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Autoimmune Alterations Induced by the New Zealand Black Lbw2 Locus in BWF1 Mice

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The New Zealand Black (NZB) Lbw2 locus (lupus NZB × New Zealand White (NZW) 2 locus) was previously linked to mortality and glomerulonephritis, but not to IgG autoantibodies, suggesting that it played a role in a later disease stage. To define its contribution, (NZB × NZW)F1 hybrids (BWF1) containing two, one, or no copies of this locus were generated. Lack of the NZB Lbw2 indeed reduced mortality and glomerulonephritis, but not serum levels of total and anti-DNA IgG Abs. There were, however, significant reductions in the B cell response to LPS, total and anti-DNA IgM and IgG Ab-forming cells, IgM Ab levels, and glomerular Ig deposits. Furthermore, although serum IgG autoantibody levels correlated poorly with kidney IgG deposits, the number of spontaneous IgG Ab-forming cells had a significant correlation. Genome-wide mapping of IgM anti-chromatin levels identified only Lbw2, and analysis of subinterval congenics tentatively reduced Lbw2 to ~5 Mb. Because no known genes associated with B cell activation and lupus are in this interval, Lbw2 probably represents a novel B cell activation gene. These findings establish the importance of Lbw2 in the BWF1 hybrid and indicate that Lbw2, by enhancing B cell hyperactivity, promotes the early polyclonal activation of B cells and subsequent production of autoantibodies. The Journal of Immunology, 2005, 174: 5065–5073.

New Zealand Black (NZB) × New Zealand White (NZW) F1 (BWF1) hybrid mice inherit a spontaneous autoimmune disease that closely resembles human systemic lupus erythematosus with multigenic contributions from both parental strains required for the full clinical picture (reviewed in Ref. 1). Genome-wide mapping of lupus-related traits using a variety of crosses and strain combinations has identified several chromosomal regions from the NZB and NZW strains that predispose to disease (2–4). In our study analyzing BWF2 intercross mice, eight loci were identified on chromosomes 17, 4, 5, 6, 7, 18, 1, and 11 (lupus NZB × NZW locus (Lbw) 1–8, respectively) that had linkage to mortality, glomerulonephritis (GN), anti-chromatin Abs, and/or splenomegaly (5). Among the most prominent was the locus on chromosome 4 (Lbw2), linked to mortality, GN, and splenomegaly, but not to IgG anti-chromatin Ab levels. This susceptible quantitative trait locus (QT) appeared to be dominantly transmitted from the NZB genome and was present in nearly all F2 mice with early mortality. Several other chromosome 4 loci, Nba1 (6), Sle2 (7), and Imh1/Mott (8, 9), have been identified by other groups, but the relationships of these loci have not been defined. The Nba1 and Sle2 loci were linked to GN and/or mortality using backcrosses of NZ mice or the NZM/Aeg2410 recombinant inbred strain, respectively. They mapped to the same general vicinity as Lbw2, although the predicted locations of the three loci differed, and in the case of Sle2, the chromosome region with the strongest linkage was NZW derived, whereas the predisposing background for Nba1, like Lbw2, was the NZB strain. Moreover, the Sle2 locus is recessively transmitted (7), whereas the Lbw2 and Nba1 loci are inherited as dominant traits. Two other NZB loci, Imh1 and Mott, were identified in separate studies limited to analysis of chromosome 4 by linkage to hyperproduction of IgM (8) or accumulation of Mott cells (9), respectively. They map to virtually the same location and probably represent a single locus. A final locus predisposing to autoimmune hemolytic anemia (Aia1) in NZB mice was also mapped to distal chromosome 4 (10).

To further probe the role of the Lbw2 locus, we used reciprocal Lbw2-interval congenic strains on the NZB or NZW backgrounds to generate BWF1 mice containing two, one, or no copies of the NZB Lbw2 interval. The absence of the NZB Lbw2 interval significantly reduced GN and mortality in the BWF1 background. This was associated with reductions in the B cell response to LPS, serum polyclonal IgM and IgM autoantibodies, and IgG deposits in the glomeruli, but not in serum levels of total IgG or IgG autoantibodies. Genome-wide composite interval mapping of IgM anti-chromatin autoantibodies in BWF2 mice identified only a single locus overlapping Lbw2.

