in Fig. 2B (\( \Delta \)neo), by deleting the enhancer in the neo cassette the transcripts are no longer detectable in the homozygous mutant mice. Thus, the enhancer in the neo cassette was the cause of the truncated \( \lambda_5 \) transcripts.

We then tested for the presence/absence of SL chain protein in the SL chain-deficient mice. Bone marrow cells from wild-type, heterozygous, and homozygous SL chain-deficient mice were cultured in vitro for 1 wk on irradiated stromal cells in the presence of IL-7. Under these conditions, it is possible to expand and maintain pre-B1 cells which express the SL chain on the cell surface together with \( \text{BILL}-\text{cadherin} \) and other proteins (31–33). Surface expression of the SL chain was analyzed using mAbs specific for VpreB and \( \lambda_5 \) (Fig. 2C). Pre-B1 cells from wild-type and heterozygous mutant mice expressed both \( \lambda_5 \) and VpreB on the surface.

**FIGURE 1.** Establishment of SL chain-deficient mice. Genomic organization of the VpreB1-\( \lambda_5 \) (top) and VpreB2 (bottom) loci are shown on the left. The partial restriction enzyme maps of the loci before (wt) and after (ko) targeting are shown. For the VpreB1-\( \lambda_5 \) locus, the map after neo cassette deletion (\( \Delta \)neo) is also shown. Restriction enzyme sites: B, BglII; Ba, BamHI; E, EcoRI; H, HindIII; K, KpnI; S, SacI; X, XbaI. The Bg/II and XbaI sites were destroyed during cloning. The arrows under the neo and hygro indicate the orientation of these genes, which are opposite to those of the SL chain genes. Southern blottings of tail DNA are shown on the right. DNA was digested with EcoRI and the membrane hybridized with probe A (top). The same DNA was digested with BamHI and the membrane was hybridized with probe B (bottom). Wild-type (wt) and homologous recombinant (ko) bands are indicated with arrows. The genotypes of the mice are shown at the top.

**FIGURE 2.** Analysis of SL chain expression. A and B, Total RNA was prepared from bone marrow cells of SL chain- and/or SL\( \Delta \)neo-deficient mice of the indicated genotype and analyzed by RT-PCR using primers specific for VpreB1 and VpreB2 (A; NC, negative control without cDNA), or \( \lambda_5 \) exon 2/3 (B). The expression of SL chain protein on the cell surface (C) and in the cytoplasm (D) of in vitro-cultured pre-B1 cells from heterozygous and homozygous SL chain-deficient mice was analyzed by FACS using Abs recognizing VpreB and \( \lambda_5 \). Histograms show isotype control (thin line) overlaid with the specific stain (thick line).
Reduction numbers of pre-BII cells in the bone marrow of SL chain-deficient mice

To investigate the effect of SL chain deficiency on B cell development, we analyzed the different bone marrow B cell compartments by FACS (Fig. 3A). As there was no obvious difference between wild-type and heterozygous mutant mice (data not shown), data from these genotypes were pooled and are shown as controls in the figures and tables. Table I summarizes the number of B lineage cells. Compared with control mice, the total number of nucleated cells in bone marrow was decreased in SL chain-deficient mice (~80%) as were B220+ cells (~50%). The B220+ B lineage cells were thereafter analyzed in detail. The pro-/pre-BI cell population (B220+c-Kit+) was increased ~2-fold compared with control mice, whereas the pre-BII cell population (B220+CD25+) was severely decreased in SL chain-deficient mice. The SL chain-mutant mice showed a 25-fold decrease in young (3 wk) and a 12-fold reduction in 8- to 9-wk-old mice as compared with control mice. In wild-type mice, pre-BII cells have productively rearranged the IgH loci and they express cytoplasmic μH chain, some of which can pair with the SL chain to form a pre-BCR and signal cell proliferation. This population can be divided into large (cycling) and small (resting) cells. By collecting a large number of bone marrow cells from 8- to 9-wk-old mice, we determined that the proportion of large cells among the B220+CD25+ cells was 26% in control and 41% in SL chain-deficient mice (data not shown). From this, we calculated the number of large and small pre-BII cells. Compared with control mice, SL chain-deficient mice showed an ~7-fold decrease in the large pre-BII cell population and an ~15-fold decrease in the small pre-BII population. These data suggested that in SL chain-deficient mice the generation of pre-BII cells and/or the proliferative expansion of large pre-BII cells is impaired.

