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Development and Selection of Edited B Cells in B6.56R Mice

Debora R. Sekiguchi,* Lenka Yunk,* David Gary,† Deepshikha Charan,* David Allman,* Martin G. Weigert,† and Eline T. Luning Prak*‡

Tolerance to dsDNA is broken in mice with a high-affinity anti-DNA H chain transgene, 56R, on the C57BL/6 background (B6.56R). B6.56R produce more anti-dsDNA Abs than BALBc.56R. To investigate how anti-DNA Abs are regulated on the B6 background, phenotypic and genetic studies were performed. B6.56R have reduced numbers of B cells and phenotypically altered B cell subsets, including relative increases in the proportions of IgM-negative bone marrow B cells, cells with a marginal zone phenotype, and cells with a transitional T3 phenotype. The peripheral B cell repertoire in B6.56R is restricted: most B cells express the 56R H chain and use a similar, limited subset of editor L chains. DNA binding is more common in B6.56R because the repertoire is shifted toward L chains that are more permissive for DNA binding. H chain editing is also observed and is increased in spontaneous as compared with LPS hybridomas. A subset of spontaneous hybridomas appears to lack H chain expression. The Journal of Immunology, 2006, 176: 6879–6887.

Autoantibodies are frequently generated at random during V(D)J recombination, but B cells that make autoantibodies are tightly regulated. Mice with transgenes encoding autoantibodies have yielded insights into several tolerance mechanisms including receptor editing, clonal deletion, anergy, follicular (Fo)3 exclusion, and ignorance (1–8). The 56R anti-dsDNA H chain knockin mouse is a useful model for studying how tolerance is maintained or broken to a ubiquitous self-Ag, DNA (9, 10). The 56R transgene creates anti-DNA Abs when paired with nearly all endogenous L chains (10). Only a handful of L chains, termed editors, allow B cells that express the 56R H chain to survive negative selection in the bone marrow. The 56R model is also useful because the H chain transgene resides in the H chain locus and can be subjected to additional rearrangements (such as VH replacement) and somatic mutation, permitting the study of central and peripheral mechanisms that alter (auto)antibody specificity.

Recently, the 56R mouse has been crossed onto various autoimmune-prone mouse models and is being used for B cell tolerance studies (11, 12). For example, in a previously published study of chronic graft vs host disease in B6.56R mice, anti-dsDNA Ab production was attributed to graft-vs-host disease (GVHD); however, B6.56R mice without GVHD also produced dsDNA Abs (12). The C57BL/6 background (hereafter B6) is known to be more susceptible to some manifestations of autoimmunity (13–17). Furthermore, the analysis of congenic mice and interval mapping studies has revealed autoimmune phenotypes in the context of the B6 background (18, 19). Collectively, these and our own studies prompted us to assess the autoimmune potential of the B6.56R mouse. To determine the influence of the B6 background on autoantibody production, we analyzed B cell development, phenotype, and Ab repertoire in B6.56R mice. This study, as well as the recent work from the Mohan laboratory (C. Mohan, unpublished observation), reveals significant phenotypic and genotypic changes in B6.56R mice and yields new insights into the selection of edited B cells in B6.56R.

The detailed characterization of B6.56R confirms that they produce anti-dsDNA Abs, have reduced numbers of B cells, exhibit reproducibly altered patterns in central and peripheral B cell subsets, and have a restricted repertoire of edited B cells. Analysis of three different hybridoma panels from B6.56R mice reveals that most B6.56R B cells retain the H chain transgene and use the same L chain editors as BALBc.56R mice, but they use these L chains in different frequencies. The altered frequencies of L chain editor usage correlate with increased DNA binding in B6.56R. Furthermore, H chain editing usually results in the abrogation of DNA binding. B6.56R, by virtue of its simplified and well-characterized repertoire, will hopefully serve as a valuable model for analyzing and tracking self- and nonself-reactive B cells in vivo.

Materials and Methods

Mice

The generation of 3H9 and 56R site-directed transgenic (sd-tg) mice has been described previously (20). 56R and 3H9 sd-tg mice, backcrossed onto the C57BL/6 background for eight generations, were produced in the laboratory of M. G. Weigert and were provided to us by R. Eisenberg (University of Pennsylvania, Philadelphia, PA). B6.56R mice were backcrossed for an additional two generations in our laboratory. The presence of the 3H9 or 56R sd-tg was determined by PCR amplification of tail DNA (6). Mice used for these experiments were bred and maintained in our mouse colony at the University of Pennsylvania Medical Center under an Institutional Animal Care and Use Committee-approved protocol.

Detection of autoantibodies in sera ELISA

Expression of IgM and IgG anti-dsDNA Abs was determined via a solid-phase ELISA as described previously (12). Serum from a diseased MRL/lpr mouse with high titer autoantibodies (provided by M. Monestier, Temple University, Philadelphia, PA) was used as a standard. DNA binding was measured at a fixed dilution of serum (1/500) for all samples including the MRL/lpr sample. All serum samples were analyzed on the same day in the same assay in duplicate.

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Flow cytometry

Cell suspensions in “FACS buffer” (Dulbecco’s PBS with 5% FBS) were prepared from the femurs, tibias, and spleens of B6, B6.56R, and B6.3H9 mice. RBC were subjected to hypotonic lysis using ACK reagent (BioSource International; 30 s incubation on ice, followed by prompt neutralization with FACS buffer and centrifugation). A total of 1 × 10^6 cells were stained with an Ab mixture in total volume of 40 μl per tube for both the primary and secondary incubations. Abs included sIgM^* FITC (clone DS-1; BD Pharmingen), sIgM^* PE (clone AF6-78; BD Pharmingen), B220-allophycocyanin Cy5 (clone 6B2, conjugated in the laboratory of D. Allman), AA4.1-allophycocyanin (conjugated in the laboratory of D. Allman), and CD43-biotin (clone S7; BD Pharmingen) revealed with neutravidin-conjugated Cascade Blue (Molecular Probes). Flow cytometry was performed on an LSRII (BD Pharmingen) and the data were analyzed with FlowJo software.

