Functional Dissection of Lupus Susceptibility Loci on the New Zealand Black Mouse Chromosome 1: Evidence for Independent Genetic Loci Affecting T and B Cell Activation

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New Zealand Black (NZB) mice develop a lupus-like autoimmune disease that is characterized by production of anti-RBC, anti-lymphocyte, and anti-ssDNA Abs, leading to hemolytic anemia and mild glomerulonephritis that develops late in life (reviewed in Ref. 1). Offspring of the cross between NZB and New Zealand White (NZW) mice (NZB/W) develop a rapidly progressive immune complex-mediated glomerulonephritis and high titer anti-dsDNA Abs. These mice are considered to be excellent models of SLE, and characterization of their immune defects has led to a number of fundamental insights into the human disease. Although NZB mice do not develop rapidly progressive glomerulonephritis, they appear to possess most of the immunologic defects required, because backcrossing of the cross between NZB and B6.H2z (the NZW MHC haplotype) mice, altered B and T cell activation with increased expression of CD69, and for B cells, costimulatory molecules and MHC. Introduction of an anti-hen egg white lysozyme Ig transgene, as a representative nonself-reactive Ig receptor, onto the B6.NZBc1(35–106) background corrected the B cell activation phenotype and led to dramatic normalization of splenomegaly and T cell activation, but had little impact on the increased proportion of memory T cells. These findings indicate that there are multiple lupus susceptibility genes on NZB chromosome 1, and that although B cell defects play an important role in lupus pathogenesis in these mice, they act in concert with T cell activation defects. The Journal of Immunology, 2003, 171: 1697–1706.

Mapping studies have revealed that susceptibility to lupus in NZB and NZB/W mice is polygenic, with multiple genes contributing to the generation of high affinity autoantibodies, nephritis, and mortality (3–11). In particular, genetic loci on NZB chromosome 1 have been shown in multiple crosses to play a prominent role in disease susceptibility (3, 5, 7, 8, 10, 11). Indeed, in one cross between NZB and B6.H2z (the NZW MHC haplotype) mice, a region on distal NZB chromosome 1 was found, in combination with H2z, to confer >90% of the susceptibility to nephritis and autoantibody production (7, 8).

The presence of lupus susceptibility genes on NZB chromosome 1 has been confirmed by Rozzo et al. (12), who showed that B6 congenic mice with an NZB interval extending from 79–109 cM produced IgG anti-nuclear Abs. Furthermore, two candidate genes in this interval have been proposed to lead to the generation of autoantibodies. Rozzo et al. (12) have proposed that a promoter polymorphism of Ifh202, an IFN-inducible transcriptional repressor, leads to increased expression of this gene in non-T cells, resulting in inhibition of B cell apoptosis following IgM cross-linking. Xiu et al. (13) in contrast have proposed that a promoter polymorphism in the NZB Fcgr2b allele leads to defective expression of FcγRIIB in germinal center B cells and impaired apoptosis of autoreactive B cells. However, work by ourselves and others suggested that it was unlikely that the 79- to 109-cM interval contained all of the lupus susceptibility genes located on NZB chromosome 1 (11). For example, Kono et al. (3) showed, in a (NZB × NZW)F2 intercross, linkage between a NZB region around 67 cM and anti-chromatin Ab production and splenomegaly. In our mapping study of (B6 × NZB)F2 intercross mice, IgG anti-ssDNA Ab production was linked to a broad region on NZB chromosome 1 with two shallow peaks at 63 and 92 cM, and a similar area of broad linkage was demonstrated for several B cell activation phenotypes, including increased expression of B7.1, B7.2, ICAM-1, and CD44 (11). Indeed, the peak lod scores for IgG...
anti-ssDNA Ab production, ICAM-1, and CD44 were all located in the 63- to 76-cM region. In this study, we have produced and characterized B6 congenic mice with a longer chromosomal interval extending from ~35 to 106 cM (denoted B6.NZBc1(35-106)) and contrasted the phenotype of these mice with congenic mice bearing shorter NZB chromosomal intervals. We confirm the presence of additional genetic loci on NZB chromosome 1 that promote autoantibody production and development of nephritis. In addition, we provide evidence that susceptibility genes within the ~85–100 interval lead to expansion of memory T cells, while those in the ~35–85 interval are associated with altered B cell activation and enhanced T cell activation. We demonstrate that correction of this abnormal B cell activation by introduction of non-self-reactive Ig transgene onto the B6.NZBc1(35–106) background has a profound effect on the autoimmune phenotype, resulting in normalization of splenomegaly and marked diminution of the abnormal T cell activation. Based upon this evidence, we hypothesize that although intrinsic B cell defects leading to abnormal activation of self-reactive B cells play an important role in driving the autoimmune process in these mice, they act in concert with T cell activation defects.

