Level of B Cell Antigen Receptor Surface Expression Influences Both Positive and Negative Selection of B Cells During Primary Development

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*J Immunol* 2002; 169:1283-1292; doi: 10.4049/jimmunol.169.3.1283

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Level of B Cell Antigen Receptor Surface Expression Influences Both Positive and Negative Selection of B Cells During Primary Development

Lynn M. Heltemes and Tim Manser

To examine the effect of B cell Ag receptor (BCR) surface density on B cell development, we studied multiple lines of mice containing various copy numbers of an IgHμδ transgene. The V_H gene in this transgene encodes multireactive BCRs with low affinity for self Ags. These BCRs promote differentiation to a B cell subpopulation that shares some, but not all of the properties of marginal zone (MZ) B cells. Surface BCR level was found to be related to transgene gene copy number in these mice. In mice containing 1–15 copies of the transgene, elevated surface BCR levels were correlated with increased numbers of B cells in the MZ-like subset. However, in mice containing 20–30 copies of the transgene, massive clonal deletion of B cells was observed in the bone marrow, few B cells populated the spleen, and B cells were essentially absent from the lymph nodes. These data support the idea that autoantigens mediate not only negative, but positive selection of developing B cells as well. More importantly, they illustrate the profound influence of BCR surface density on the extent to which either of these selective processes take place. The Journal of Immunology, 2002, 169: 1283–1292.

B cell development and tolerance induction occur in a series of distinct steps in the central and peripheral lymphoid organs. The pre-B cell Ag receptor (BCR) and BCR play critical regulatory roles in these processes (1, 2). B cells lacking pre-BCR or BCR expression do not survive in the bone marrow or the periphery (3, 4). Recognition of self Ag by the BCR has been shown to result in death, receptor editing, developmental arrest, or anergy (5). Recent evidence suggests that self ligand engagement by the BCR may also lead to positive clonal selection (6–8). Self-reactive specificities may also influence the B cell subset and microenvironmental locale in which a clone resides (6, 9–11).

Multiple mature B cell subsets have been identified in the mouse periphery. The B cells in each subset are characterized by their size, cell surface phenotype, functional activity, and anatomical location (12, 13). These subsets include B1, marginal zone (MZ), and B2 (follicular) B cells. Two immature transitional B cell subsets have also been defined in the spleen and blood that differ in activity and surface markers (14). A “layered” immune system where each B cell subset has a distinct function has been proposed (15). B1 cells express higher frequencies of self-reactive and polyreactive specificities than B2 cells. Expression of BCR specificities to common self and pathogen structures by B1 cells suggests they are involved in immunoregulation, promotion of damaged or dead cell clearance, and priming of the memory B cell response (16–20). MZ cells appear to be responsible for initial responses to T cell-independent Ags encountered in the general circulation (18). B2 cells are involved in T cell-dependent Ag responses leading to the formation of germinal centers and the memory compartment (21, 22).

The factors influencing differentiation to these distinct B cell subsets have yet to be fully defined. Several groups have hypothesized that B cell subsets are programmed from the stem cell to differentiate along separate lineages. Another hypothesis suggests that the separate lineages are determined by Ag-BCR interactions (11, 23). For example, expression of an antiphosphatidyl choline BCR influences the development of B cells to the B1 subset (23–25). Other studies also support the conclusion that certain autoreactive BCR specificities promote development to the B1 subset (13, 23–27). In addition, some self- or multireactive BCR specificities may influence development to the MZ subset (10). Finally, more recent and limited evidence supports the idea that surface (s)BCR density may also regulate the formation of the B1 and B2 subsets (8, 28).

In A/J mice, immunization with p-azophenylarsonate (Ars) results in the induction of a B cell clonotype that is a minor participant in the primary response but dominates anamnestic responses. A single combination of V_H, D, J_H, V_H, and J_H gene segments forms the BCR of this clonotype and is termed “canonical” (29, 30). Primary B cells expressing canonical Abs are multireactive, binding to both Ars and a variety of self Ags (31). Due to their low precursor frequency, it has been difficult to study the nature of the canonical Ab-expressing cells that are by definition memory precursors. Therefore, we generated transgenic mouse lines expressing a canonical Ig H chain in an attempt to overcome the low precursor frequency.

We have previously studied a single IgHμδ transgenic mouse line of this type with a high transgene copy number. These mice contain a subpopulation of splenic B cells that are functionally and phenotypically distinct from mature B1 and B2 cells. They share some characteristics of the recently described “T2” transitional population (7, 32), although they most closely resemble MZ cells.
These B cells are slgD<sup>hi</sup>-slgM<sup>hi</sup>-CD21<sup>hi</sup>/35<sup>hi</sup>-CD23<sup>lo</sup>- larger in size than “resting” B cells and express elevated levels of CD1d and CD24 (33). They appear to be slowly cycling, have a heightened response to LPS, are hyporesponsive to anti-IgM, and are not short lived. Additionally, they efficiently colonize both the splenic follicles and MZ, but they are inefficient at colonizing the lymph nodes and Peyer’s patches. We have termed this novel B cell subset “MZ/T2”. In light of the recent evidence suggesting that differentiation to the B1 subset may be influenced by BCR surface density (34), we chose to study additional transgenic lines containing various numbers of copies of the canonical Ars I<sub>gs</sub> expression.