Hybridomas

LPS hybridomas were generated as described previously (21). Spontaneous hybridomas were fused in polyethylene glycol to Sp2/Ag8 myeloma cells (22) at a ratio of 1:5.1 and plated after overnight culture at 1 × 10^5 and 5 × 10^5 cells/well in 96-well flat-bottom plates and selected for 10 days in TGA TAA TGA GCC CTC TC-3

To estimate the likelihood of obtaining 19 or more successes in 43 trials was the likelihood of obtaining 19 or more successes in 43 trials was <1 × 10^{-6}. A two-tailed Student’s t test was performed to evaluate the significance of differences between the average serum IgG or IgM anti-dsDNA reading.

Results

B6.56R mice produce anti-dsDNA Abs

B6.56R mice spontaneously produce anti-dsDNA Abs (11, 12), in contrast to what has been reported previously in BALB/c.65R (10). To determine whether the difference in serum anti-DNA production is due to the B6 background, sera from BALB/c, B6, BALB/c.56R, B6.56R, and B6.3H9 were tested in parallel for dsDNA binding activity using a solid-phase ELISA. B6.56R mice exhibited significantly elevated levels of IgM and IgG anti-dsDNA Abs, when compared with wild-type controls and nontransgenic littersmates (p < 0.001, Fig. 1). IgM anti-dsDNA Ab levels were increased from a background reading of under 0.25 OD units to an average value of over 1.5 in B6.56R (Fig. 1a). IgG anti-dsDNA Ab levels increased from a background level of under 0.1 OD units in B6 and BALB/c controls to an average value of 0.15 in B6.56R (Fig. 1b). BALB/c.56R mice also exhibited increased levels of IgM

Figure 1. Anti-dsDNA serology

Sera were obtained from BALB/c (n = 7), B6 (n = 26), BALB/c.56R (n = 8), B6.56R (n = 20), B6.3H9 (n = 4), and MRL/lpr (n = 2) mice that ranged in age from 3 wk to 11 mo. Average OD values (of duplicate readings) for individual mice are indicated with dashes (each dash represents a single mouse). Vertical lines represent the confidence interval for each OD. *, p < 0.01, compared with B6 × two-tailed Student’s t test; **, p < 0.001 as compared with B6 × two-tailed Student’s t test. IgG anti-dsDNA Ab OD values were 0.93 for MRL/lpr (n = 2) and 3.02 for MRL/lpr.56R (n = 3). All ELISA measurements shown as well as the MRL/lpr controls (data not shown) were performed in the same assay at the same dilution (1/500) on the same day.
Negative selection of anti-DNA B cells in the bone marrow

To gain further insight into how anti-DNA B cells develop and are regulated on the B6 background, flow cytometry was performed on bone marrow from B6.56R, B6.3H9, and nontransgenic (B6) controls. Fig. 2 shows flow cytometric profiles from a representative mouse for each genotype. The data shown come from a single flow cytometry experiment in which the staining and analysis conditions were uniform. Similar results have been obtained in separate flow cytometry experiments that collectively have analyzed a total of eight B6.56R mice, three B6.3H9, and eight nontransgenic littermate or wild-type B6 mice (data not shown). The mice used in the flow cytometry experiments shown ranged in age from 3 to 5 mo. Mice that fall outside of this age range have not been studied in adequate numbers to fully evaluate the effects of aging on B cell numbers and phenotype.