Materials and Methods

**Mice**

B6 and NZB mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and Harlan Sprague Dawley (Indianapolis, IN), respectively. Congenic mice were produced by backcrossing mice with NZB chromosome 1 intervals onto the B6 genetic background, using the speed congenic technique. Mice were typed at each successive generation using polymorphic microsatellite markers that discriminate between NZB and B6 DNA, spaced at ~20-cM intervals throughout the genome. Fully backcrossed mice were obtained in six to seven generations and intercrossed to produce congenic mice that were homozygous for NZB chromosome 1 intervals. B6.NZBc1(35–106) mice expressing transgenes encoding IgM3dG H and L chains specific for hen egg white lysozyme (anti-HEL Ig transgenic (Tg)) (14) (MD4) were produced by backcrossing the anti-HEL Ig transgene from B6 anti-HEL Ig Tg mice (purchased from The Jackson Laboratory) onto the B6.NZBc1(35–106) background. Offspring were genotyped by PCR, using primers specific for the V region of the Ig H chain. All of the mice were housed in microisolators in the animal facility at the Toronto Western Hospital (Toronto, Canada) and were specific pathogen free.

**Flow cytometry staining and analysis**

A total of 1 × 10⁸ RBC-depleted spleen cells was incubated with 10 µg/ml mouse IgG (Sigma-Aldrich, St. Louis, MO) for 15 min to block Fc receptors and then stained with various combinations of directly conjugated mAbs. Following washing, aliphococyanin-conjugated streptavidin (BD Pharmingen, San Diego, CA) was used to reveal biotin-conjugated Ab staining. Dead cells were excluded by staining with propidium iodide (PI; Sigma-Aldrich), 0.6 µg/ml. Flow cytometry of the stained cells was performed using a dual laser FACSCalibur (BD Biosciences, Mountain View, CA) and analyzed using CellQuest (BD Biosciences) software. The following directly conjugated mAbs were purchased from BD Pharmingen: biotin anti-CD4 (L3T4), biotin anti-CD8 (53-6.7), biotin anti-IgM* (DS-1), PE anti-B220 (16-10A1), PE anti-B7.2 (GL1), PE anti-ICAM-1 (3D12), PE anti-CD69 (B3H6), PE anti-CD45R (IC2), PE anti-CD44 (IM7), PE anti-MHC (I-A*), PE anti-NK1.1 (PK136), PE anti-IgM* (DS-1), FITC anti-CD21 (7G6), and FITC anti-CD5 (53-7.3). Biotin-, PE-, and FITC-conjugated anti-B220, as well as FITC-conjugated anti-CD62L mAbs were purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). FITC-conjugated anti-IgM and Mac-1 Abs were purchased from Jackson ImmunoResearch (West Grove, PA) and Serotec (Oxford, U.K.), respectively. Biotinylated HEL was prepared using an EZ-Link Sulfo-NHS-LC Biotinylation kit (Pierce, Rockford, IL). All isotype controls were purchased from Cedarlane, except for hamster IgG controls, which were obtained from BD Pharmingen.

**Measurement of Ab production**

IgM and IgG anti-ssDNA, anti-dsDNA, anti-chromatin, and anti-histone Abs were measured by ELISA. dsDNA was prepared from calf thymus DNA (Sigma-Aldrich), and ssDNA was prepared by boiling dsDNA for 10 min and quick cooling on ice. H1-stripped chromatin was prepared from chicken RBCs, as described (15). Bovine histones (a mixture of H1, H2A, H2B, H3, and H4) were purchased from Roche (Basel, Switzerland). ELISA plates were coated overnight with Ag diluted in PBS (dsDNA, 20 µg/ml; dsDNA, 10 µg/ml; chromatin, 2 µg/ml; histones, 2.5 µg/ml), washed with PBS/Tween 20 (0.5%), and blocked with PBS/BSA (2%). After further washing, serum samples, diluted 1/100 in PBS/BSA/Tween 20, were added. Bound IgM or IgG Abs were detected using alkaline-phosphatase-conjugated anti-IgM or anti-IgG as a secondary reagent (Catlog, Burlingame, CA).

**Immunoﬂuorescence staining of tissue sections**

Spleens and kidneys were snap frozen in OCT compound (Sakura Finetek, Torrance, CA) at the time of sacrifice. Cryostat sections (6 µm) were fixed in acetone, washed with PBS, and blocked with PBS/5% normal goat serum. Kidney sections were stained with FITC-conjugated anti-IgG or IgM F(ab’2) (Catlog). Spleen sections were stained with biotinylated PNA (to reveal germinal centers; Sigma-Aldrich) and FITC-conjugated anti-IgM (to reveal B cell follicles; Jackson ImmunoResearch). Biotin staining was revealed using rhodamine (tetramethylrhodamine isothiocyanate)-conjugated streptavidin as a secondary reagent (Molecular Probes, Eugene, OR). Stained sections were mounted with Mowiol (a generous gift from M. Fehlings, Toronto, Canada), and tissue fluorescence was visualized using a Zeiss (Oberkochern, Germany) Axiosplan 2 imaging microscope. Digital images were obtained using the manufacturer’s imaging system.