Materials and Methods

Mice

Mice were bred and maintained at The Scripps Research Institute. Reciprocal NZB and NZW interval-specific congenic mice (NZB.NZW-Lbw2 and NZW-NZB-Lbw2) containing the Lbw2 interval (D4Mit17 to D4Mit126, 31.4–71.0 cM) were generated by six backcrosses using a speed-congenic approach with 85 outside markers (11). This region overlapped with Sle2 (38.0 cM) (7) and Agn21 (6.5–71.0 cM) (12). Littermate BWF1 mice, heterozygous or homozygous for the NZB Lbw2 locus, were generated by crossing NZW with (NZB × NZW-NZW-Lbw2)F1, mice, then selecting BWF1 mice that were either fully heterozygous or NZW at the Lbw2 interval. Similarly, littermate BWF1 homozygous for the NZB Lbw2 locus and heterozygous control BWF1 mice were obtained by crossing...
NZB with (NZW × NZW.NZB-Lbw2)F1, mice. To generate subinterval BWF1.NZW-Lbw2 congenic mice, (NZB.NZW-Lbw2 × NZB)F1 with the desired crossovers were mated with NZB, then offspring containing the NZW interval were intercrossed to yield homozygous subinterval NZB congenics. These mice were then crossed with NZW mice to produce the BWF1 subinterval lines.

**Immunopathology**

Serum was obtained at regular intervals, and autopsies were performed at 10–12 mo (5). Proteinuria was measured by Bio-Rad protein assay. Tissues were fixed in Bouin’s or zinc formalin solution, and sections were stained with periodic acid-Schiff reagent and hematoxylin. Glomerular IgM and IgG deposits were detected by staining frozen kidney sections with either anti-IgM- or anti-IgG-FITC (Vector Laboratories) (13). The severity of GN and deposits was blindly graded on a 0–4 scale (14).

Ig, autoantibody, and immune complex assays

ELISAs for IgM and IgG total and autoantibodies were performed as previously described (5, 15, 16). IgG subclasses were measured using alkaline phosphatase-conjugated goat anti-IgG subclass-specific Abs (Caltag Laboratories). Standard curves were generated using a calibrated mouse serum (The Binding Site). For Matrigel matrix ELISA, wells were first coated with 50 µg/ml poly-L-lysine overnight, then with 425 µg/ml well growth factor-reduced Matrigel matrix (BD Biosciences). For the glomerular binding ELISA, wells were coated with sonicated rat glomerular extract as previously described (17, 18). Circulating immune complex levels were determined by IgG ELISA of 2.5% polyethylene glycol serum precipitants (19).

**Flow cytometry**

Cells, isolated by standard procedures, were stained with combinations of fluorescent dye- or biotin-conjugated Abs to CD3, CD4, CD5, CD8, CD19, IgM, IgD, and IgM (BD Pharmingen) was added to the final incubation. Data were acquired on a Vantage SE equipped with OminiComp Dual Beam Enterprise krypton/argon lasers (50,000–200,000 events) and analyzed with CellQuest software (BD Biosciences). Voltage was normalized with calibration particles (Sperotech). For FACS sorting, splenocytes were stained with Abs to CD21, CD23, and B220, and three populations were collected: B220<sup>+</sup>CD21<sup>+</sup>CD23<sup>−</sup> (follicular B cells), and B220<sup>−</sup>CD21<sup>−</sup>CD23<sup>−</sup> (marginal zone (MZ) B cells), and B220<sup>−</sup>CD21<sup>−</sup>CD23<sup>+</sup> (follicular B cells).

**Proliferation assays**

*Escherichia coli* LPS (O55:B5) was obtained from Difco. Thymidine incorporation was performed as previously described (20).