The gating strategy for dividing developing B cells into Hardy fractions D (L chain rearrangement), E (immature IgM+), and F (IgM+) is shown for three mice in Fig. 2a. B cell numbers found in the different fractions are summarized in Table I. Three-month-old B6.56R mice have ∼3- to 5-fold fewer B220− lymphocytes in the bone marrow than B6 mice. Part of this decrease is due to the overall decrease in bone marrow cellularity found in B6.56R and B6.3H9 compared with nontransgenic B6 mice. As B cell maturation proceeds, the B6.56R B cells appear to fall further behind, and then for B220−CD43− cells, which include Hardy fractions D, E, and F. Resolution of these fractions is achieved by assessing AA4 (CD93) and surface IgM (sIgM) levels, as shown. Fraction D is sIgM−AA4high, fraction E is sIgM−AA4low, and fraction F is sIgM−AA4− (55). Representative plots are shown in a. Transitional B cell subsets in spleens of anti-DNA mice. Plots were gated for lymphocytes based on forward vs side scatter profiles. Transitional B cells were identified based on B220 and AA4.1 expression (left plots). Transitional subsets, using the Allman classification scheme (20), were analyzed based on CD23 and IgM expression (right plots). Representative plots from the same mice described in the a are shown. c, Splenic B cell subsets in B6, B6.3H9, and B6.56R mice. Plots were gated for lymphocytes based on forward vs side scatter profiles and on mature B cells (AA4−B220−). B cells were further subdivided into FoB and MZB fractions based on CD23 vs CD21/35 staining. The FoB and MZB fractions were further analyzed for H chain allotype usage. IgMh is the allotype of the knockin H chain allele and IgMc is the allotype of the endogenous B6 allele. Representative plots from the same mice described in the Fig. 2a legend are shown. d, Numbers of B cells in different splenic subsets. B cell numbers (in millions) are shown for B6 (n = 3 56R, B6.3H9, and B6.56R (IgM−negative littermates), B6.56R (n = 4) and B6.3H9 (n = 2) mice. Cell counts were obtained by multiplying the number of viable splenocytes in each mouse by the fraction of B220− lymphocytes (B ly). Transitional cell numbers (Tr) were obtained by multiplying the B cell number by the fraction of AA4+ positive cells. The number of marginal zone (MZ) cells was approximated by multiplying the B cell number by the fraction of IgMh, CD21low, and CD23high cells. The number of Fo B cells (Fo) was estimated by multiplying the B cell number by the fraction of IgM−, Cd21high cells. Error bars represent a fixed percentage that corresponds to one SD of the estimated B cell number for each age range. The degree of variability may be higher as these estimates do not account for variability in the flow cytometry data acquisition or analysis.
dropping below 10% of wild-type B cell numbers. There is also a shift in the proportions of B cells found in fractions D, E, and F, with fewer cells in fraction E in B6.56R. The decreased representation of fraction E is also seen in B6.3H9, but to a lesser degree (12 vs 7%, Fig. 2a). Finally, it appears that most of the cells in fraction F in B6.56R and B6.3H9 exhibit a lower intensity of IgM staining compared with B6. The IgM reagent that was used in this experiment specifically recognized the IgM<sup>a</sup> allotype (which corresponds to the transgenic allele). In 3- to 5-mo-old mice, ~10% of IgM<sup>a</sup> B cells in the bone marrow expressed the IgM<sup>a</sup> allotype (data not shown). In an 11-mo-old mouse the fraction of IgM<sup>a</sup> cells increased to ~25% (data not shown). Determining whether increased frequencies of H chain-edited B cells are characteristic of older B6.56R mice will require further analysis of more old mice. Overall, the presence of fewer bone marrow B cells in B6.56R than in nontransgenic littermates favors models in which the 56R H chain and have a tendency to bind dsDNA (see hybridoma analysis below). Consistent with this possibility, the increase in IgM<sup>b</sup> cells was less pronounced in the lower affinity 3H9 mouse than in the 56R mouse (Fig. 2c). Also consistent with negative regulation of the 56R transgene, the fluorescence intensity of IgM<sup>b</sup> was lower in B6.56R than in B6.3H9. In summary, both B6.56R and B6.3H9 mice exhibit alterations in their peripheral B cell subsets. To determine whether there are significant and reproducible differences between B6.56R and B6.3H9 peripheral B cell subsets and phenotypes will require a more detailed analysis than the one shown here. To further evaluate how anti-dsDNA Abs are created in B6.56R mice, hybridomas were produced and subjected to genotypic and phenotypic analysis.

### Altersations in peripheral B cell subsets in B6.56R

Flow cytometry was also performed to evaluate the composition of the peripheral B cell compartment in B6.56R. Gating strategies and the analysis of transitional and mature cell subsets are shown in Fig. 2, b and c, respectively. Splenic B cell numbers are decreased in transitional cells (here defined as B220<sup>+</sup>F4-80<sup>−</sup>CD23<sup>−</sup>) lymphocytes) in B6.56R. When transitional B cells are further subdivided according to the scheme of Allman and Hardy (25), there appears to be a progressive increase in the relative number of transitional cells with a T3 phenotype and a corresponding decrease in the relative number of cells with a T2 phenotype (as one surveys B6, B6.3H9, and finally B6.56R mice (Fig. 2b). Among the more mature B cell subsets, FcO cells are decreased while cells with a marginal zone (MZ) phenotype are increased in relative and absolute numbers in B6.56R (Fig. 2, c and d). These findings are consistent with the expanded MZ population reported in BALBc.56R mice (10, 26). Of note, a similar shift in the relative numbers of FcO and MZ cells, though possibly to a lesser degree, is observed in B6.3H9 (Fig. 2, c and d).

To determine whether the alterations in Fo and MZ-like cells in B6.56R are related to the autoreactive potential of the H chain, we analyzed these cells for H chain allele usage (Fig. 2c). The targeted allele expresses the IgM<sup>a</sup> allotype and untargeted B6 allele expresses the IgM<sup>b</sup> allotype. Most splenic B cells in B6.56R express IgM<sup>a</sup>. The presence of IgM<sup>b</sup> among peripheral B cell subsets could be due to negative regulation of cells that express the 56R H chain and have a tendency to bind dsDNA (see hybridoma analysis below). Consistent with this possibility, the increase in IgM<sup>b</sup> cells was less pronounced in the lower affinity 3H9 mouse than in the 56R mouse (Fig. 2c). Also consistent with negative regulation of the 56R transgene, the fluorescence intensity of IgM<sup>b</sup> was lower in B6.56R than in B6.3H9. In summary, both B6.56R and B6.3H9 mice exhibit alterations in their peripheral B cell subsets. To determine whether there are significant and reproducible differences between B6.56R and B6.3H9 peripheral B cell subsets and phenotypes will require a more detailed analysis than the one shown here. To further evaluate how anti-dsDNA Abs are created in B6.56R mice, hybridomas were produced and subjected to genotypic and phenotypic analysis.