**Grading of kidney sections**

Kidneys were fixed in Formalin, paraffin embedded, sectioned (3 µm), and stained with PAS. Grading was performed by a renal pathologist (G. LaJoie), who was blinded as to the strain of origin of the tissue section. The grading scale used for light microscopy was as follows: grade 0, normal glomeruli; grade 1, minimal expansion and/or proliferation; grade 2, focal segmental (endocapillary) proliferative glomerulonephritis; grade 3, diffuse (endocapillary) proliferative glomerulonephritis; and grade 4, diffuse proliferative glomerulonephritis with crescents. Glomerular staining of kidney sections stained with FITC anti-IgM or anti-IgG was graded by immunofluorescence microscopy. Sections with no or only trace deposits were graded as 0; those with mesangial deposits, grade 1; those with mesangial and segmental capillary wall deposits, grade 2; those with diffuse mesangial and capillary wall deposits, grade 3; and those with crescents, grade 4.

**Statistical analysis**

Comparisons of differences between groups of mice for continuous data were performed using the Student’s t test with Welch’s correction. χ² analysis was used for comparisons of proportions between groups of mice.

**Results**

**Generation of B6.NZBc1 congenic mice and characterization of their serologic phenotype**

A variety of immunologic and clinical phenotypes, including elevated levels of costimulatory molecules on B cells, IgM and IgG autoantibody production, splenomegaly, and nephritis, have been linked to NZB chromosome 1 in various crosses (3–11). To begin to characterize the immunogenetic basis for these abnormalities, we backcrossed NZB chromosomal regions derived from distal chromosome 1 onto the nonautoimmune B6 genetic background, using the speed congenic technique. These mice were then intercrossed to produce congenic mice that were homozygous for NZB chromosome 1 intervals because our previous mapping study indicated that chromosome 1 susceptibility loci function in an additive or recessive fashion (11). Three different mouse strains with NZB chromosome 1 intervals extending from ~35 to 106 cM, ~85 to 106 cM, and ~100 to 109 cM were produced, designated B6.NZBc1(35–106), B6.NZBc1(85–100), and B6.NZBc1(100–109), respectively (Fig. 1). The largest chromosomal region encompasses a broad area of linkage for elevated levels of costimulatory molecules and IgG anti-ssDNA Ab production that we have previously defined in a mapping study (11). The ~85- to 106-cM interval is similar to the ~79- to 109-cM interval studied by Rozzo et al. (12) and contains the putative candidate genes, If202 and...
These two genes, Fcgr2b (12, 13), whereas the 35- to 106-cM interval excludes these two genes.

Cohorts of male and female congenic B6.NZBc1 mice were then followed for up to 7 mo for development of autoantibodies and nephritis. As shown in Fig. 2, by 6–7 mo of age, B6.NZBc1(35–106) and B6.NZBc1(85–106), but not B6.NZBc1(100–109), produced significant titers of anti-nuclear autoantibodies. However, both the penetrance and level of autoantibodies produced by B6.NZBc1(35–106) mice were significantly higher than for B6.NZBc1(85–106) mice. Similar differences between these two congenic mouse strains were observed for both male and female mice, and were already apparent in young 14- to 16-wk-old mice (Fig. 2). In contrast to the findings for IgG, the levels of IgM anti-nuclear autoantibodies in these mice were either normal or only slightly elevated when compared with B6 controls (data not shown). These data extend previous work indicating that there are genetic loci between 35 and 85 cM that promote autoantibody production.

**Contribution of NZB chromosome 1 genetic loci to lupus nephritis**

To further assess the possibility that IgG autoantibody production differs between B6.NZBc1(35–106) and B6.NZBc1(85–106) mice, we investigated whether the incidence of nephritis differs between the two mouse strains. Mice were followed for up to 7 mo for the development of proteinuria and sacrificed, and their kidneys were harvested.

Three mice died during the 7-mo period, one male and two female B6.NZBc1(35–106) mice. It is not known whether these mice died of renal disease. Although low grade proteinuria was frequently seen in experimental mice, none of the remaining mice developed high grade proteinuria (>3 g/L, based upon testing with Albustix). Deposition of Ig in the kidneys of sacrificed mice was assessed by immunofluorescence microscopy following staining with FITC anti-IgM or anti-IgG, and graded using a four-point scale, as outlined in Materials and Methods. The majority of experimental mice had deposition of Ig within the kidneys; however, as shown in Fig. 3A, the pattern of IgG deposition differed significantly between B6.NZBc1(35–106) and B6.NZBc1(85–106) congenic mouse strains (p = 0.03, female; p = 0.0013, male; χ2 test for trend). Although the majority of male and female B6.NZBc1(85–106) mice had no or only weak segmental mesangial IgG deposition, most male and female B6.NZBc1(35–106) mice had diffuse mesangial + capillary wall deposits. B6.NZBc1(35–106) mice also had more severe renal lesions on light microscopy (Fig. 3B) (p = 0.0006, female; p = 0.0030, male; χ2 test for trend). Over 70% of B6.NZBc1(35–106) mice had focal segmental or diffuse proliferative glomerulonephritis. In contrast, proliferative lesions were seen infrequently in B6.NZBc1(85–106) mice (7%). Although there was a trend to more severe renal lesions in male than female congenic mice, this did not achieve statistical significance.