**ELISPOT assay**

The method reported by Sedgwick and Holt (21) was used with ELISA plates coated with anti-IgM or IgG Abs (5 µg/ml), or calf thymus ssDNA or dsDNA (25 µg/ml). DNA plates were precoated with poly-L-lysine (60 min, 37°C). Matrigel plates were purchased from BD Biosciences.

**Elution of Abs**

Kidney and liver sections were weighed, homogenized, then washed four times with PBS to eliminate unbound Igs. Pellets were resuspended in 0.02 M citrate buffer, pH 3.2, then incubated at 37°C for 2 h with mixing. After centrifugation (30 min, 2000 rpm), neutralized supernatants (0.1 M NaOH) were dialyzed overnight at 4°C against PBS and concentrated by ammonium sulfate precipitation.

**Microsatellite analysis**

PCR of tail DNA using microsatellite-specific primers was performed as previously described (5). Markers defining the Lbw2 interval included D4Mit9, D4Mit28, D4Nds2, D4Mit16, and D4Mit48. D4Tsri44, located at 12.1 Mb, was identified by the following primers: 5'-CCACCACTAG-GCTTAGCTAAA and CCCACCTGGTAGAAGAGATT. One hundred four markers encompassing all autosomal chromosomes were used in the genome-wide scan (5) and are available by request.

**Composite interval mapping**

This approach combines multiple regression and interval mapping to detect QTLs (22). The BWF2 linkage map was constructed with Mapmaker/exp version 3 (23), and composite interval mapping of QTL for IgM anti-
dsDNA autoantibody levels was performed with QTL Cartographer version 1.13g (24). The order of the markers is tentative, however, because of the incompleteness of available genome maps. Model 6 of the Zmapqtl package was used with 2-cM intervals, 10-cM window size, and four background parameters. Significance levels were determined by 1000 random permutations of IgM anti-chromatin levels and genotypes for α = 0.1 and 0.05, which, respectively, correspond to the suggestive and significant threshold criteria proposed by Lander and Kruglyak (25) for genome-wide linkage.

**Statistics**

Survival was analyzed by the Kaplan-Meier statistic with censored events, and significance was determined by log-rank test. Unless indicated, the unpaired t test was used to compare groups, and Fisher’s r to z was used for correlation tests. A value of p < 0.05 was considered significant.

**Results**

**Survival of BWF1.NZW-Lbw2 and BWF1.NZW-Lbw2 subinterval mice**

NZB.NZW-Lbw2 interval-specific mice were crossed with the NZW strain to generate littermate BWF1 hybrids with either one or no copies of the lupus-promoting NZB-Lbw2 locus. Cumulative survival was significantly increased in mice lacking the NZB chromosome 4 fragment (Fig. 1A). Twelve-month survival was 53% compared with 7% for Lbw2 heterozygous animals (p < 0.0002), and the 50% mortality age was extended by at least 4 mo.

Three additional BWF1.NZW-Lbw2 subinterval hybrids with smaller NZW genomic regions, designated lines A, B, and C (Fig.

**FIGURE 1.** Cumulative survival of BWF1 mice with one or no copies of the NZB chromosome 4 interval containing the Lbw2 locus and BWF1.NZW-Lbw2 subinterval hybrids. A, BWF1 and BWF1.NZW-Lbw2 mice were generated by crossing NZW mice with (NZB × NZW.NZW-Lbw2)F<sub>1</sub> mice and selecting for mice with no (dashed line) or one (bold line) NZB-derived chromosome 4 interval containing the Lbw2 locus. Survival at 1 year was 7 of 15 and 1 of 15 for mice with no or one copy of the NZB Lbw2 chromosome 4 interval, respectively (p < 0.0002). BWF1.NZW-Lbw2 sublines were generated by crossing NZB.NZW-Lbw2 sublines (A, B, and C) to NZW mice. Mice were followed for 10–12 mo (n = 12–20 mice/group). A value of p ≤ 0.01 for each of the three BWF1 sublines vs BWF1. B, Lbw2 subinterval lines. The NZW chromosome 4 genomic intervals present in NZB, NZW-Lbw2 mice and lines A, B, and C are shown (black lines). Line A contains a small fragment of NZB genome within the NZW interval. Mortality appears to map to a region between D4Mit203 and D4Mit70 (gray line). The map is based on mouse genome assembly NCBI build 33 ([www.ensembl.org]) with polymorphic markers used to determine the introgressed intervals indicated.
Pathology of BWF1 mice lacking the NZB-derived Lbw2 interval