Reduced numbers of immature and mature B cells in bone marrow and spleen of SL chain-deficient mice

The number of both immature (IgM⁺IgD⁻) and mature (IgM⁺IgD+) B cells in bone marrow was markedly reduced in SL chain-deficient mice. Depending on age, the difference in IgM⁺ cells was 20- and 9-fold in young (3 wk) and old (8–9 wk) mice, respectively. However, the ratio of small pre-BII to immature B cells, as analyzed in 8- to 9-wk-old animals, was 2:1 in control and 1:1 in SL chain-deficient mice (Table I). This suggested that the absence of the SL chain, while impairing the transition from pre-BI to pre-BII cells, does not influence the transition from pre-BI to immature B cells.

Immature B cells generated in the bone marrow migrate to the spleen where they mature. Therefore, spleen cells of SL chain-deficient mice were analyzed to study the effect of the mutations on

Table I. Absolute numbers of B lineage cells in bone marrow and spleen

<table>
<thead>
<tr>
<th>Organ</th>
<th>Age (wk)</th>
<th>Genotype</th>
<th>No.</th>
<th>Total</th>
<th>B220⁺</th>
<th>B220⁺c-Kit⁺</th>
<th>B220⁺CD25⁺</th>
<th>B220⁺IgM⁺</th>
<th>B220⁺IgD⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>3</td>
<td>Control</td>
<td>5</td>
<td>22.41 ± 2.19</td>
<td>8.33 ± 1.14</td>
<td>1.00 ± 0.12</td>
<td>5.75 ± 1.04</td>
<td>2.14 ± 0.29</td>
<td>0.91 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>8–9</td>
<td>5</td>
<td>18.77 ± 0.76</td>
<td>3.27 ± 0.39</td>
<td>2.27 ± 0.24</td>
<td>0.23 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>3</td>
<td>Control</td>
<td>5</td>
<td>214.42 ± 17.4</td>
<td>102.71 ± 6.83</td>
<td>83.34 ± 6.77</td>
<td>84.16 ± 7.90</td>
<td>3.82 ± 0.63</td>
<td>3.52 ± 0.57</td>
</tr>
<tr>
<td></td>
<td>8–9</td>
<td>5</td>
<td>57.06 ± 3.42</td>
<td>6.04 ± 0.94</td>
<td>1.76 ± 0.23</td>
<td>0.32 ± 0.09</td>
<td>0.44 ± 0.11</td>
<td>0.25 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

*The mean and SEM of the absolute numbers (×10⁶) are shown for indicated populations in bone marrow (two femurs) and spleen of control and homozygous SL chain-deficient (−/−) mice.*
the peripheral B cell populations (Fig. 3B and Table I). The total number of nucleated cells was 4- and 2-fold decreased in young and old SL chain-deficient mice, respectively. The B220<sup>+</sup> cell population was also affected. In young mice, an almost 15- to 20-fold reduction was observed whereas in older mice the difference was less pronounced (5-fold). The majority of IgM<sup>+</sup> cells were also IgD<sup>+</sup> (mature B cells) independent of genotype. The number of mature B cells was also decreased 20- to 25-fold in 3-wk-old and 5-fold in 8- to 9-wk-old SL chain-deficient mice. This suggested that the SL chain does not affect the differentiation from immature to mature B cells. The ratio of Igκ vs Igλ did not vary between the genotypes.