### Production of LPS and spontaneous B6.56R splenic hybridomas

Three splenic hybridoma panels were produced and analyzed for this study: an LPS panel from a 4-mo-old B6.56R female (LPS; n = 92 clones of which 5 expressed IgG and the remainder IgM), an LPS panel from an 8-mo-old B6.56R female (“LPS old”; n = 48 clones of which three expressed IgG and the remainder IgM), and a spontaneous panel from the same 4-mo-old B6.56R mouse as the 4-mo LPS panel (n = 58 clones). The spontaneous panel was divided into two panels, named “Spont” (n = 32 clones) and “Spont NS” (n = 26 clones).

The Spont NS (nonsecretor (NS)) hybridomas were striking in that none had retained the 56R transgene DNA and none manifested serologic evidence of an IgG or IgM Ab. To further investigate the nature of these clones, we analyzed the supernatants in ELISAs for κ and λ L chain secretion by coating the plates with anti-Ig total (H plus L), applying hybridoma supernatant and interrogating bound Ab with anti-κ or anti-λ. Using this assay, 12 clones were positive for κ and two were positive for λ. This observation was corroborated by PCR genotyping for L chain rearrangements (L. Yunk, D. Charan, and E. T. Luning Prak, unpublished data). Thus, the majority of these clones appear to be B cells. To determine which H chains were used by these κ- or λ-secretory clones, supernatants were screened for the expression of IgA and IgE. One clone secreted IgE. None of the 14 clones expressed IgA. All Spont NS hybridomas were tested for H chain rearrangements using MH1 (a primer that binds to ~50% of V<sub>H</sub> gene segments, Ref. 23) and reverse primers in J<sub>H</sub>knock as well.

### Table I. Distributions of bone marrow B cells in B6, B6.3H9, and B6.56R mice<sup>a</sup>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>B Ly</th>
<th>Fraction D</th>
<th>Fraction E</th>
<th>Fraction F</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td></td>
<td>9.93 ± 1.67</td>
<td>2.04 ± 0.37</td>
<td>0.75 ± 0.21</td>
</tr>
<tr>
<td>B6.3H9</td>
<td></td>
<td>3.5 % (B220&lt;sup&gt;+&lt;/sup&gt;CD43&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>58%</td>
<td>21%</td>
</tr>
<tr>
<td>B6.56R</td>
<td></td>
<td>2.82 ± 0.62</td>
<td>0.53 ± 0.13</td>
<td>0.05 ± 0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup>Distributions of bone marrow B cells. Shown are the numbers of B cells in the different bone marrow fractions in millions (±SD) and the percentages of cells in Hardy fractions D, E, and F. The number of bone marrow B cells was obtained by multiplying the number of bone marrow cells obtained from a single mouse for each genotype by the fraction of B220<sup>+</sup> lymphocytes (B Ly). The percentages are calculated by dividing the numbers of cells in the individual fractions by the total number of cells in fractions D, E, and F and multiplying by 100. The numbers of cells in fractions D (H chain rearrangement), E (L chain rearrangement), and F (naive) were obtained by multiplying the number of B cells by the fraction of CD43<sup>−</sup> cells using the gating scheme shown in Fig. 2a. The resulting number of B220<sup>+</sup> cells was multiplied by the fractions of cells falling into the appropriate gates (see Fig. 2a). Three B6 mice, four B6.56R mice, and two B6.3H9 mice were analyzed in the same experiment. The two B6.3H9 mice gave very similar results (<5% difference between mice).
Table II. 56R H chain usage and dsDNA binding in different B6.56R hybridoma panels

<table>
<thead>
<tr>
<th>Panel</th>
<th>dsDNA Binding</th>
<th>n Clones in Panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>67</td>
<td>92</td>
</tr>
<tr>
<td>LPS</td>
<td>2</td>
<td>92</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>LPS old</td>
<td>30</td>
<td>48</td>
</tr>
<tr>
<td>LPS old</td>
<td>3</td>
<td>48</td>
</tr>
<tr>
<td>Subtotal</td>
<td>117</td>
<td>172</td>
</tr>
<tr>
<td>Subtotal</td>
<td>6</td>
<td>172</td>
</tr>
</tbody>
</table>

*H chain rearrangements and DNA binding of B6.56R hybridomas.

shown are the numbers of hybridomas that exhibit dsDNA binding (see Materials and Methods for DNA ELISA description). Hybridomas are classified by 56R transgene status and panel type. The three B6.56R hybridoma panels are: LPS (in vitro LPS panel from a 4-mo-old B6.56R mouse), spontaneous splenic hybridomas derived from the same 4-mo-old B6.56R mouse, LPS old (in vitro LPS panel from an 8-mo-old B6.56R mouse). For analysis purposes, only the IgG- or IgM-secreting clones from the spontaneous panel (not tabulated) lack the 56R knockin DNA and none secretes IgM or IgG or anti-dsDNA (see Materials and Methods).

as JH4-2 (data not shown). To date, no evidence of any functional H chain rearrangement has been observed in any Spont NS hybridoma. Furthermore, no complete V to DJ rearrangement has been obtained in any Spont NS hybridoma. Furthermore, no complete V to DJ rearrangement has been obtained in any Spont NS hybridoma.

Analysis of H chain editing in B6.56R

In all three panels except Spont NS, most of the hybridomas have the 56R Ig DNA (149 of 172, ~87%, Table II). These findings are consistent with the previously documented frequency of VH revision of 17% in BALB/c.56R LPS hybridomas (10). These findings are also consistent with the flow cytometry analysis presented here of B6.56R, in which ~10% of splenic B6.56R B cells expressed the IgM* allotype, corresponding to the endogenous (untargeted) H chain allele (data not shown).