**Characterization of cellular phenotypes in B6.NZBc1 congenic mice**

Given the more severe manifestations of disease in B6.NZBc1(35–106) mice, we were interested in determining whether these mice demonstrated a distinct cellular phenotype when compared with B6.NZBc1(85–106) mice. To this end, spleens isolated from 14-wk-old mice were weighed and divided for tissue immunofluorescence and flow cytometric analysis of splenocyte populations. As shown in Table I, both B6.NZBc1(35–106) and B6.NZBc1(85–106) mice had larger spleens, increased numbers of splenocytes, and a decreased proportion of CD8− T lymphocytes when compared with B6 mice. In addition, consistent with results reported by Rozzo et al. (12), B6.NZBc1(85–106) mice had an increased proportion of B lymphocytes as compared with B6 mice. However, similar altered proportions of B and CD8+ T lymphocytes were seen in the B6.NZBc1(100–109) mouse strain, indicating that the immune mechanisms leading to these alterations are insufficient to induce autoimmunity. In contrast, expansion of the CD4+ T cell population was unique to the B6.NZBc1(35–106) mouse strain, and thus more likely to be related to the autoimmune phenotype. Overall, B6.NZBc1(35–106) mice had significantly larger spleens, increased proportions of CD4+ T lymphocytes, and decreased proportions of B lymphocytes when compared with B6.NZBc1(85–106) mice.

Investigation of activation markers on B and T cells revealed that both B6.NZBc1(35–106) and B6.NZBc1(85–106) mice had evidence of increased lymphocyte activation, but again appeared to have distinct phenotypes. In B6.NZBc1(85–106) mice, there was evidence of altered T cell activation with an increased proportion of memory CD44highCD62Llow CD4+ T cells (see Table II) and a corresponding decrease in the proportion of naive CD44low CD62Lhigh CD4+ T cells (data not shown). Similar findings were observed for B6.NZBc1(35–106) mice, but were accompanied by an increase in the proportion of recently activated CD69+ CD4+ T cells. B6.NZBc1(35–106) mice also showed evidence of increased B cell activation with an increased proportion of B cells with elevated levels of CD69, costimulatory molecules, and MHC. Taken together, these data strongly suggest that lupus susceptibility genes located within the 85–100 NZB chromosome 1 region are associated predominantly with altered T cell activation, while those within the 35–85 region affect B cell activation.
To further explore the nature of altered T and B cell activation in congenic mice, splenic sections were stained with anti-IgM and PNA to reveal splenic B cell follicles and germinal centers, respectively. As shown in Fig. 4, spleens from B6 and B6.NZBc1(85–106) mice had few or no germinal centers. In contrast, spleens from B6.NZBc1(35–106) mice had a large number of germinal centers. Increased numbers of germinal centers were already apparent in 6-wk-old B6.NZBc1(35–106) mice, although not to the same extent as 14-wk-old mice. Although germinal centers could be detected in 6-mo-old B6.NZBc1(85–106) mice, they never achieved the numbers seen in B6.NZBc1(35–106) mice. Thus, the combination of lupus susceptibility genes in B6.NZBc1(35–106) mice appears to lead to markedly enhanced T-B collaboration, an observation that is compatible with the presence of high titer predominantly IgG autoantibodies in these mice.

**FIGURE 2.** Autoantibody levels in chromosome 1 congenic mouse strains. Serum samples from 14- to 16-wk (A) and 6- to 7-mo-old (B) congenic mice and age-matched control B6 and NZB mice were assayed for the presence of anti-ssDNA, anti-dsDNA, anti-chromatin, and anti-histone Abs by ELISA. Congenic mouse strains are denoted by their NZB interval. Each circle represents the determination from an individual mouse, with ♂ indicating female mice and ○ male mice. Horizontal lines indicate the mean for each population examined.
levels of costimulatory molecules, such as marginal zone B cells or B1 cells. To assess this possibility, freshly isolated splenocytes were stained with a combination of anti-B220 and anti-CD5, or anti-B220, anti-CD21, and anti-CD23 Abs to identify the various B cell subsets. Although the proportion of CD5⁺ B1 B cells was not increased in B6.NZBc1(35–106) mice (data not shown), altered proportions of several other splenic B cell subsets were identified (Fig. 5). Specifically, B6.NZBc1(35–106) mice had decreased proportions of T2 and marginal zone B cells, and increased proportions of T1 (B220high Syndecanlow-intermediate (int); data not shown) and follicular B cells when compared with B6 mice. However, only the altered proportion of T1 and T2 B cells was unique to B6.NZBc1(35–106) mice, as similar changes in the proportion of follicular and marginal zone B cells were seen in B6.NZBc1(85–106) and B6.NZBc1(100–109) mice (data not shown).

The lack of expansion of B cell populations that normally express elevated levels of costimulatory molecules suggested that there is enhanced activation of other B cell subsets in B6.NZBc1(35–106) mice. To further characterize which B cell subsets are abnormally activated, splenocytes were stained with Abs directed against B220, CD21, and costimulatory molecules. As shown in Fig. 6, increased expression of costimulatory molecules was already apparent in CD21low T1 cells, the earliest peripheral B cell developmental stage, and with the exception of B7.1, remained elevated in CD21int (predominantly follicular) and CD21high (T2 and marginal zone) B cell populations. Thus, enhanced B cell activation is an early developmental event in B6.NZBc1(35–106) mice, and accompanied by altered proportions of transitional B cells.