**SLΔNeo-deficient mice**

The data presented in Fig. 2B demonstrate the presence of a truncated λ5 transcript (exon 2/3) in the bone marrow of homozygous SL chain-deficient mice, but this was not detectable in bone marrow from SLΔNeo-deficient mice. The sequence data indicated that it would not be possible to make a truncated protein from this RNA. In addition, the FACS analyses in Fig. 2 showed that no λ5 protein was detected in the homozygous SL chain-deficient pre-BI cells. Even so, it may be argued that a truncated λ5 protein is produced that is not recognized by the LM34 Ab. We therefore analyzed the SLΔNeo-deficient mice, where we performed the same analyses as above both on bone marrow and spleen cells from homozygous mutant and wild-type mice. We did not detect any obvious difference in any of these analyses when compared with those described above for the SL chain-deficient mice (data not shown). We therefore conclude that the SL chain-deficient mice are “true” knockouts and show the phenotype of mice lacking the entire SL chain, i.e., VpreB1, VpreB2, and λ5. Therefore, with the exception of Igκ vs Igλ, all Igκ-expressing B cells expressed IgM<sup>+</sup> and in SL chain-deficient mice this percentage remains intact in bone marrow immature and mature B cells expressed two IgH allotypes on the cell surface was analyzed. SL chain-deficient and control mice heterozygous for the IgM<sup>+</sup> and IgM<sup>+</sup> alleles were selected. Bone marrow cells from these mice were stained with Abs recognizing B220, IgD, IgM<sup>+</sup>, and IgM<sup>+</sup> and analyzed by FACS (Fig. 5). Both IgD<sup>+</sup> and IgD<sup>+</sup> cells were analyzed and no difference was observed (data not shown). In both control and SL chain-deficient mice, ~55–60% of the IgM<sup>+</sup> cells expressed IgM<sup>+</sup> and ~40–45% expressed IgM<sup>+</sup>. In control mice, 0.7% of the IgM<sup>+</sup> cells expressed both IgM<sup>+</sup> and IgM<sup>+</sup> and in SL chain-deficient mice this percentage was 0.5%. In a separate experiment, bone marrow cells from these mice were stained for cytoplasmic IgM<sup>+</sup> and IgM<sup>+</sup>. The results showed IgH allelic exclusion independent of the genotype (data not shown). This demonstrated that in the absence of SL chain allelic exclusion of IgH remains intact in bone marrow immature and mature B cells. We also analyzed the SLΔneo-deficient mice in the same way and similar results were obtained. Thus, the lack of the entire SL chain does not affect the mechanism of IgH allelic exclusion.

**IgM serum levels and immune responses**

To investigate whether the decreased number of B lymphocytes in the periphery had any effect on the level of IgM in the serum, mice of different genotypes were bled and the sera analyzed by ELISA to determine the amount of IgM. Wild-type and heterozygous mutant mice both demonstrated levels of 1.4 mg/ml and homozygous mutant mice reached levels of 1.6 mg/ml (three animals per group). Thus, there was no significant difference in the total IgM concentration when comparing control and homozygous mutant mice.

The responses against T cell-dependent (TD) and -independent (TI) Ags were also analyzed. Eight-week-old mice were immunized with either NP-chicken γ-globulin (TD Ag) or NP-Ficoll (TI Ag). Two weeks later, serum samples were prepared and Ag-specific Abs were measured by ELISA. Immune responses to TD Ags, both against the hapten and the carrier, were similar in SL chain-deficient and control mice (Fig. 4 and data not shown). The Ab response against the TI Ag was reduced 5- to 10-fold in mice lacking the SL chain as compared with control mice (Fig. 4). Thus, despite reduced numbers of B lymphocytes in the periphery, the SL chain-deficient mice were able to mount an immune response against both T cell-dependent and -independent Ags, although not at the same level as the control, implying that the peripheral B cells are functionally normal.