BWF1 mice with no copies of the NZB-Lbw2 had a lower incidence of edema by the time of autopsy (43 vs 89%; \( p = 0.04 \)) and reduced glomerular pathology (\( p < 0.03 \); Table I). To further define the nature of the kidney pathology, an additional set of 8- to 9-mo-old littermates was compared. Mice lacking the NZB-Lbw2 interval had significantly less proteinuria (\( p < 0.008 \); Table I), reduced GN scores (\( p = 0.029 \); Fig. 1A), and, importantly, lower amounts of IgM and IgG deposits (\( p < 0.05 \); Fig. 2B).

In the initial BWF2 study (5), splenomegaly (12-mo-old spleen weight) also strongly mapped to the Lbw2 interval. In the current analysis, however, only 1 of 15 wild-type BWF1 mice was alive at this time point, precluding statistical comparison of spleen weights. When younger cohorts at 2–3, 3–4, 7, and 9 mo of age were examined, no difference in spleen weights between the two groups was observed (Table I and data not shown; \( p > 0.05 \)).

Serological findings in BWF1 mice with and without the NZB-derived Lbw2 interval

When total serum IgM levels were compared, control wild-type BWF1 mice showed a substantial increase between 5 and 7 mo of age, whereas the congenic mice had a slower rise, resulting in significantly lower levels at 7 and 9 mo of age (\( p = 0.007 \) and 0.03, respectively; Fig. 3A). In contrast, levels of total IgG increased only slightly over the same period, and although average levels in congenic mice were lower at all time points compared with those of wild-type littermates, differences did not reach statistical significance (\( p > 0.05 \)).

IgM anti-dsDNA and anti-chromatin Abs increased and were at similar levels between 5 and 7 mo in the two BWF1 groups, but thereafter were lower in mice lacking the NZB-Lbw2 interval (\( p = 0.03 \) and 0.003 at 9 mo of age for anti-dsDNA and anti-chromatin, respectively; Fig. 3B). IgG autoantibodies to dsDNA and chromatin, in contrast, increased to a similar degree regardless of the genotype of the Lbw2 region (\( p > 0.05 \); Fig. 3B).

Although Abs to dsDNA and chromatin are associated with pathogenicity, Abs to ssDNA are more cross-reactive, less pathogenic, and associated with polyclonal activation. Nevertheless, significantly lower levels of IgM anti-ssDNA were also observed in mice without the NZB-Lbw2 interval (\( p = 0.003 \) at 7 and 9 mo of age), whereas virtually identical increases were found in the IgG anti-ssDNA levels (Fig. 3B). Differences in IgM autoantibodies appeared to be due to the inability of congenic mice to increase levels of IgM autoantibodies rather than to a decrease in existing levels. Notably, the effect of Lbw2 on IgM anti-ssDNA Abs occurred at the same time as total IgM, but before IgM anti-dsDNA and anti-chromatin Abs, consistent with IgM anti-ssDNA Ab levels reflecting polyclonal activation. IgM Abs to an irrelevant foreign Ag, BSA, however, were at low levels throughout (data not shown), indicating that the IgM response was nevertheless to some extent Ag dependent. Overall, these findings demonstrate that