Constriction of the B cell repertoire by introduction of a nonspecific transgene results in normalization of B and T cell activation in B6.NZBc1(35–106) mice

Because up-regulation of costimulatory molecules in B6.NZBc1(35–106) mice is seen on recent bone marrow emigrants, and self-reactive B cells have been shown to up-regulate costimulatory molecules in response to Ag receptor engagement at an early developmental stage (16), we questioned whether altered expression of costimulatory molecules was seen on all B cells, regardless of specificity or a more restricted subset of presumably self-reactive B cells. To assess this possibility, we crossed an Ig transgene recognizing hen egg white lysozyme (anti-HEL Ig Tg) (14) onto the B6.NZBc1(35–106) background. In these mice, the transgene is integrated centromeric to the MHC locus on B6 chromosome 17 in a region that had not been linked to development of lupus, ensuring that differences observed between B6.NZBc1(35–106) anti-HEL

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Table I. Comparison of splenic cell populations in various NZB chromosome 1 congenic mouse strains

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>cl1(100–109)</th>
<th>cl1(85–106)</th>
<th>cl1(35–106)</th>
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<tbody>
<tr>
<td></td>
<td>n = 13</td>
<td>n = 6</td>
<td>n = 11</td>
<td>n = 12</td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
<td>84.1 ± 8.6</td>
<td>78.8 ± 22.4</td>
<td>112.3 ± 25.4*</td>
<td>158.5 ± 61.5***</td>
</tr>
<tr>
<td>No. splenocytes per ½ spleen × 10⁶</td>
<td>45.2 ± 8.1</td>
<td>57.8 ± 6.4**</td>
<td>63.5 ± 8.9***</td>
<td>72.7 ± 17.1***</td>
</tr>
<tr>
<td>% B cells</td>
<td>58.6 ± 1.3</td>
<td>63.3 ± 2.3**</td>
<td>62.4 ± 1.7**</td>
<td>59.3 ± 2.6</td>
</tr>
<tr>
<td>% CD4⁺ cells</td>
<td>18.2 ± 1.5</td>
<td>15.8 ± 1.0**</td>
<td>17.1 ± 1.6</td>
<td>20.1 ± 1.7**</td>
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<tr>
<td>% CD8⁺ cells</td>
<td>13.4 ± 0.9</td>
<td>11.2 ± 1.1**</td>
<td>11.1 ± 1.0***</td>
<td>10.8 ± 1.2***</td>
</tr>
<tr>
<td>% Mac-1⁺ cells</td>
<td>3.28 ± 0.51</td>
<td>2.92 ± 0.12</td>
<td>3.00 ± 0.76</td>
<td>3.67 ± 1.05</td>
</tr>
</tbody>
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* Results are mean ± SD. Significance level determined by Student’s t test with Welch’s correction for comparison with B6 mice.

* Number of 14- to 16-wk-old female mice in each group.

* Comparison of B6.NZBc1(85–106) and B6.NZBc1(35–106).

*, p < 0.05.
** p < 0.01.
*** p < 0.001.
Ig Tg and non-Tg littersmate control mice result solely from expression of the Ig transgene (14) (unpublished observations). In both B6 and B6.NZBc1(35–106) hemizygous anti-HEL Ig Tg mice, greater than 95% of B cells expressed the IgM transgenic H chain with less than 2.5% of these cells demonstrating reduced binding to HEL, suggesting that they expressed endogenous L chains.

As shown in Fig. 7, introduction of the anti-HEL Ig Tg onto the B6.NZBc1(35–106) background resulted in marked normalization of the altered costimulatory molecule expression on B cells. For B7.1 and B7.2, costimulatory molecule expression in B6.NZBc1(35–106) anti-HEL Ig Tg mice was similar to that seen in B6 anti-HEL Ig Tg mice, whereas for ICAM-1, expression on CD21int and CD21high cells remained slightly elevated. Similar findings were obtained when splenocytes were stained with anti-IgM, instead of anti-B220, to permit gating on B cells expressing the anti-HEL Ig H chain. These results are consistent with the hypothesis that the increased B cell activation seen in B6.NZBc1(35–106) mice reflects enhanced

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Table II. Comparison of B and T cell activation markers in various NZB chromosome 1 congenic mouse strains

<table>
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<tr>
<th></th>
<th>B6</th>
<th>c1(100–109)</th>
<th>c1(85–106)</th>
<th>c1(35–106)</th>
<th>p Valuec</th>
</tr>
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<tr>
<td>% B220+ CD69+</td>
<td>8.27±2.92</td>
<td>9.38±3.73</td>
<td>8.45±2.55</td>
<td>11.64±3.96*</td>
<td>0.0324</td>
</tr>
<tr>
<td>% B220+ B7.1+</td>
<td>8.76±2.67</td>
<td>8.57±1.29</td>
<td>7.89±2.48</td>
<td>11.27±1.89*</td>
<td>0.0018</td>
</tr>
<tr>
<td>B220+ MFI ICAM-1</td>
<td>196±23</td>
<td>202±22</td>
<td>208±26</td>
<td>264±22***</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>B220+ MFI A1</td>
<td>1594±220</td>
<td>1912±172**</td>
<td>1446±217</td>
<td>2330±293***</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>% CD4+ CD69+</td>
<td>16.6±2.4</td>
<td>22.8±7.82</td>
<td>19.0±3.72</td>
<td>26.7±6.26***</td>
<td>0.0003</td>
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<tr>
<td>% CD4+ CD44high CD62Llow</td>
<td>27.5±4.39</td>
<td>27.1±5.46</td>
<td>38.2±4.78***</td>
<td>39.4±4.44***</td>
<td>0.54</td>
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</table>

Results are mean ± SD. Significance level determined by Student’s t test with Welch’s correction for comparison with B6 mice.