**Allelic exclusion in SL chain-deficient mice**

To investigate whether allelic exclusion of the IgH locus was functioning in the absence of the SL chain, the frequency of single immature and mature B cells expressing two IgH allotypes on the cell surface was analyzed. SL chain-deficient and control mice heterozygous for the IgM<sup>+</sup> and IgM<sup>+</sup> alleles were selected. Bone marrow cells from these mice were stained with Abs recognizing B220, IgD, IgM<sup>+</sup>, and IgM<sup>+</sup> and analyzed by FACS (Fig. 5). Both IgD<sup>+</sup> and IgD<sup>+</sup> cells were analyzed and no difference was observed (data not shown). In both control and SL chain-deficient mice, ~55–60% of the IgM<sup>+</sup> cells expressed IgM<sup>+</sup> and ~40–45% expressed IgM<sup>+</sup>. In control mice, 0.7% of the IgM<sup>+</sup> cells expressed both IgM<sup>+</sup> and IgM<sup>+</sup> and in SL chain-deficient mice this percentage was 0.5%. In a separate experiment, bone marrow cells from these mice were stained for cytoplasmic IgM<sup>+</sup> and IgM<sup>+</sup>. The results showed IgH allelic exclusion independent of the genotype (data not shown). This demonstrated that in the absence of SL chain allelic exclusion of IgH remains intact in bone marrow immature and mature B cells. We also analyzed the SLΔneo-deficient mice in the same way and similar results were obtained. Thus, the lack of the entire SL chain does not affect the mechanism of IgH allelic exclusion.

**Discussion**

At the transition from DJ<sub>4</sub>/DJ<sub>H</sub>-rearranged pre-BI to VDJ<sub>H</sub>-rearranged pre-BII cells, pre-BCRs are deposited on the surface membrane of those cells in which a productive rearrangement has given rise to a μH chain capable of pairing with the SL chain. This

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**Table II. Reduction in B-1a B cells in SL chain-deficient mice**

<table>
<thead>
<tr>
<th>Age</th>
<th>Population</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 days</td>
<td>B1-a</td>
<td>0.72 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;CD5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.96 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;CD5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td>9 wk</td>
<td>B1-a</td>
<td>24.43 ± 3.79</td>
</tr>
<tr>
<td></td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;CD5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>17.30 ± 2.52</td>
</tr>
<tr>
<td></td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;CD5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>13.50 ± 1.40</td>
</tr>
</tbody>
</table>

<sup>a</sup>The mean and SEM of absolute numbers (×10<sup>6</sup>) are shown for B-1a (B220<sup>+</sup>CD5<sup>+</sup>), B220<sup>+</sup>CD5<sup>+</sup> and B220<sup>+</sup>CD5<sup>+</sup> lymphocyte populations in the peritoneum of mice (three to five per group) of indicated genotype and age.

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**FIGURE 4.** Immune responses. Mice of the indicated genotype were immunized with TD or TI Ags, and the amount of Ag-specific Abs in the sera were analyzed by ELISA. The titers of hapten (NP)-specific IgM and IgG are shown. CGG, Chicken γ-globulin.
surface expression of pre-BCR promotes between two to five rounds of division, thus expanding the number of cells producing μH chain between 4- and 32-fold. When the pre-BCR cannot be deposited on the surface, there is no proliferative expansion of the pre-BI compartment (17, 34). As we have predicted from our previous observations (17, 21, 26), the SL chain-deficient, VpreB1−/−VpreB2−/− mice also lack pre-BII cell proliferative expansion and show a B cell immunodeficiency (Fig. 3 and Table I). Since the SL chain and the SL immunodeficient mice show the same phenotype, they are throughout the discussion termed SL chain-deficient mice. In terms of kinetics of development, defective cellularity of the immature and mature B cell compartments (Table I) and response to T cell-dependent Ags (Fig. 4), all seem indistinguishable in the single A5−/−, the double VpreB1−/−VpreB2−/− (20, 26), and the SL chain-deficient mice. The pre-BI population, as in the other SL chain-immunodeficient mice, is 2- to 3-fold larger than in wild-type littermates, probably because the cells cannot exit this compartment by further B cell differentiation (Table I). The ratio of the sizes of the small pre-BII compared with the immature B cell compartment, as well as that of the small pre-BII compared with the mature B cell compartment is similar in wild-type, A5−/−, VpreB1−/−VpreB2−/−, and SL chain-deficient mice (Table I) (21, 26). All these observations suggest that the major measurable defect introduced by mutations of the SL chain subunits is the lack of proliferative expansion at the transition from pre-BI to pre-BII cells.