Most B6.56R hybridomas retain the 56R transgene and bind dsDNA

A consistent finding among all of the IgM and/or IgG secreting B6.56R hybridomas is that most (117 of 172, ~68%) of the clones bind dsDNA (Table II). When dsDNA binding is analyzed as a function of H chain transgene status, 78% of 56R* hybridomas bind dsDNA, whereas only 26% of 56R* hybridomas do. In contrast, a previous study in BALB/c.56R documented that a minority of the 56R* hybridomas produced dsDNA (10). The difference in frequency of DNA binding between B6.56R and BALB/c.56R hybridomas therefore must rest with the L chain.

In previously published studies, the L chain repertoire of BALB/c.56R hybridomas was found to be highly restricted: nearly all of the non-DNA binders in 56R* BALB/c.56R hybridomas harbored a Vκ21D (Vκ21-4) L chain rearrangement (10). In contrast, B6.56R hybrids had relatively few Vκ21D rearrangements (8% of all Vκ rearrangements were to Vκ21D, Table III). Instead, rearrangements to Vκ20 (26%) and Vκ38c (26%) dominated in B6.56R (Table III). The representation of Vκ20, Vκ38c, and Vκ21D rearrangements is similar in mice at 4 and 8 mo of age (Table III). The number of spontaneous B6.56R clones that secrete IgM or IgG Abs is too small to make a definitive comparison in terms of specific Vκ frequencies. B6.56R hybridomas therefore appear to express the same L chains as BALB/c.56R hybridomas, but in differing proportions. These data do not rule out that B6.56R may also exhibit a broader range of L chains. A significant fraction (39%) of B6.56R hybridomas have “other” rearrangements (Vκ-Jκ, but not Vκ21D, Vκ20, or Vκ38c; Table III).

To analyze the L chain contribution to dsDNA binding, selected hybridomas had to fulfill three criteria: 1) they had to amplify in the 56R transgene PCR; 2) they had to have genetic evidence of a single Vκ21D, Vκ20, or Vκ38c rearrangement; 3) they had to lack genetic or serologic evidence of an additional H or L chain rearrangement. Using these criteria, 55 hybridomas were analyzed for dsDNA binding as a function of κ (data not shown). As predicted, the usage of these L chains correlated with dsDNA binding. The Vκ21D L chain abrogated dsDNA binding, consistent with previous findings in BALB/c.56R (10). Vκ38c and Vκ20, in contrast, allowed dsDNA binding (data not shown). The Jκ segment can also influence DNA binding: 56R* hybrids with Vκ20-Jκ4, Vκ20-Jκ2, and Vκ20-Jκ1 rearrangements secreted anti-dsDNA Abs while two independent hybrids with Vκ20-Jκ5 rearrangements did not (data not shown). Sequence analysis of one of the hybrids with a Vκ20-Jκ5 rearrangement revealed that Vκ20 did not harbor any somatic mutations.

B6.56R* hybridomas exhibit a restricted diversity of Vκ-Jκ rearrangements

Four molecular features of the L chain rearrangements suggest a very limited diversity of L chain usage in 56R* hybridomas. First, the L chain usage, as determined by Vκ-specific PCR using primers in Vκ20, Vκ38c, and Vκ21 reveal a restricted usage of Vκs (Table III). The Vκ38c primer is specific for a single germine Vκ (gj38c; data not shown). The Vκ21 primer, while able to amplify more than one Vκ21 family member (E. T. Luning Prak, unpublished data), recovers a single-specific Vκ21 family member (21D or 21–4) in 56R mice (10). Similarly, the Vκ20 primer can bind to

Table III. κL chain rearrangements in 56R* hybridomas

<table>
<thead>
<tr>
<th>L Chain</th>
<th>LPS</th>
<th>LPS Old</th>
<th>Spontaneous</th>
<th>B6.56R Total</th>
<th>BALB/c.56R (from Ref. 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vκ21D</td>
<td>2 (2)</td>
<td>11 (17)</td>
<td>1 (6)</td>
<td>14 (8)</td>
<td>54 (78)</td>
</tr>
<tr>
<td>Vκ20</td>
<td>16 (19)</td>
<td>25 (40)</td>
<td>2 (12)</td>
<td>43 (26)</td>
<td>3 (4)</td>
</tr>
<tr>
<td>Vκ38c</td>
<td>26 (31)</td>
<td>11 (17)</td>
<td>6 (38)</td>
<td>43 (26)</td>
<td>7 (10)</td>
</tr>
<tr>
<td>Other</td>
<td>40 (48)</td>
<td>16 (26)</td>
<td>7 (44)</td>
<td>63 (39)</td>
<td>5 (7)</td>
</tr>
<tr>
<td>Rearrangements</td>
<td>84 (100)</td>
<td>63 (100)</td>
<td>16 (100)</td>
<td>163 (100)</td>
<td>69 (100)</td>
</tr>
</tbody>
</table>

*Vκ gene usage in 56R* hybridomas from B6.56R.

Genomic DNA from LPS and spontaneous hybridomas was analyzed for L chain gene rearrangements using a series of PCR assays (see Materials and Methods). Only hybridomas that are 56Rtg* are shown. The analysis does not distinguish productive (in-frame) from nonproductive rearrangements. The other category consists of rearrangements that amplify with generic Vκ-Jκ primers, not with any of the Vκ-specific primers (see Materials and Methods). Percentages (given in parentheses) are calculated based on the total numbers of κ rearrangements shown.
many different Vk, yet only one Vk20 gene (bt20) is amplified in
five of five 56R.B6 LPS hybridomas (data not shown). Third, most
Vk38c and Vk21D rearrangements in hybridomas and in B6.56R
spleen appear to have the same CDR3 length. This point is illus-
trated in Fig. 3, which shows CDR3 spectratyping for two B6.56R
hybridomas with Vk38c-Jk5 rearrangements. Both hybridomas
have the same 300-bp amplicon. 56R.B6 spleen DNA shares the
same 300-bp amplicon. In contrast, wild-type B6 spleen has a dif-
ferent, more diverse collection of Vk38c-Jk5 CDR3 lengths, some
of which appear on the basis of size to be out of frame. Similar data
were obtained for Vk21 (data not shown). Fourth, the analysis of
Jk distributions among LPS hybridomas revealed that most 56R
transgene-retaining hybridomas used distal (Jk4 or Jk5) segments.