Materials and Methods

Mice

Transgenic mice were generated as previously described (33). Two transgene constructs (a μβ construct containing the canonical anti-Ars (36–65) V(D)J gene and an HS1234 construct containing four 3′ α H chain enhancer elements) were injected into fertilized FVB/nJ eggs. Four founder lines were obtained in which three founders (Ars10, Ars20, Ars30) displayed good exclusion of expression of the endogenous IgH locus. The fourth founder line, which appeared to lack the HS1234 construct (Ars37) had poor allelic exclusion and was omitted from the experiments. The mice were maintained by back-crossing to A/J and/or A/JxFVB mice and transgene status was determined by Southern blot analysis. Ars20 mice were bred to J<sub>α</sub>2 knockout mice (a kind gift of Dr. R. Hardy, Fox Chase Cancer Center, Philadelphia, PA). Transgene status for the H chain knockout was determined by PCR as previously described (35). All mice were housed under specific pathogen-free conditions, and given autoclaved food and water. The use of mice in these studies was conducted in compliance with Institute guidelines and all protocols using animals were approved by the Institutional Animal Care and Use Committee.

Southern blot analysis

To isolate genomic DNA, tail tissue was digested with StuI and electrophoresed through a 0.8% agarose gel. Southern blots were performed according to standard procedures. Ten micrograms of DNA was digested at 37°C overnight with StuI and electrophoresed through 0.8% agarose and transferred to nylon membranes (Hybond N⁺; Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization with either an intronic enhancer (IE) region probe or with a J14B probe (just 5′ of the μ switch region) was conducted at 65°C overnight. Hybridization washes were done at high stringency conditions. Some membranes were analyzed using a Typhoon PhosphorImager (Amersham Pharmacia Biotech) and band densities were determined using ImageQuant version 5.2 software (Molecular Dynamics; Amersham Pharmacia Biotech).

Flow cytometry

Single-cell suspensions were prepared from lymphoid organs of 8–20 wk old, naive transgenic, age-matched transgene-negative littermates or A/J mice. Cells were stained with different combinations of the following Abs: α-IgM-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA), α-IgD-biotin and -PE (clone 11-26; Southern Biotechnology Associates, Birmingham, AL), α-IgD<sup>hi</sup>-biotin (clone AF4-73.3), α-IgM<sup>α</sup>-FITC (clone DS-1), α-BP-1-PE (Ly-51, clone 6C5), α-CD14-PE (clone 1B1), α-CD3-biotin and -FITC (clone 145-2C11), α-CD4-FITC (clone H129.19), α-CD8-PE (clone 53-6.7) (eBioscience, San Diego, CA), α-CD19-PE (clone 1D3), α-CD21/35-FITC (clone 7G6), α-CD23-biotin and -PE (clone B3B4), α-CD24-biotin (HSA, clone M1/69 and clone 30-F1), α-CD25-FITC (clone 7D4), α-CD43-biotin (clone S7), α-CD45r-biotin, -FITC, and -PE (B220, clone RA3-6B2) (biotin; eBioscience), α-CD80-biotin (B7-1, clone 16-10A1), or α-idiotype biotinylated Abs 107 and E4. All Abs were purchased from ascites and biotinylated using standard methods. CyChrome (BD Biosciences and BD PharMingen) or R670-Streptavidin (Life Technologies, Rockville, MD) was used as a secondary reagent. Cells were either fixed in 1% paraformaldehyde or analyzed immediately. Cells were assayed on a Coulter Epics Elite and data were analyzed using FLOWJO software (Treestar, San Carlos, CA).

Immunohistochemistry

Spleens from naive mice were frozen and cryosections prepared as previously described (36). Sections were stained with the following reagents: 1) biotin-107 or biotin-E4, branched streptavidin–alkaline phosphatase (DAKO, Glostrup, Denmark) and HRP-peanut agglutinin (Sigma-Aldrich, St. Louis, MO); 2) biotin-CD3 (BD Biosciences and BD Pharmingen) or R670-Streptavidin (Life Technologies, Rockville, MD) was used as a second step reagent. Cells were either fixed in 1% paraformaldehyde or analyzed immediately. Cells were assayed on a Coulter Epics Elite and data were analyzed using FLOWJO software (Treestar, San Carlos, CA).