### Table I. Pathology of BWF1 mice with one or no copies of the NZB chromosome 4 interval containing the Lbw2 locus

<table>
<thead>
<tr>
<th>Disease Traits</th>
<th>F1</th>
<th>NZW</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edema(^a)</td>
<td>89% (8/9)</td>
<td>43% (6/14)</td>
<td>0.04(^b)</td>
</tr>
<tr>
<td>GN score</td>
<td>3.1 ± 0.16 (9)(^c)</td>
<td>2.5 ± 0.23 (11) ()</td>
<td>0.04(^d)</td>
</tr>
<tr>
<td>Proteinuria(^a)</td>
<td>357 ± 54 μg/ml (29)</td>
<td>175 ± 44 μg/ml (23)</td>
<td>0.008(^e)</td>
</tr>
<tr>
<td>Spleen (9 mo)(^a)</td>
<td>259 ± 50 mg (5)</td>
<td>227 ± 30 mg (7)</td>
<td>&gt;0.05(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Percentage of animals with edema at the time of autopsy (number with edema/total number).

\(^b\) Comparisons made with the Fisher exact test.

\(^c\) Mean ± SE (number of mice). Edema and GN score results are from mice studied for cumulative survival.

\(^d\) Comparisons made with the unpaired \( t \) test.

\(^e\) Total protein in urine from 8- to 9-mo-old mice.

\(^f\) Comparisons made with the Mann-Whitney \( U \) test.

\(^g\) Spleen weights of mice surviving 9 mo.
Lbw2 plays an important role in the early autoimmune activation of B cells.

Although the reduced GN and mortality in BWF1 mice lacking the NZB-Lbw2 interval cannot be accounted for by differences in serum levels of IgG anti-DNA autoantibodies, the lower levels of total and autoantibody IgM in this group suggest that the increased serum levels of IgG anti-DNA autoantibodies, the lower levels of the NZB-Lbw2 interval is caused by some possibility is that Lbw2 modifies the levels of autoantibody IgG subclasses. Indeed, BWF1 mice develop increases in autoantibody production or characteristics. One such alteration in autoantibody production or characteristics. One such possibility is that Lbw2 modifies the levels of autoantibody IgG subclasses. The mean and SE for BWF1 mice heterozygous at the NZW-Lbw2 interval is caused by some possibility is that Lbw2 modifies the levels of autoantibody IgG subclasses.

Levels of circulating immune complexes were also determined in the second set of littermates examined for kidney pathology, because at this time point there was significantly less glomerular Ig deposits in BWF1.NZW-Lbw2 mice. No difference, however, was detected between wild-type BWF1 and congenic mice. The mean and SE for BWF1 mice heterozygous at the NZW-Lbw2 interval (no copies; Fig. 4) and BWF1 mice homozygous for the NZW-Lbw2 interval (no copies; ) are shown. n = 8 – 22.

Spleen and peritoneal cell numbers and composition

No differences were observed in the numbers of splenic B cells and B1 (CD5+) naive (CD21+ CD23+), follicular (CD21hi CD23lo/hi), MZ (CD21hi CD23lo) or activated (CD69+) B cell subsets or in the numbers of T cells and certain T cell subsets, including CD4+, CD8+, activated CD4+ CD69+, and memory/effector CD4+ CD44hi T cells at 1–2, 3–4, and 7–8 mo of age (p > 0.05; data not shown). Similarly, no significant differences were found in the number of memory (CD27+) B cells at 7 mo or in the numbers of peritoneal T, B, and B1 subsets at 1–2 and 3–4 mo (p > 0.05; data not shown).

Response to LPS

Total spleen cells from BWF1 mice lacking NZB-Lbw2 had a reduced response to LPS compared with wild-type mice (mean ± type and congenic BWF1 mice and compared the relative proportions of autoantibodies to total IgG in the kidney eluates. Matrigel consists of extracellular matrix proteins secreted by the Engelbreth-Holm-Swarm mouse sarcoma and includes predominantly laminin, type IV collagen, and entactin. Autoantibodies to unfractionated Matrigel and to these specific Ags have been associated with GN and, in some instances, with cross-reactivity to dsDNA (28–31). Similarly, anti-glomerular binding Abs have been associated with human and murine lupus nephritis (18, 32).