The basis for this skewing could be receptor editing (via leapfrog-
ging rearrangements) and/or selection of particular Vk-Jk combi-
nations. In support of the latter alternative, Jk can influence the Ag
binding characteristics of the Ab and certain Vk gene segments
were found more commonly with specific Jk gene segments (Table
IV). For example, 29 of 42 (69%) Vk20+ hybridomas harbored
Vk20-Jk4 rearrangements. Vk-Jk skewing was also observed in
previously published studies of the BALB/c.56R mouse, in which
nearly all Vk21D rearrangements were to Jk2 (10). Most of the
Vk21D rearrangements in B6.56R hybridomas were also to Jk2.
The reproducible finding of specific Vk-Jk combinations in hy-
bridomas independently derived from different mice favors the hy-
pothesis that specific Vk-Jk combinations are selected.

The recurrence of specific Vk-Jk combinations could be due to
the recovery of expanded clones of B cells. To evaluate the hy-
bridoma panels for evidence of clonal expansion, the extended
genotypes of clones with editor L chain rearrangements were an-
alyzed. Different hybridomas having identical genotypes (profiles
of rearrangements at the k, l, and H chain loci) could be derived
from the same progenitor. Sixty hybridomas (of a total of 198)
belong to six groups having at least four members with identical
genotypes and phenotypes, suggestive of clonal expansion of ed-
ted B cells (data not shown). A definitive demonstration of clonal
expansion will require further analysis.

Table IV. Vk-Jk distributions in B6.56R hybridomas

<table>
<thead>
<tr>
<th></th>
<th>56R</th>
<th>Jk1</th>
<th>Jk2</th>
<th>Jk4</th>
<th>Jk5</th>
<th>total</th>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>4</td>
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</tr>
<tr>
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<td>Neg</td>
<td>9</td>
<td>11</td>
<td>16</td>
<td>14</td>
<td>43</td>
</tr>
</tbody>
</table>

*Jk usage in B6.56R hybridomas.
Shown are Jk distributions for rearrangements to different Vk. Hybrids (pooled
from all three B6.56R panels) are grouped according to whether they have the 56R tg
DNA (Pos or Neg, respectively). Percentages of rearrangements are given in paren-
theses. These data do not address the functional status of the L chain rearrangement.
Probable inversions (clones in which Jk1 or Jk2 rearrangements amplify with the Jk2
primer but not with the Jk5 primer) are excluded from this analysis.
To further explore the role of selection in shaping the κ repertoire, hybridomas that lacked the 56R transgene were analyzed for L chain usage. We reasoned that these hybridomas should lack L chain editor rearrangements. We were surprised to find that 19 of 43 Vκ rearrangements in 56R negative hybridomas involved Vk20, Vk38c, and Vk21D (Table IV). Given that there are 93 functional Vκ genes (24) and assuming that rearrangements to the different Vs are random, finding rearrangements to three specific Vs accounting for 44% of the rearrangements is highly improbable (see Materials and Methods). This suggests that these Vs were selected for, raising the possibility that H chain revision occurred after L chain rearrangement in at least a subset of H chain-edited clones.

**Discussion**

B6.56R mice produce anti-dsDNA Abs. Anti-dsDNA Abs are not normally encountered in nonautoimmune strains of mice such as BALB/c or B6. Even B6.3H9 mice (which have a lower affinity anti-DNA H chain) do not spontaneously produce elevated titers of serum anti-dsDNA Abs (6). The level of anti-dsDNA in B6.56R is also higher than in BALBc.56R and a higher fraction of B6.56R hybridomas (71% vs <20%) bind dsDNA. These results imply that biasing the repertoire toward DNA binding and the B6 strain both contribute to the loss of tolerance to DNA.

There is a growing body of evidence that the B6 background is more conducive to the development of autoimmune than other strains such as BALB/c. For example, ANA production in FcγRIIB knockout mice has been linked to two loci, sshb2 and sshb3, on the B6 background (13, 27). Splenomegaly and anti-DNA Ab production have been linked to the imb1 locus in B6.Fas<sup>lo</sup> mice (19). Mice expressing altered signaling molecules or anti-apoptotic molecules are susceptible to autoimmune disease manifestations on the B6 background (14–17). B6 mice are also more susceptible than BALB/c mice to experimentally induced myasthenia gravis (28) and uveitis (29). MHC and non-MHC linked factors have been reported to influence autoimmune responses in B6 including increased numbers of CD4 T cells, a greater tendency to develop Th1 immune responses, the presence of fewer regulatory T cells and that B6 may be more resistant to the inhibitory effects of regulatory T cells than BALB/c (30–33). Finally, there are differences in the germline encoded Ab repertoire that may contribute to the enhanced formation of anti-DNA Abs in B6 (12, 34). A greater propensity to develop autoimmune in B6 therefore arises from B cell intrinsic and extrinsic factors in the B6 background.