FIGURE 1. IgH allelic exclusion in the spleen and lymph nodes of the transgenic mouse lines. A. A/J, FVB, and a 50:50 mixture of A/J and FVB spleen cells were labeled with anti-CD45R (B220), anti-IgM<sup>α</sup> (transgene allotype), and anti-IgD<sup>α</sup> (endogenous allotype), and analyzed by flow cytometry. Two gates were drawn, one around a minor double positive subpopulation and another around events that were IgM<sup>α</sup>-<sup>hi</sup>, IgD<sup>α</sup>-<sup>hi</sup>. Numbers next to all gates indicate the percentage of events in that gate relative to all events in the lymphocyte gate. B, Spleen or lymph node cells were isolated, and labeled and analyzed as described above. All populations were gated on B220 expression. Gates were set in the littermate control plots around the major IgM<sup>α</sup>-<sup>hi</sup>, IgD<sup>α</sup>-<sup>hi</sup> populations and then copied onto the plots obtained from the transgenic mice. Values next to each gate indicate the percentage of events in that gate relative to the total number of events in the lymphocyte gate. The data represent at least two separate experiments consisting of three mice (pooled) per experiment.
of donkey anti-mouse IgM (Jackson ImmunoResearch Laboratories). The Abs were detected using the Vector Blue Alkaline Phosphatase Substrate kit III and the Vector NovaRed Substrate kit for peroxidase (Vector Laboratories, Burlingame, CA).

Results

Using canonical μδ H chain and HS1234 3′ enhancer constructs (see Materials and Methods for details), several lines of conventional transgenic mice were generated and back-crossed to A/J mice. To evaluate transgene expression and allelic exclusion in adult mice of these lines, we took advantage of the allelo type differences between A/J mice (Igh<sup>a</sup>) and the Igh<sup>a</sup> allotype encoded by the transgene. An anti-IgD<sup>e</sup> Ab was used to analyze endogenous IgH expression because no anti-IgM<sup>e</sup> Ab is available. Spleen cells from A/J and FVB mice (Igh<sup>e</sup>) were used to test the cross-reactivity of the Abs. As shown in Fig. 1A, there is slight cross-reactivity of the anti-IgD<sup>e</sup> Ab with FVB B cells, as indicated by the fact that ~10% of these cells stain at levels equivalent to A/J B cells. Therefore, a 50:50 mixture of A/J and FVB splenocytes cells was analyzed to determine whether in a mixed population the different allotypes could be distinguished. As shown in Fig. 1A, the right panel shows that the Igh<sup>e</sup> (IgM<sup>e</sup>+, IgD<sup>e</sup>−) and IgH<sup>a</sup> (IgM<sup>a</sup>−, IgD<sup>a</sup>−) populations of cells are distinct. As shown in Fig. 1B, there are negligible numbers (i.e., less than observed in the FVB control samples) of B220 positive cells corresponding to the IgH<sup>e</sup> population in all of the transgenic lines (Fig. 1B). This is true in both the lymph nodes and spleen, which indicates good transgene exclusion of the endogenous locus by all three mouse lines with no obvious differences among the lines.

Influence of transgene copy number on IgM and IgD surface expression levels

To estimate the number of copies of the μδ transgene in each of the transgenic lines, Southern blot analyses were performed. DNA

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Transgene copy number and sBCR expression. A, Tail DNA from Ars10, Ars20, and Ars30 mice was subjected to Southern blot analysis and probed with the IE probe as described in Materials and Methods. A representative image of results obtained from the phosphoimager is presented demonstrating the differences in gene copy number (band intensity) between the transgenic lines. Spleen (B) or bone marrow (C) cells were isolated, pooled, and stained with anti-CD45R(B220), anti-IgM, and anti-IgD. Histogram plots from transgenic mice (open histograms) are overlaid on those obtained from littermate mice (filled histograms) to demonstrate sIg levels on B cells (B220-gated) from the spleen and the bone marrow. B. Mean fluorescence intensities for each distribution in the histograms were calculated and are shown. C. Gates were set over the major IgD<sup>+</sup> peak in each distribution and the percentage of events in those gates relative to the total number of events in the lymphocyte gates are indicated. The data represent at least three separate experiments performed on cells from three mice (pooled) per experiment.
samples were screened using a probe specific for a region starting 700 bp upstream of and including the IE. This region is contained in the transgenic construct. Gene copy number was quantitated by taking the ratio of band intensities between the endogenous and transgenic bands. Although we previously estimated that the Ars20 line contained four to five copies of the transgene by visual inspection of blot autoradiograms (33), the far more accurate phosphoimaging approach revealed that this line has between 10 and 15 copies of the transgene. The Ars30 lines have two to three copies of the transgene, while the Ars10s have one to two copies (Fig. 2A).