Comparison of B6.NZBc1(85–106) and B6.NZBc1(35–106).

MFI, mean fluorescence intensity.

*, p < 0.05

**, p < 0.01

***, p < 0.001

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**FIGURE 4.** Quantitation of splenic germinal centers. A. Spleens from 14- to 16-wk-old female mice were stained for dual-color immunofluorescence. Green and red colors depict staining for IgM and PNA, respectively. Note the marked increase in both the number and size of germinal centers in B6.NZBc1(35–106) mice. B. Scatterplot showing the average number of germinal centers per lymphoid follicle in splenic sections. Congenic mouse strains are denoted by their NZB interval. Each ○ represents the score for an individual female mouse. Horizontal lines indicate the mean for each population examined.

**FIGURE 5.** Distribution of B cells in different B cell subsets. A. Freshly isolated splenocytes from 14- to 16-wk female mice were stained with anti-B220, anti-CD21, and anti-CD23. Shown are dot plots gated on PIexcluding B220+ cells. Regions used to determine the proportion of follicular (FC), T1, T2, and marginal zone (MZ) B cells are indicated. Numbers within the box indicate the proportion of B cells in each population. B, Scatterplot showing the proportion of B cells in each B cell subset, gated as indicated in A. Each ○ represents the determination for an individual mouse. Horizontal lines indicate the mean for each population examined.
activation of self-reactive B cells. In further support of this hypothesis, the increased proportion of CD69+ B cells was also normalized in anti-HEL Ig Tg B6.NZBc1(35–106) mice. We next sought to determine whether constriction of the B cell repertoire and/or normalization of B cell activation abrogated the other cellular abnormalities in B6.NZBc1(35–106) mice. As shown in Table III, both splenic weight and the number of splenocytes were reduced to B6 levels in anti-HEL Ig Tg B6.NZBc1(35–106) mice. Consistent with this observation, the number of germinal centers was dramatically reduced (data not shown). In contrast, although the proportion of recently activated CD69+ cells or mean fluorescent intensity for B7.2 and ICAM-1 in each of the three B cell subsets (CD21low, CD21int, CD21high), examined as indicated by the vertical lines. B, Scatterplots, gated on the indicated B220+ populations, show the percentage of B7.1+B cells and mean fluorescence intensity for staining with anti-B7.2 and ICAM-1. For the percentage of B7.1+B cells, background staining with a relevant isotype control was subtracted. Each circle represents the determination from an individual mouse. Horizontal lines indicate the mean for each population examined. Costimulatory molecule expression was significantly increased (p ≤ 0.002) in all B cell subsets, with the exception of B7.1 expression in CD21low and CD21high populations.

Discussion
The presence of lupus susceptibility genes on NZB chromosome 1 outside the 85–106 interval was predicted by our previous gene mapping experiments (11). In this study, we confirmed this prediction by showing that within the 35–85 interval there are genetic loci that significantly enhance autoantibody production, splenomegaly, and development of proliferative glomerulonephritis, in a substantial proportion of mice by 7 mo of age. Although we have not yet identified the precise gene or genes within this interval that promote this autoimmune phenotype, we show that these genes mediate their effects predominantly through abnormal activation of self-reactive B cells, and that this abnormal activation plays an integral role in driving the autoimmune process.