Despite the production of anti-dsDNA Abs, which are a specific hallmark of autoimmune diseases such as systemic lupus erythematosus, unmanipulated B6.56R mice do not seem to get sick. They appear to live normal life spans, and Mohan’s group does not find evidence of proteinuria or nephritis in B6.56R (C. Mohan, unpublished observation). Nevertheless, the production of anti-dsDNA Abs is not commonly encountered in normal individuals. In patients with SLE, anti-DNA Abs are sometimes found years before other manifestations of systemic autoimmunity (35).

We prepared hybridoma panels from 4- and 8-mo-old B6.56R mice in hopes of finding age-related increases in autoantibody production and autoimmune symptoms, but failed to find significant differences between the panels. Mice may not have aged to a sufficient degree in these experiments to develop overt disease without further provocation. Mohan’s findings of age-related increases in anti-nuclear Ab titers, but only significantly after the age of 9 mo in B6.56R mice, support this contention (C. Mohan, unpublished observation). Furthermore, B6.56R mice may produce relatively “nonpathogenic” anti-DNAs. Clearly, not all anti-dsDNA Abs are created equal in terms of their ability to contribute to disease pathogenesis (36, 37). Anti-DNA B cells, despite surviving negative selection in the bone marrow, appear to be kept in check in B6.56R.

Several lines of evidence point to the negative regulation of B cells in B6.56R mice. First, B6.56R have significantly reduced numbers of B cells. In the B6.56R bone marrow, there is a relative increase in the number of B cells found in Hardy fraction D and decreased numbers of cells in more mature fractions. Fraction D corresponds to where L chain rearrangement takes place in wild-type mice. The accumulation of cells in fraction D could be due to negative selection of B cells with newly formed (auto)antigen receptors. Cell surface levels of IgM could be decreased due to autoantigen encounter (Ag engagement could lead to receptor internalization) or to L chain editing (such that cells from fraction E “fall back” into fraction D when they edit). Second, B6.56R mice reproducibly exhibit decreased levels of IgM<sup>+</sup> compared with wild-type mice. Decreased levels of 56R expression are reminiscent of the anergic IgM<sup>low</sup> phenotype seen in B cells with anti-nuclear Abs (6, 38–40). This decrease in surface IgM levels appears to be more pronounced in B6.56R than it is in B6.3H9, implying that the decrease is influenced by the affinity of the H chain for DNA. Conversely, B6.56R cells expressing IgM<sup>+</sup> express levels that are comparable to wild-type B6. Third, there is a relative expansion of transitional cells with a T3 phenotype in B6.56R. Whether the T3 subset consists of precursors for a Fo or extrafollicular B cell compartment is at present unresolved, but it is tempting to speculate that these cells are excluded from further maturation or fail to become fully functional in B6.56R. Fourth, B6.56R, like BALB/c.56R, has increased numbers of MZB cells (10). The expansion of the MZ can be seen in mice with B cell deficiency (41). It has been reported recently that T cells with autoreactive potential can undergo homeostatic proliferation in the setting of lymphopenia (42). The MZ could be a major source for autoreactive or multireactive IgM production. B6.56R exhibit more significant increases in IgM anti-DNA (~6-fold) than IgG Abs (~1.5-fold). Thus, there may be a block in the production of high titer, isotype-switched, high-affinity anti-dsDNA Abs in B6.56R.

By performing a detailed analysis of the H and L chain repertoire in B6.56R, we were able to gain insights into why anti-DNA production differs between B6.56R and BALB/c.56R. When we began these experiments, we predicted that negative selection was relaxed on the B6 background. Given that 56R H chain usage is comparable in B6.56R and BALB/c.56R, we expected to find a wider diversity of L chains in 56R<sup>+</sup> B cells. What we found, instead, was a shift in L chain usage. Like BALB/c.56R, B6.56R B cells use a restricted subset of L chains termed editors (10). Some of these L chains (such as Vk21D) abrogate DNA binding, probably due to their low isoelectric points, which allow them to cancel out positively charged amino acids in the 56R H chain (10). However, other L chains, notably Vk38c and Vk20, can permit DNA binding. These DNA-permissive L chains account for a significantly higher proportion of the hybrids in B6.56R than in BALB/c.56R. In addition, a substantial fraction of B6.56R hybridomas did not amplify with Vk21D-, Vk20-, or Vk38c-specific primers.

Why B6.56R uses DNA-permissive L chains more often than BALB/c.56R is unknown. One possibility is that B6 has a lower threshold for positive selection of L chain editors. Alternatively, negative selection is less stringent in B6.56R. It is striking how similar the L chain editor repertoire is in different 56R mice. LPS panels from different mice use the same editor L chains in similar proportions. Furthermore, particular Jκ segments are found with particular Vκ segments (e.g., Vk20 with Jκ4, Vk38c with Jκ5 or Vk21D with Jκ2). These Vκ-Jκ associations are reproducible from mouse to mouse and between B6.56R and BALB/c.56R strains (10, 11). When individual 56R<sup>+</sup> hybridomas are analyzed, it is
clear that Jκ segments can influence the specificity of a given Vκ segment for dsDNA binding. These findings suggest that B6.56R B cells expressing these editor L chains are selected. The driving force behind the selection of editors is not clear, although it has been proposed that low-affinity interactions with self-Ags could provide tonic survival signals (43, 44). Thus, the environment in B6 may be more permissive for the expansion of weakly autoreactive cells than BALB/c.