Levels of total, anti-dsDNA, and anti-Matrigel IgG Abs in serum and kidney eluates from BWF1 mice with one or no copies of the NZB-Lbw2 locus. A, Serum levels of IgG Abs. B, Levels of IgG Abs in kidney eluates. Ab concentrations from individual BWF1.NZW-Lbw2 (W; ○) and BWF1 (F; □) are shown. Mean values are indicated by horizontal bars. n = 8–13 mice. p < 0.05.
Table II. Total IgG and IgG autoantibodies in sera and kidney eluates from BWF1 with one (F1) or no copies (NZW) of the NZB Lbw2 locus*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lbw2</th>
<th>No. of Mice</th>
<th>Total (mg/ml)</th>
<th>Anti-dsDNA (µg/ml)</th>
<th>Anti-Matrigel (µg/ml)</th>
<th>Anti-Glomerular (OD)</th>
<th>Ratio Anti-dsDNA/Total</th>
<th>Ratio Anti-Matrigel/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sera</td>
<td>NZW</td>
<td>11</td>
<td>21.8 ± 3.4</td>
<td>343 ± 114</td>
<td>0.047 ± 0.02</td>
<td>0.380 ± 0.060</td>
<td>0.016</td>
<td>2.2 × 10⁻⁵</td>
</tr>
<tr>
<td>F1</td>
<td></td>
<td>11–13</td>
<td>22.4 ± 2.7</td>
<td>430 ± 124</td>
<td>0.065 ± 0.03</td>
<td>0.218 ± 0.054</td>
<td>0.019</td>
<td>2.9 × 10⁻⁵</td>
</tr>
<tr>
<td>p Value</td>
<td></td>
<td></td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>NZW</td>
<td>8–11</td>
<td>0.15 ± 0.02³</td>
<td>0.08 ± 0.03³</td>
<td>0.014 ± 0.001³</td>
<td>0.084 ± 0.012</td>
<td>0.533</td>
<td>0.093</td>
</tr>
<tr>
<td>F1</td>
<td></td>
<td>8–13</td>
<td>0.38 ± 0.07³</td>
<td>0.17 ± 0.03³</td>
<td>0.026 ± 0.002³</td>
<td>0.054 ± 0.011</td>
<td>0.447</td>
<td>0.068</td>
</tr>
<tr>
<td>p Value</td>
<td></td>
<td></td>
<td>0.002</td>
<td>0.03</td>
<td>&lt;0.0001</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>NZW</td>
<td>6</td>
<td>0.025 ± 0.004</td>
<td>Undetectable</td>
<td>Undetectable</td>
<td>ND³</td>
<td>ND³</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td></td>
<td>5</td>
<td>0.039 ± 0.006</td>
<td>Undetectable</td>
<td>Undetectable</td>
<td>ND³</td>
<td>ND³</td>
<td></td>
</tr>
<tr>
<td>p Value</td>
<td></td>
<td></td>
<td>&gt;0.05</td>
<td></td>
<td></td>
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</table>

*Amount of IgG in eluates is expressed as micrograms per milligram of organ wet weight which is considered equivalent to a microgram per milliliter concentration.

²Micrograms per milligram (µg/mg).
³ND = not done.

SEM, 113.278 ± 8,032 vs 144.862 ± 7,474 cpm; p = 0.02; n = 4–6 in 3- to 4-mo-old mice. To determine whether specific subsets of B cells were primarily affected, flow cytometry-sorted splenetic B cell subpopulations from 7- to 8-mo-old littermates were examined for LPS responsiveness. Interestingly, there was a reduced response to LPS primarily in the MZ B cell population from BWF1.NZW-Lbw2 mice compared with wild-type littermates (35,550 ± 5,685 vs 63,437 ± 8,726 cpm; p = 0.019; n = 6–7) as well as a smaller difference in the follicular B cell population that did not reach statistical significance (congenic, 35,997 ± 14,150; wild type, 49,479 ± 15,341 cpm; p > 0.05; n = 4–7).