To examine the effect of transgene copy number on expressed Ig surface levels, polyclonal anti-IgD and anti-IgM Abs were used to examine surface density of the transgenic BCR in the bone marrow and spleen in the three mouse lines. As can be seen in Fig. 2B, there is an increase in surface expression of IgD in the spleen in all Ars lines when compared with littermates. The most notable increase is in the Ars20 lines with a lesser increase in the Ars30s. This is also apparent in Fig. 1B, where the increased levels of sIgD expression on Ars20 and 30 splenic B cells result in higher levels of staining with the slightly cross-reactive anti-IgD$^\text{E}$ reagent. The Ars10 splenic B cells have a small increase in sIgD expression. In both Ars20s and 30 mice, there is also an increase in sIgM expression in the spleen with the greatest increase in the Ars20s (Fig. 2B). The Ars10 splenic B cells express near normal, or perhaps slightly reduced levels of sIgM. In summary, there are varying levels of BCR expression on splenic B cells among the different lines with Ars10 mice expressing close to normal levels of total sBCR.

As can be seen in Fig. 2C, there is a small population of IgD positive cells in the bone marrow in all the transgenic lines. This increase relative to littermates (Tg-; Tg, transgene) may result from early expression of the IgD portion of the transgene. By analogy to what was found in the spleen, the Ars20 mice have the highest levels of this sIgD$^\text{E}$ population, with lower levels observed in the Ars30s and the Ars10s. In contrast to IgD, bone marrow IgM levels are not elevated in any of the lines and the number of sIgM$^\text{E}$ cells may actually be decreased.

**B cell development in the bone marrow**

We next examined B cell development in the bone marrow of the transgenic mice using three-color flow cytometry and anti-IgM, anti-B220, and anti-CD43 (S7) as markers (1). As illustrated in Fig. 3, expression of the $\mu\delta$ construct does not appear to overtly disturb pro to immature B cell development in the Ars10 and 30 lines. However, the percentage of B220$^+$ cells in the marrow is reduced in the Ars30 line. In Ars20 bone marrow, this reduction in percentage of B220$^+$ cells is also apparent. In addition, there seems to be a perturbation in the early stages of development, as indicated by the single population of B220$^+$, CD43$^{\text{int}}$ cells. These alterations may result from “accelerated development” due to early onset of IgH expression, as documented in other lines of IgH transgenic mice (37).

The markers CD25 (IL-2R$\alpha$) and CD80 (B7.1) were used to further evaluate B cell development in the bone marrow (data not shown). All the transgenic mice revealed increased percentages of CD25$^+$ positive B cells, suggesting decreased development beyond the pre-B stage to the immature stage. The highest numbers of CD25$^{\text{high}}$ B cells were found in the Ars20 mice which also displayed the greatest reduction in immature/recirculating B cells. Increased numbers of bone marrow B cells expressing CD80 were found in all three transgenic lines, suggesting the presence of activated cells. Again, the largest number of CD80-expressing cells were found in the Ars20 mice (~7%). This was followed by the

**Phenotypic differences in peripheral B cell compartments**

We next examined what effect transgene-encoded sBCR levels played in influencing the development of peripheral B cell compartments. Flow cytometric analysis was done on all three transgenic lines to assess the size of the MZ/T2 population, previously observed in the Ars20 mice. As was seen for the immature/mature B cell subpopulation in bone marrow, total B cell numbers in the spleen follow the trend that Ars10 mice have nearly normal levels of splenic B cells, followed by Ars30 mice with a ~20% decrease in B cell numbers and then Ars20 mice with a ~40% decrease in cell numbers (Table I). We also assessed the percentage of splenic B cells that stained with the E4 monoclonal anti-idiotypic Ab, which is highly specific for the “canonical” BCRs that dominate the anti-Ars response in A/J mice (38). These differences in percentages paralleled those of the total B220$^+$ populations. As seen in Fig. 4A, the CD21$^{35\text{high}}$, CD23$^{10\text{low}}$ MZ/T2 population exists in the spleens of all three transgenic lines but is most abundant in the

**Table 1. B cell numbers in the spleen and lymph nodes of Ars transgenic mice**

<table>
<thead>
<tr>
<th>Ars Line</th>
<th>Spleen</th>
<th>Lymph Nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B220$^+$</td>
<td>E4$^+$</td>
</tr>
<tr>
<td>Littermate</td>
<td>59.5$^a$</td>
<td>NA$^a$</td>
</tr>
<tr>
<td>Ars10</td>
<td>59.2</td>
<td>13.5</td>
</tr>
<tr>
<td>Ars20</td>
<td>42.5</td>
<td>8.2</td>
</tr>
<tr>
<td>Ars30</td>
<td>50.7</td>
<td>11.5</td>
</tr>
</tbody>
</table>

$^a$ Numbers represent percentage of lymphocytes.