Expansion of edited B cell clones in B6.56R is suggested by the presence of hybridomas with similar genotype-phenotype profiles. The expansion of B cells with H chain editing rearrangements is supported by the B6.56R model in the setting of chronic GVHD (cGVHD; Ref. 12). In this study, cGVHD was induced by the injection of bm12 splenocytes (which differ at a single class II allele from B6) into B6.56R. Spontaneous hybridomas recovered from recipient mice undergoing cGVHD confirmed the presence of expanded clones with shared somatic mutations. Most of these expanded clones bound DNA yet lacked the 56R H chain (12). In spontaneous and LPS hybridomas characterized in the present study, H chain editing typically abrogated rather than reinstated DNA binding: 26% of 56R− hybridomas bound dsDNA, whereas 78% of 56R+ hybridomas bound dsDNA. These findings are consistent with the observations by Ravetch and colleagues (11) and Mohan’s group (C. Mohan, unpublished observation) that most serum anti-DNA Abs in B6.56R express the allotype of the transgenic allele. Therefore, cGVHD may not stimulate H chain editing to produce anti-DNA B cells. Rather, edited B cells, including those that happen to bind dsDNA, may be more readily activated in the setting of cGVHD. Consistent with this interpretation, B cells that have successfully edited the 56R H chain are overrepresented in the autoimmune response that accompanies cGVHD.

According to this model, successfully edited B cells predominate within a B cell pool that can be activated and undergo expansion (as is the case during GVHD). Conversely, B cells that retain the 56R allele languish, but are able to persist, at least in a B cell-deficient environment. Another possibility is that only edited 56R B cells are diverse enough to express a crucial pathogenic specificity needed for the initiation or propagation of autoimmunity during cGVHD. Autoantibodies against a variety of self-Ags such as DNA, histones and other nuclear Ags can be created during cGVHD (45), but whether multireactivity or polyclonality is an inciting cause or a consequence of evolving autoimmunity is unresolved. The mechanistic basis for autoimmunity in cGVHD is complex and incompletely understood. The development of anti-dsDNA Abs appears to require a diverse repertoire of CD4+ T cells (46). Donor APC, host APC, and recipient regulatory T cells can influence the timing and severity of GVHD (47–49).

In an effort to understand what became of anti-DNA B cells if they made it through differentiation and potentially activation, we produced spontaneous hybridomas from B6.56R. We reasoned that these spontaneous B cells might provide a snapshot of what functionally mature B cells looked like in B6.56R. The spontaneous panel differed in three important respects from the LPS panels. First, more clones had edited the 56R H chain (41% retained the 56R transgene compared with 89% of LPS hybridomas). Second, fewer spontaneous hybridoma Abs bound dsDNA (36 vs 69% of LPS hybridomas). These hybridomas reveal that H chain editing often abrogates DNA reactivity. Third, a large fraction of these spontaneous hybridomas (45%) did not secrete detectable quantities of Abs. In particular, no evidence of a functional H chain was obtained in 26 of 58 spontaneous hybridomas. Given that a majority of these “nonsecretor” clones have molecular and/or serologic evidence of L chain rearrangement or expression, most are B cells. It is possible that our genotyping and serologic assays failed to detect the H chain. For example, the Vκ may not have been recognized by our PCR primers and the H chain ELISAs may have failed to detect very low levels of Abs or a variant Ab (the requisite convergence of both of these technical issues would imply a limited Vκ diversity). The alternative is that these cells lack a functional H chain.

If these “nonsecretor” hybridomas lack a functional H chain, how did the B cells that gave rise to them survive while lacking a functional BCR? One possibility is that the chromosome carrying the 56R transgene was lost during fusion or the culture period. Hybridomas can occasionally lose chromosomes (22), but, in our experience with LPS hybridomas, this happens infrequently. Thus, this is either a peculiarity of spontaneous hybridoma panels or 56R was lost while the B cell was still in the mouse. The acquired loss of H chain functionality implies that nonsecretor hybridomas should be commonly encountered when hybridomas are produced during immunization. However, as such hybridomas are usually screened for Ag binding before being further characterized, clones lacking functional H chains may have been overlooked. In contrast, loss of the H chain in vivo would seem at odds with data that demonstrate that a functional BCR is required for the development (50) and survival of B cells (51). One possibility is that these B cells have recently mutated or inactivated the 56R allele and the fusion rescued them from death or apoptosis. One interesting published example of an inactivating H chain rearrangement is provided by the murine pseudo-D1 segment. This D segment lacks a functional 5′RSS and can therefore undergo DJ but not V to DJ rearrangement (52).

A more intriguing explanation for the loss of the H chain in nonsecretor hybridomas is that a population of B cells undergoes exhaustive H chain editing in vivo to suppress autoimmunity, ultimately resulting in the loss of a functional H chain. It is striking in this connection that editor L chains comprise 44% of the Vκ rearrangements in 56R− hybridomas. Some of these 56R− hybridomas produce IgM or IgG Abs. The abundance of editor L chain rearrangements implies that 56R loss could have occurred after the expression of the L chain editor. If this were the case, some of these 56R− Ab secreting hybridomas could be intermediates on their way to becoming L chain-only B cells. The enrichment of H chain editing and H chain loss among spontaneous hybridomas could mean that H chain editing is occurring in peripheral B cells. Peripheral receptor editing, while controversial, has been proposed to serve various functions including the limitation of autoimmunity during an immune response, the diversification of the repertoire and the rescue of B cells with lethal somatic mutations (53, 54). These varied and seemingly contradictory roles for editing could limit or enhance autoreactivity. We hope that the B6.56R mouse, by virtue of its simplified repertoire, will serve as a useful model for tracking clones of edited B cells through development and determining the causes and consequences of receptor editing.

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