Spontaneous splenic IgM- and IgG-producing Ab-forming cells (AFCs)

The absence of the NZB-Lbw2 interval in BWF1 mice was associated with reduced numbers of IgM and, at later ages, IgG AFCs in the spleen (Fig. 5). Similar reductions in splenic IgM and IgG anti-dsDNA-producing AFCs were also detected (Fig. 5). Interestingly, a marked increase in IgM anti-dsDNA AFCs was observed in wild-type, but not congenic BWF1, mice between 3 and 4 mo of age, a time preceding the increase in IgG anti-dsDNA AFCs. Significantly, lack of the NZB-Lbw2 interval lowered the ratio of IgM anti-dsDNA AFCs to total IgM AFCs for all ages tested (p < 0.05), particularly between 3 and 4 mo of age, whereas the ratio of IgG anti-dsDNA to total IgG AFCs was not significantly altered at any age (p > 0.05; Fig. 5, lower panels). Similarly, splenic IgG anti-Matrigel AFCs were also significantly reduced in 7- to 10-mo-old congenic mice (5.4 ± 2.9 vs 19.2 ± 2.6/10⁶ cells; n = 5–7 mice; p = 0.004), and this was also related to the reduction in total IgG AFCs, because the proportion of IgG anti-Matrigel AFCs to total IgG AFCs was similar in BWF1 congenic and wild-type mice (6.4 ± 3.3 vs 11.8 ± 2.7; p > 0.05). Thus, there was a specific reduction in the proportion of spontaneous IgM anti-dsDNA-producing AFCs in BWF1 mice lacking the NZB-Lbw2 locus, whereas the decrease in IgG anti-dsDNA and anti-Matrigel AFCs was commensurate with the overall reduction in the number of spontaneous IgG-producing AFCs.

To determine whether these differences were associated with changes in the underlying B cell repertoire, total and anti-dsDNA IgM AFCs were assessed after in vitro LPS stimulation of spleen cells from 1.5- and 3.5-mo-old animals (Table III). The number of IgM AFCs after LPS stimulation was similar in wild-type and congenic BWF1 mice consistent with our observation that Lbw2 did not alter the percentages of B cells or their subpopulations. In contrast, IgM anti-dsDNA AFCs were significantly reduced (p ≤ 0.002) in mice lacking the NZB-Lbw2 locus at both time points, indicating alterations in the B cell repertoire by Lbw2 as early as 1.5 mo of age.

Because previous studies have suggested that the number of AFCs is a better indicator of B cell activation and Ab production than serum Ab levels (33, 34), we examined correlations among IgG AFCs, serum Ab levels, and kidney deposits. The number of total IgG AFCs correlated positively with both numbers of IgG anti-dsDNA AFCs (r = 0.77; p = 0.002) and anti-Matrigel (r = 0.62; p < 0.03) AFCs, but not with serum levels of these Abs (r ≤ 0.5; p > 0.05). Importantly, numbers of IgG AFCs correlated with kidney deposits of total IgG (r = 0.78; p = 0.002), anti-dsDNA (r = 0.68; p = 0.01), and anti-Matrigel (r = 0.77; p = 0.002) Abs, whereas, in contrast, IgG serum levels did not correlate with any type of kidney deposit (r ≤ 0.35; p > 0.05). These findings indicate that the number of AFCs more accurately reflects the production of IgG and IgG autoantibodies than serum levels. Similarly, the number of anti-Matrigel AFCs significantly correlated with anti-Matrigel Abs in kidney (r = 0.67; p = 0.01), but not serum (r = 0.21; p > 0.05), whereas the number of anti-dsDNA AFCs had greater correlation with anti-dsDNA Abs in kidney (r = 0.42; p > 0.05) than in serum (r = 0.19; p > 0.05), although this did not reach statistical significance.