Although we have not yet measured the kinetics of in vitro differentiation of surface IgM+ B cells from SL chain-deficient pre-BI cells, we would predict from our observations of the B cell developmental defects that these kinetics, as in A5−/− pre-BI cells (21), would also be indistinguishable between SL chain-deficient and wild-type pre-BI cells. Hence, expression of the SL chain before expression of the μH chain in pre-BI cells (35, 36), possibly with BILL-cadherin (32, 33) and other associated proteins, appears not to influence the capacity of these cells to enter Vh to DJh rearrangements and later to Vh to Jh rearrangement and differentiate to pre-BII, immature, and mature B cells. From an unaltered differentiation capacity of the A5−/− pre-BI cells in vitro (21), we conclude that they have the same unaltered capacity to differentiate in vivo. Hence, the developmental defect of SL chain-deficient pre-BI cells is most likely their inability to proliferate, leading to a reduction in the number of μH chain-producing pre-BI cells in which subsequent L chain gene rearrangement can take place. Furthermore, those cells producing μH chains which best fit the SL chain are expanded most, and these μH chains should also fit best to the conventional L chain which is generated later (37, 38). Therefore, in SL chain-mutant mice, many more pre-BI cells would have to enter Vh to DJh rearrangements to produce an equal number of cells (as compared with wild-type mice) producing well-fitting μH chains in a given period of time. According to this view, B cell differentiation from the SL chain-deficient cells is not leaky, but simply inefficient.

The original repertoire of Vh domains expressed in μH chains as a consequence of Vh to DJh rearrangements at the transition of pre-BI to pre-BII cells in the bone marrow is influenced in a major way by the SL chain (16). The Vh domains that cannot pair with SL chains, notably Vh81X within the Vh7183 family, the VhQ52 domains, and nearly half of all VhJ558 domains are suppressed from the μH chain repertoire of pre-BI and all subsequent B lineage cells; the cells that express them cannot form a pre-BCR and therefore are not expanded in the same way as those μH chain producers that contain pairing Vh domains. In A5−/− mice, the pre-BII compartment still contains all of these nonpairing μH chain producers. We predict that future single-cell PCR/pairing analyses of the μH chain repertoires expressed in single pre-BII cells of the SL chain-deficient mice will also show the same non-suppressed Vh repertoire as that observed in A5−/− pre-BII cells. As L chain becomes expressed at the transition from pre-BII cells to mature B cells, the Vh repertoire of μH chain-producing cells becomes suppressed in A5−/− cells, as it is in wild-type cells when the SL chain is testing the μH chain at the transition from pre-BI to pre-BII cells. A future single-cell PCR/pairing analysis is likely to show the same Vh repertoire change for SL chain-deficient cells at the transition from pre-BI to mature B cells.