Our investigation confirms and extends the work of Rozzo et al. (12), who produced congenic mice with a NZB chromosome 1 interval extending from ~79 to 110 cM (B6.Nba2). Although we confirm that there are genetic loci on distal NZB chromosome 1 that promote anti-chromatin, anti-dsDNA, and anti-histone Ab production, we show that these serologic abnormalities do not result solely from the effects of the candidate gene, Ifh202 at 96 cM, as proposed by these authors. Interestingly, comparison of the phenotype of B6.NZBc1(85–106) mice, with that reported for B6.Nba2 mice, reveals some differences. Although the penetrance and titer of anti-chromatin and anti-dsDNA autoantibody production appear similar between the mouse strains, anti-histone Abs were not produced at significant titer by B6.NZBc1(85–106) mice. This could simply result from differences between the two laboratories in the sensitivity or the specificity of the assays used to detect anti-histone Abs. Alternatively, there may be an additional lupus susceptibility gene located between 79 and 85 cM that promotes production of anti-histone Abs in B6.Nba2 mice. Comparison of the reported cellular phenotype of B6.Nba2 mice with that for B6.NZBc1(85–106) mice does not permit discrimination between these two possibilities. Rozzo et al. do not provide data on T cell activation in B6.Nba2 mice. However, they do show that B6.Nba2 mice have an increased proportion of CD69+ and Fas-expressing B cells at 6 mo of age, suggestive of increased B cell activation. Although this phenotype was not observed in 14-wk-old B6.NZBc1(85–106) mice, we have found that 6-mo-old
FIGURE 7. Levels of B cell costimulatory molecule expression in splenic B cell subsets of anti-HEL Ig Tg mice. Freshly isolated splenocytes from 14- to 16-wk-old female mice were stained with anti-B220, anti-CD21, and anti-B7.1, anti-B7.2, or anti-ICAM-1. Scatterplots, gated on the indicated PI-excluding B220high populations, show the percentage of B7.1− B cells and mean fluorescence intensity for staining with anti-B7.2 and anti-ICAM-1. For the percentage of B7.1− cells, background staining with a relevant isotype control was subtracted. Each circle represents the determination from an individual mouse. □, Indicate non-Tg mice, and ○, anti-HEL Ig Tg mice. Differences between non-Tg and anti-HEL Ig Tg B6.NZBc1(35–106) mice was significant (p < 0.05), with the exception of B7.2 and ICAM-1 for the CD21low population and B7.1 for the CD21high population.

B6.NZBc1(85–106) mice have mildly increased B cell activation with increased Fas expression (unpublished observations).

If there is a lupus susceptibility locus in the 79–85 interval, it is unlikely that this accounts for all of the differences between B6.NZBc1(85–106) and B6.NZBc1(35–106) mice, because the serologic phenotype of B6.NZBc1(35–106) mice differs from that reported for B6.Nba2a mice. In particular, the penetrance and titer of anti-chromatin and anti-histone Abs appear to be much higher in B6.NZBc1(35–106) mice. It is therefore probable that there is an additional lupus susceptibility locus more proximal to the centromere than 79 cM. This is supported by the results of previous studies by ourselves and others demonstrating linkage for autoantibody production, and two of the prominent phenotypes in B6.NZBc1(35–106) mice, splenomegaly and increased costimulatory molecule expression on B cells, to a region at ~64–66 cM on NZB chromosome 1 (3, 11).

Multiple lupus susceptibility loci have also been localized to chromosome 1 in BXSB and NZM lupus-prone mice (17–23). Although these findings point to an important role of chromosome 1 genes in the pathogenesis of lupus, it is unlikely that the genetic polymorphisms that promote disease are the same in all three mouse strains. In NZM mice, an inbred mouse strain derived from the NZB and NZW mouse strains, there are at least four susceptibility loci on chromosome 1 (21); however, all of these are derived from the NZW parent and located distal to 85 cM. Of the four loci, one (Sle1c) has been tentatively identified as a polymorphism affecting expression of complement receptor 2, and is not found in NZB mice (24). Studies of congenic mouse strains with NZM intervals containing the other chromosome 1 susceptibility loci indicate that only one mouse strain, B6.Sle1a, has increased production of anti-chromatin autoantibodies and an increased proportion of memory T cells similar to that observed in our B6.NZBc1(85–106) mice (21).

In BXSB mice, four susceptibility loci have also been mapped to chromosome 1 (22, 23). Although the majority of these loci are located in different regions than those observed in NZM mice, three of the susceptibility loci, located at ~35, ~63, and ~100 cM, are encompassed within the NZB 35–106 interval. These loci have been linked to autoantibody production, glomerulonephritis, and splenomegaly, however a B cell phenotype similar to that observed in the B6.NZBc1(35–106) mice has not been described for BXSB mice. Thus, the NZB susceptibility genes, located within the 35–85 chromosome 1 interval, that lead to altered B cell activation and enhanced autoimmunity, appear to be unique to this mouse strain. These findings have relevance for linkage studies in human lupus and suggest that within the outbred human population there is likely to be extensive genetic heterogeneity in the susceptibility to lupus. Furthermore, the findings confirm the results of Morel et al. (21), indicating that positive linkage results in a particular chromosomal region can result from the presence of multiple different susceptibility alleles that are located within close proximity.

Table III. Normalization of the autoimmune phenotype in B6.NZBc1(35–106) mice by introduction of an Ig transgene

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>B6.NZBc1(35–106)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NTg</td>
<td>Tg</td>
</tr>
<tr>
<td>Spleen weight</td>
<td>75.3 ± 7.8 (7)</td>
<td>63.0 ± 10.6 (4)</td>
</tr>
<tr>
<td>Splenocytes per ½ spleen × 10⁶</td>
<td>35.8 ± 11.0 (7)</td>
<td>24.1 ± 6.3 (4)</td>
</tr>
<tr>
<td>% B220low CD69low</td>
<td>6.5 ± 1.3 (4)</td>
<td>3.6 ± 0.3 (2)*</td>
</tr>
<tr>
<td>% CD4+ CD69low</td>
<td>17.1 ± 3.0 (5)</td>
<td>15.6 ± 1.6 (2)</td>
</tr>
<tr>
<td>% CD4+ CD44high CD62Llow</td>
<td>20.2 ± 1.0 (5)</td>
<td>20.5 ± 2.3 (2)</td>
</tr>
</tbody>
</table>

* Results are mean ± SD. Numbers in brackets denote the number of 14- to 16-wk-old female mice examined in each group. Significance level for comparison of Tg mice with NTg controls for each stain, as determined by Student’s t test with Welch’s correction.