$^b$ NA, not applicable.
FIGURE 4. Cell surface phenotypic analysis of Ars10, Ars20, and Ars30 B cells in the spleen and lymph nodes. Spleen or lymph node cells were pooled and stained with various fluorescent Abs, as described in Materials and Methods. In these analyses, all data were obtained from the live lymphocyte and B220" gates. A, In the CD21/35 by CD23 plots, the numbers next to the gates represent the percent of B220" cells characterized as CD21/35highCD23low (MZ and MZ/T2). Cross hairs in the IgD by IgM plots are set at the middle of the littermate population to serve as a point of reference when comparing with the data from the transgenic mice. In these plots, quadrant statistics are presented as percentages of B220" cells. In the CD1d by CD24 plots, gates are drawn around the major subpopulations of CD1dhigh, CD24high cells, and numbers next to these gates are percentages relative to B220" cells. These data are representative of three separate experiments using three mice per group.

B Spleen J H Knockouts

C Lymph Nodes

These data are representative of three separate experiments using three mice per group.
The Ars10 line has a ∼2-fold increase in the amount of MZ/T2 cells in the spleen when compared with littermates. The Ars20 mice demonstrate a 6-fold increase in the MZ/T2 cells with an ∼3-fold increase for the Ars30s.

Further examination of splenic B cells demonstrated that the majority of these cells are IgD\textsuperscript{high} IgM\textsuperscript{high} in the Ars20 and 30 mice. In the Ars10 mice only sIgD levels appear elevated on splenic B cells (Fig. 4A). To further examine the surface phenotype of splenic B cells, the CD21 and CD24 (HSA) markers were used. In Fig. 4A, the HSA by CD1d plots show subpopulations of B220\textsuperscript{+} cells in all the transgenic lines that express elevated levels of both these markers. The Ars20 mice have the largest increase in HSA\textsuperscript{high}CD1d\textsuperscript{high} cells when compared with the littermates (8-fold), while the Ars10 and Ars30 lines display 2- to 3-fold increases. Examination of CD5 and CD43 expression by splenic B cells in the lines revealed negligible levels, indicating that the MZ/T2 population is distinct from B1 B cells (data not shown). In total, the results of the analysis of the splenic B cell compartment parallel those obtained from the bone marrow in revealing the greatest deviation in B cell development and B cell numbers as compared with littermates in the Ars20 line, followed by the Ars30 line and then the Ars10 line.

Despite our evidence for good allelic exclusion in these three transgenic lines (Fig. 1B), we considered the possibility that low levels of endogenous IgH expression might be influencing peripheral development to the MZ/T2 phenotype. To investigate this question, Ars20 transgenic mice were crossed to JH knockout mice to preclude an influence of the endogenous IgH locus. As can be seen in Fig. 4B, the size of the CD21/33\textsuperscript{high}, CD23\textsuperscript{low} MZ/T2 populations are similar in the Ars20 and the Ars20/J\textsuperscript{H−/−} mice.

Immunohistochemical analysis of lymphoid microarchitecture and B cell local in the spleens of the transgenic mice showed normal compartmentalization and subcompartmentalization of B and T cell regions of the white pulp, and that transgene and canonical BCR expressing B cells were present in all B cell areas (Fig. 5, \textit{top three rows}). In these studies, the anti-idiotypic mAbs 107 and E4 were used. 107 is specific for the transgenic V\textsubscript{H} in combination with a variety of L chains (33). No apparent expansion of the MZ, or enrichment of transgene encoded or canonical BCR expressing B cells in this locale was observed in any of the lines of mice.

Similar to the results obtained from spleen, a substantial decrease in lymph node B220\textsuperscript{+} B cell numbers is seen in the Ars20 line (Table I). The Ars10 and 30 lines display reduction of lymph node B cells that is less severe. Evaluation of E4\textsuperscript{+} B cells in lymph nodes revealed reductions that paralleled those of B220\textsuperscript{+} cells. Moreover, Fig. 4C shows that the MZ/T2 population, as defined by expression of elevated levels of CD21 and CD1d, and low levels of CD23 is absent in the lymph nodes of all three lines. Nonetheless, elevated sIgM levels are seen in the lymph nodes of all the mice, with the largest increase in the Ars20 line. sIgD levels are near normal in all lines of mice in the lymph node (data not shown). Finally, CD24 (HSA) levels are slightly elevated on lymph node B cells in the transgenic lines when compared with littermates, suggesting that these cells may not be completely “mature” (Fig. 4C).

Further increases in transgene copy number result in dramatic B cell loss in homozygous Ars20 transgenic mice

To evaluate the influence of further increases in sBCR levels on B cell development, Ars20 mice were intercrossed to create a homozygous subline called AX20, containing 20–30 copies of the transgene. To distinguish heterozygous mice from homozygous mice, Southern blots of tail DNA were analyzed by phosphoimager. The band density numbers were determined as compared with the endogenous band and then a ratio was used to determine the status of the mice. Fig. 6A illustrates the Ig band intensity differences between the Ars20 and the AX20 mice.