A second functional role has been proposed for the pre-BCR, namely, to signal a pre-BII cell, which has made one productive VDJh rearrangement and produced a μH chain that can pair with the SL chain and form a pre-BCR on the surface, to turn off the rearrangement machinery (recombination-activating gene 1, recombination-activating gene 2, and TdT), and to close the second DJh-rearranged allele (39). Thereby, the resulting VDJh/DJh pre-BII cell is allelically excluded and prevented from possible VDJ rearrangement at the second allele. Since Vh to DJh rearrangements occur randomly in- and out-of-frame, two-thirds of all original rearrangements are out-of-frame and unable to produce a μH chain. Consequently, no pre-BCR is made and the cell rearranges the second allele. Of these, one-third will be productive, thus generating a VDJh/VDJh-rearranged cell. This cell is allelically excluded without the need of suppression of any further VDJ rearrangement, but might have to be suppressed for secondary Vh replacements (40). Therefore, it cannot be excluded that the machinery involved in Vh replacements should be turned off in these cells, and the VDJh-rearranged loci should become inaccessible for Vh replacements.

A defect in signaling for allelic exclusion should be readily visible as an increase in the number of "double producers" which are immature and mature B cells which deposit IgM with μH chain produced from both alleles on a single cell. If the defect is complete, the VDJh/DJh-rearranged cells should continue to rearrange the second allele. Hence, they should disappear from the repertoire of pre-BII and all subsequent cells. It was already surprising that A5−/− pre-B and B cells had a ratio of VDJ/DJ to VDJ/VDJ cells which was indistinguishable from that in wild-type B cells (19). Furthermore, newly generated B cells of F1 IgM+IgM− mice of wild-type as well as A5−/− mice had <0.5% double producers (27). It should be noted that both wild-type and A5−/− pre-BII and mature B cells contained comparable numbers (between 4 and 8% of all VDJ/VDJ cells) of VDJ+/VDJ− double μH chain-producing cells. However, in these cells it is always the case that only one of

![FIGURE 5. Allelic exclusion of the Igh locus. Bone marrow cells were prepared from control and SL chain-deficient mice heterozygous for IgM and IgM+. Surface expression of the IgM allotypes was analyzed by FACS. B220+IgD− cells were gated and the expression of IgM+ and IgM− was plotted. The percentages of single- and double-expressing cells, as a percentage of total IgM− cells, are shown.](http://www.jimmunol.org/Downloadedfrom/6291)
the two μH chains produced could be deposited on the surface as a pre-BCR (19). This might be expected from the previous finding that although approximately half of all originally produced μH chains can pair with the SL chain, the other half cannot (16). This suggests that nonpairing μH chains which cannot form a pre-BCR are also unable to turn off the rearrangement machinery and close the second allele.

Although it was still conceivable that a partially defective pre-BCR, composed of VpreB and μH chain, might signal allelic exclusion (23), the results obtained from VpreB1−/−VpreB2−/− double-mutant mice now rule out this possibility. These double-B cells still allelically exclude the IgH locus as well as wild-type B cells (26).

Although it has been observed previously that A5 protein cannot bind μH chain in the absence of VpreB protein, as it appears incapable of forming a disulfide-bonded complex (23), the possibility existed that a noncovalent, weak interaction would suffice to form a pre-BCR-like complex with the capacity to signal allelic exclusion. The results reported here for SL chain-deficient mice now rule this out: immature and mature B cells are still allelically excluded in F1 IgM+/IgM− SL chain-deficient mice. Although analysis of the Vμ repertoire in the developing B cells and the contribution of pairing and nonpairing μH chains in these repertoires has yet to be conducted, we conclude from the results presented in this paper that the SL chain, and hence the pre-BCR, is not required for allelic exclusion of the IgH locus.

Allelic inclusion is observed in B cells, in which one IgH allele has been mutated in the transmembrane portion of the μH chain to prevent its membrane deposition (9). Hence, membrane-bound μH chain signals allelic exclusion, but not together with the SL chain. Three other possible partners have been suggested: the heat shock protein 70 chaperon H chain binding protein (41), the 8HS20-protein 70 chaperon H chain binding protein (41), the 8HS20-


2. ten Boekel, E., F. Melchers, and A. G. Rolink. 1995. The status of Ig loci rear-


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