NTg, nontransgenic.

*, p < 0.05.

**, p < 0.01.

***, p < 0.001.
proximity, making identification of individual susceptibility alleles difficult.

Although the autoimmune phenotype in NZB and NZM chromosome 1 congenic mice arises, at least in part, from different susceptibility genes, the functional defects that lead to the generation of this phenotype may be similar. Both mouse strains have genetic loci that predominantly affect T cell activation and distinct genetic loci that lead to altered B cell activation (21). In addition, both strains have B cell defects that lead to increased expression of costimulatory molecules on B cells, and further enhance T cell activation, autoimmune production, and development of glomerulonephritis. Thus, the functional defects leading to lupus may be much less heterogenous than the genetic defects leading to lupus, a finding that suggests that therapies directed toward correction of specific functional defects may be more broadly effective than gene-targeted therapy.

What defect is leading to the abnormal B cell activation in B6.NZBc1(35–106) mice? We show that recent bone marrow emigants have an activated phenotype in these mice and that this activated phenotype is associated with an increased proportion of TL1 cells. It is unlikely that this B cell activation results from interaction with activated T cells because in NZB mice the increased proportion of B cells with up-regulated costimulatory molecules is CD40L independent (V. Roy and J. Wither, unpublished observations). Furthermore, altered expression of costimulatory molecules is already seen in B cell precursors in the bone marrow. Previous work by others indicates that self-reactive B cells can up-regulate costimulatory molecules following Ig receptor engagement in the bone marrow (16). Consequently, we postulated that the altered expression of costimulatory molecules in B6.NZBc1(35–106) mice results from altered signaling of B cells within the bone marrow following Ig receptor engagement with self Ags. The observation that a nonself-reactive Ig receptor transgene normalizes the B cell activation phenotype is consistent with this hypothesis. At present it is unclear whether the altered response to Ig receptor engagement results from a decreased threshold for B cell activation or decreased apoptosis of self-reactive B cells; however, the altered proportions of various peripheral B cell subsets in B6.NZBc1(35–106) mice would tend to support the latter possibility. In general, a decreased threshold for B cell activation is associated with expansion rather than contraction of the marginal zone B cell subset (25). In addition, a central B cell apoptosis defect could lead to an increased proportion of immature B cells emerging from the bone marrow, such as observed in B6.NZBc1(35–106) mice. Our preliminary analysis of bone marrow in B6.NZBc1(35–106) mice, in which we found a significantly increased proportion of B cell precursors and expansion of the pre-B cell compartment, provides support for a B cell developmental abnormality in these mice, but does not permit discrimination between these two possibilities.

One of the most dramatic findings in this study was that correction of the B cell defect by introduction of an Ig transgene dramatically normalized the manifestations of autoimmune disease. We demonstrate that not only are autoantibody production and nephritis corrected, but also splenomegaly, the increased proportion of germinal centers, and to a large extent T cell activation. Thus, autoreactive B cells play a pivotal role in driving the autoimmune process in B6.NZBc1(35–106) mice, as has been demonstrated for MRL. lpr/lpr lupus-prone mice (26). Nevertheless, several observations suggest that there are also B cell-independent immune defects in B6.NZBc1(35–106) mice. In young B6.NZBc1(85–106) mice, expansion of memory T cells occurs in the absence of B cell phenotypic changes or an increased number of germinal centers, implicating other APC populations in the activation of these cells. This finding is somewhat surprising in view of previous work suggesting that both proposed NZB candidate lupus susceptibility genes located within this interval, Ifi202 and Fcgr2b, are predominantly expressed in B cells (12, 13). Nevertheless, the observation that introduction of the anti-HEL Ig transgene onto the B6.NZBc1(35–106) background failed to completely normalize the expansion of CD4+ T cells to the levels seen in B6 mice supports a role for a B cell-independent T cell activation defect in these mice. It is notable that in NZM chromosome 1 congenic mice, which are phenotypically similar to our NZB chromosome 1 congenic mice, T cell activation resulted from an intrinsic T cell defect (27, 28).

The presence of abundant germinal centers in B6.NZBc1(35–106) mice in the absence of immunization or infection suggests aberrant T-B collaboration between self-reactive B and T cells. We have recently demonstrated, by adoptively transferring anti-HEL Ig Tg B cells into soluble HEL recipient mice, that in contrast to normal B cells, naive self-reactive NZB B cells can solicit and receive help from self-reactive NZB T cells, leading to recruitment of self-reactive B cells into germinal centers and differentiation into Ab-forming cells. Given the abundance of germinal centers in B6.NZBc1(35–106) mice, it is tempting to speculate that this defect will localize to chromosome 1. In ongoing experiments, using the anti-HEL Ig Tg model, we are examining this possibility together with the possibility that other B cell tolerance defects contribute to the genesis of the autoimmune phenotype in these mice.

Acknowledgments

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1705

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