In the bone marrow, the number of B220\textsuperscript{+} B cells in AX20 mice is drastically reduced when compared with the Ars20 mice. Three-color flow cytometric analysis using anti-IgM, anti-CD43 (S7), and anti-B220 revealed a slight perturbation in the pro/pre-B1 to pre-BII transition, similar to that observed in Ars20 heterozygous mice (Table III). More strikingly, immature and mature B cells

![CD3 / IgM](image_url)

![107 / PNA](image_url)

![E4 / PNA](image_url)

**FIGURE 5.** Splenic lymphoid microarchitecture and location of transgene-expressing B cells. Three adjacent spleen sections from Ars10, Ars20, Ars30, and AX20 mice were stained with anti-CD3 and anti-IgM, 107 and PNA, and E4 and PNA as described in Materials and Methods. In the first column, the CD3\textsuperscript{+} cells appear blue and the IgM\textsuperscript{+} cells appear orange. In columns two and three, PNA staining appears orange and 107 and E4 staining appear blue. The bright orange cells outside of the white pulp areas in some images are red pulp macrophages that stain due to endogenous peroxidase activity. Original magnification of images was ×100. These data are representative of those obtained from several spleens from each transgenic line.
were almost absent from the marrow of AX20 mice. This decrease in immature and mature B cells was accompanied by the appearance of a population of larger and more granular B220/H11001 cells (Fig. 6B). This population is the dominant population in the bone marrow of AX20 mice. In the Ars20 mice, only a small subpopulation of slighter more granular cells are present as compared with littermates.

Due to these observations, further characterization of the bone marrow, spleen, and lymph node cells in AX20 mice was done. The extreme decrease in B cell numbers in these mice is also evident in the spleen and lymph node (Table III, Fig. 6C) with very few, if any, B cells detectable in the lymph nodes. IgM and IgD levels were examined on the small number of B cells in the bone marrow, spleen, and lymph nodes. In the bone marrow, B cells appear to express elevated levels of IgD but only very low levels of IgM. The same pattern is seen in the spleen (Fig. 6C). There may be a few IgDhigh, IgMhigh cells in the AX20 spleen, but such cells are absent in the lymph node. Table III quantitatively illustrates the dramatic reduction in B cell numbers in the periphery of AX20 mice as compared with Ars20 mice.

Immunohistochemical analysis of AX20 spleens showed normal populations of T cells in the white pulp parietoportal lymphoid sheath surrounded by a thin layer of B cells expressing transgene-encoded BCRs (Fig. 5, bottom row). Nothing resembling mature B cell follicles was observed in the AX20 spleens. Few, if any, E4+/H11001 canonical BCR-expressing B cells were observed in AX20 spleens. Comparison of the data on AX20 splenic B cells shown in Fig. 5 and Fig. 6, B and C, reveals an apparent discrepancy in B cell number. This may have resulted from many of these B cells being excluded from the viable lymphocyte gate in the flow cytometric analysis. However, we also observed a discrepancy between the numbers of B220+/H11001 and IgM+/H11001 cells as evaluated by histology of spleen sections (data not shown). Future studies will be required to determine whether most AX20 splenic B cells are short lived, perhaps due to activation-induced death, and whether B220 expression is lost before sIgH expression.

T cell development, numbers, and microenvironmental locale appear normal in all of the IgH transgenic lines

The splenic T cell compartment was analyzed by flow cytometry and immunohistochemistry to determine what effect increased IgH transgene expression and accompanied alterations in B cell numbers and development had on this compartment. T cell numbers, CD4, and CD8 subset ratios and the size and organization of splenic T cell zones in the Ars10, Ars20, and Ars30 transgenic mice were comparable to littermates (Fig. 5 and data not shown). The AX20 T cell compartment also appeared to be fairly normal with T cell numbers and CD4 to CD8 ratios similar to those observed in the Ars20 mice (Fig. 5 and data not shown). These histology data demonstrate that despite massive B cell loss in AX20 mice, the T cell compartment appears unaffected.

Table II. B cell development in the bone marrow of homozygous (AX20) and heterozygous Ars 20 mice

<table>
<thead>
<tr>
<th></th>
<th>B220+</th>
<th>Pre/PreB</th>
<th>Large</th>
<th>Small</th>
<th>Immature</th>
<th>Recirculating</th>
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<td>Littermate</td>
<td>54.5+</td>
<td>6.3</td>
<td>4.0</td>
<td>16.5</td>
<td>13.3</td>
<td>3.8</td>
</tr>
<tr>
<td>Ars20</td>
<td>23.8</td>
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<td>4.2</td>
<td>5.0</td>
<td>1.6</td>
</tr>
<tr>
<td>AX20</td>
<td>8.4</td>
<td>3.7</td>
<td>1.3</td>
<td>1.5</td>
<td>0.1</td>
<td>0.007</td>
</tr>
</tbody>
</table>

* B220+IgM+/CD43+.
* B220+IgM+/CD43+-.
* B220-IgM-.
* B220IgMhigh.
* Numbers represent percentage of lymphocytes.
Discussion

Evidence from other transgenic mouse models has indicated that BCR specificity influences development to the MZ and B1 subpopulations (6, 8, 10, 11, 23, 28). The Ars10 mice, with near normal levels of sBCR expression, allowed us to focus on the influence of BCR specificity on B cell development to the MZ/T2 subset, previously discovered in the high copy number Ars20 mice. Ars10 mice have a substantially increased number of MZ/T2 splenic B cells compared with littermate controls. This reinforces the idea that differentiation to the MZ/T2 subset is influenced by BCR specificity. However, the most significant conclusion of our studies is that sBCR level strongly influences degree of B cell development in general and development to the MZ/T2 subset in particular. Recent reports have indicated that BCR density may play a role in development to other B cell subsets (8, 28, 39, 40). The Ars30 mice, with two to three copies of the transgene, have somewhat elevated BCR levels (2- to 3-fold) that correlate with a slightly increased representation of the MZ/T2 subset relative to the Ars10 line, and reductions in total and canonical B cell numbers in the bone marrow, spleen, and lymph nodes. In Ars20 transgenic mice, with 10–15 copies of the transgene, we observed the highest sBCR levels (5-fold increased) and further increases in number of MZ/T2 cells and reductions in bone marrow and peripheral B cell numbers. In total, these results suggest that the amount of self ligand that can be engaged by a given B cell is directly related to the level of negative selection and the extent of promotion of development to the MZ/T2 subset.

Previous data on hybridomas obtained from polyclonally activated Ars20 splenic B cells showed that the majority of these produce low-affinity anti-DNA mAbs, with half also producing mAbs that are reactive with arsonate (33). Several of these mAbs were also identified that did not bind DNA or arsonate, but when two were tested in an antinuclear Ab assay, they were found to be weakly reactive with cytoplasmic structures. A diverse V_{H} L chain repertoire was collectively expressed by these preimmune Ars20 hybridomas, but each hybridoma appeared to express only one endogenous L chain gene. The particular L chain coexpressed with the transgenic H chain in a given B cell could influence both levels of sBCR expression, due to differences in V_{H}V_{L} pairing efficiency, and degree of self-reactivity. Because we have not characterized the L chain repertoires in the Ars10, 30, and AX20 lines, it is possible that differences among these repertoires influenced our results. However, the correlation between total sBCR levels and representation of the MZ/T2 subset (Ars10, 20, and 30 lines) and the reduction in numbers of splenic and bone marrow B cells (all lines) suggests that such influences were minor in these two compartments. Therefore, it seems that BCR self-reactivity is predominantly determined by the canonical transgenic H chain.

Recent reports suggest that autoreactive Ig transgenic B cells may survive as a result of inefficient allelic exclusion leading to transgenic Ag receptor dilution or dual receptor expression (34, 41, 42). By studying transgene-encoded BCR expression and B cell development in an Ars20 subline lacking a functional endogenous H chain locus, we were able to demonstrate continued predominance of the MZ/T2 B cell subset in the spleen. Furthermore, there was no apparent influence of lack of a functional endogenous IgH locus on B cell development in the bone marrow (data not shown). This would suggest that survival and development of these multi-reactive cells is not a result of transgenic Ag receptor dilution. Moreover, because Ars20 hybridomas each appear to express only one functional L chain, reduction of autoreactive Ag receptor avidity due to expression of multiple L chains also seems unlikely.

Many Ig transgenic mouse models expressing self-reactive specificities have revealed that autoantigen engagement via the BCR results in tolerance induction (5). In the bone marrow of the Ars10 mice, despite predominant self-reactive BCR expression, no overt alterations in B cell development were identified. However, as BCR density increased in Ars20 and Ars30 mice, there was a corresponding decrease in B cell numbers at the immature stages of development. This is consistent with the induction of tolerance via autoantigen-induced clonal deletion (43–47). In homozygous AX20 transgenic mice, B cells develop fairly normally until the pre-B to immature stage, at which point most are lost. Deletion is commonly seen among developing B cells expressing high-affinity autoantibodies, yet the BCR autospecificity encoded by the canonical H chain are, in general, of low affinity. Therefore, even low-affinity autoreactive BCRs expressed at high enough levels result in central tolerance via clonal deletion. Interestingly, we noted that many bone marrow B cells in the Ars mice, particularly the Ars20 line, displayed elevated levels of expression of CD80 and some had increased granularity. In AX20 bone marrow, the majority of B cells are enlarged and also have increased intracellular granularity. Therefore, it is possible that the central deletion taking place in these lines results from activation-induced cell death. It has been suggested that tolerance induction requires multiple antigenic encounters (48). This would be consistent with our data in suggesting that tolerance acts as a function of the total amount of autoantigen engaged.

The phenotype of the B cells in the lymph nodes and peritoneal cavity (data not shown) of Ars10, Ars20, and Ars30 mice is relatively normal in comparison to the situation in the spleen. Total sBCR levels are still raised on lymph node B cells in the Ars20 mice and to some extent in the Ars30 mice, but there is no elevation of CD1d expression on lymph node B cells in any of the transgenic lines. Additionally, lymph node B cells express normal levels of CD21/35 and CD23, maintain good allelic exclusion, and uniformly express transgene-encoded BCRs. Furthermore, experiments on the Ars20/JH knockout line demonstrated no phenotypic differences among lymph node B cells as compared with Ars20 mice (data not shown). Therefore, B cells exclusively expressing the transgenic H chain can colonize the lymph nodes and acquire what appears to be largely a follicular (B2) phenotype. In

### Table III. B cell numbers in homozygous (AX20) and heterozygous Ars20 mice

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>% Lymphocytes (% B220^+)</th>
<th>Lymphocytes</th>
<th>% Lymphocytes (% B220^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Littermate</td>
<td>62.9 (97.3) 60.6 (96.6)</td>
<td>64.2 (97.3)</td>
<td>45.6 (96.6) 39.3 (96.9)</td>
</tr>
<tr>
<td>Ars20</td>
<td>52.6 (95.6) 34.5 (93.6)</td>
<td>61.1 (96.9)</td>
<td>13.6 (96.4)</td>
</tr>
<tr>
<td>AX20</td>
<td>46.4 (91.5) 2.5 (95.6)</td>
<td>57.4 (98.6)</td>
<td>0.1 (98.1)</td>
</tr>
</tbody>
</table>

* NC. Not enough cells to calculate.
regard, it should be noted that an analogous population of B cells is present to various extents in the spleens of Ars10, 20, and 30 mice.

However, the Ars20 mice have a large reduction in lymph node B cell numbers. In the Ars10 and 30 lines, lymph node B cell numbers are also reduced as compared with the spleen. These data suggest that like splenic B cell differentiation, transgene-encoded BCR surface density influences lymph node B cell differentiation and that tolerance “checkpoints” may be operative in the periphery. The action of such checkpoints may preclude access of B cells with high autoantigen avidity from colonizing the lymph nodes and perhaps the splenic “follicular” compartment as well. Moreover, because Ars20 lymph node B cells express elevated sBCR levels, but exhibit a follicular phenotype, it is possible that the BCRs they express have reduced or distinct autoreactivity as compared with their splenic MZ/T2 counterparts. We are currently investigating whether this is the case and if so, whether the expression of particular L chain variable regions is responsible.

In total, our data support a model invoking a hierarchical regulatory role for self Ag avidity at all stages of primary B cell differentiation. This regulation is first affected at the immature stage of development in the bone marrow, where high avidity B cell clones are deleted, perhaps via an activation-induced death pathway. This is exemplified most dramatically by the AX20 mice, in which the antibody conferred to the majority of developing clones is largely due to the high level of transgenic H chain expression. When avidity is reduced due to lower levels of transgene expression, B cell loss in the bone marrow is reduced in a commensurate fashion, as seen in the Ars20, 30, and 10 lines. Less autoreactive clones then populate the spleen, but their differentiative fate is strongly influenced by their level of avidity for self ligands. In the Ars10, 20, and 30 mice, this level of avidity translates to degree of differentiation to the MZ/T2 subcompartment. The process continues as B cells attempt to populate the lymph nodes. Apparently, those with the MZ/T2 phenotype are precluded from entering this pathway, resulting in a substantial reduction in lymph node B cell numbers in mice in which a major fraction of splenic B cells are resident in the MZ/T2 subset. From a practical perspective, our data underscore a major caveat in the use of conventional IgH transgenic mice for the study of B cell positive and negative selection and differentiation. This regulation is a conserved checkpoint focuses V_{H}12 B cell differentiation toward a single B-1 cell specificity. BCRs with low avidity are deleted, perhaps via an activation-induced death pathway.

Whether the MZ/T2 state of differentiation represents “arrested development” or “abortive activation” as has been invoked to explain the phenotypic and functional state of autoreactive B cells in other transgenic systems (5, 47, 49–52) remains to be completely investigated. Our previous studies on Ars20 splenic B cells revealed that most were not short lived. Nonetheless, Ars20 splenic B cells do not efficiently give rise to Ab-forming cells either in vitro or in vivo. Interestingly, this is also a property of primary canonical clonotypes in nontransgenic A/J mice. We previously speculated that this behavior was due to chronic self-ligand engagement, precluding the receipt of the high levels of CD4 T cell help required to drive AFC differentiation. In addition, we suggested that this situation might promote entry into the germinal center/memory pathway by preventing terminal differentiation of the cells. Because the transgene used to construct the Ars10, 20, and 30 lines is incapable of normal class switching and perhaps V_{H} gene somatic hypermutation rigorously testing these ideas will require the construction of new lines of mice in which a fully functional canonical V_{H} gene is introduced directly into the endogenous Igh locus.

References