Mortality and immunopathology of BWF1 mice with two copies of the NZB-Lbw2 interval

Littermate BWF1 mice with one or two copies of the NZB-Lbw2 chromosomal segment were also generated and compared. The cumulative survival of mice in the two groups was nearly identical over a 10-mo period (p > 0.05; Fig. 6A), as were the average GN scores at 10 mo (2.8 ± 0.2 and 2.9 ± 0.3, respectively, for BWF1 hybrids with one or two copies of the NZB Lbw2 interval; p > 0.05). The average spleen weights were also similar in mice with one or two copies of the NZB-Lbw2 locus (417 ± 64 and 405 ± 43 mg, respectively; p > 0.05), but were significantly greater than those of the BWF1 mice described above that lacked the NZB-Lbw2 interval (p < 0.02 and p < 0.008, respectively). There was no significant difference (p > 0.05) between the two groups in either polyclonal IgM and IgG levels or levels of IgM and IgG anti-dsDNA Abs (Fig. 6B). These findings are consistent with dominant expression of the NZB-Lbw2 interval.
levels of circulating immune complexes, the number or proportion of T and B cells, or their activation and memory/effector subsets in bone marrow, spleen, or peritoneal cavity. Analysis of subinterval BWF1 congenic mice tentatively localized Lbw2 to a common ~5-Mb interval, and genome-wide mapping indicated that Lbw2 is the major QTL for hyperIgM. These findings now define the component role of the NZB-Lbw2 locus in promoting autoimmunity in the BWF1 hybrid.

An unexpected finding in the initial BWF2 mapping study (5), which we now confirm with congenic mice, was the association of Lbw2 to mortality and GN, but not to levels of IgG anti-chromatin autoantibodies, a trait considered pathogenically related to the development of GN. Because of this, we initially proposed that Lbw2 might act at a disease stage later than autoantibody production. Indeed, unaltered autoantibody levels and glomerular deposits, but reduced kidney damage, presumably from inadequate inflammatory mechanisms, were subsequently reported for lupus-prone mice with complete deletions of the FcRγ-chain (35) or MCP-1 (36), or heterozygous deletion of IFN-γ (37). It is now evident, however, that Lbw2 acts at an early stage of disease pathogenesis, because it promotes in vivo and in vitro activation of B cells, elevated levels of both IgM and IgG autoantibodies, and glomerular deposits of IgG autoantibodies. Furthermore, although serum levels were not significantly altered, reduced numbers of spontaneous IgG AFCs in BWF1.NZW-Lbw2 mice were found, indicating a reduction in total and autoantibody IgG production. This conclusion is supported by the correlation of total and anti-Matrigel IgG AFCs with the amount of kidney deposits and the lack of correlation of serum levels with either number of AFCs or kidney deposits. This lack of correlation is probably due to the dependence of circulating Ab levels on both production and catabolism. Thus, serum levels are a less accurate gauge of Ig production or B cell activation than the number of AFCs, which previous studies have estimated is ~20 times more sensitive (33, 34). Furthermore, the rate of Ig catabolism increases with higher serum levels (38), and in the case of autoantibodies, serum levels may be reduced by the binding of Abs to self-Ags.

The underlying basis for the increased spontaneous AFCs induced by the NZB-Lbw2 locus appears to be the development of hyperresponsive B cells, a well-known feature of NZB mice (39, 40) that we now clearly document to play a major role in disease. In contrast, several other significant B cell alterations observed in NZB mice, including expansion of MZ B cells (CD21high,CD23low,IgMhigh) (41, 42), increased costimulatory molecule expression (42), and increased B1 cells (43), were not affected and are therefore caused by other susceptibility loci. Some of these B cell traits have, in fact, been linked to chromosome 1 (44). Enhancement of B cell responsiveness and development of lupus-like manifestations have been reported with overexpression of Cd19 (45) or lack of Cd22 (46, 47), Hcph (SHP-1) (48, 49), Lyn (50, 51), or Fcgr2b (FcγRIIb) (52). None of these genes, however, is located within the Lbw2 interval.

Our findings also show a greater proportion of anti-dsDNA AFCs after LPS stimulation in BWF1 compared with mice lacking the NZB-Lbw2 locus despite similar numbers of total IgM AFCs (Table III). As noted above, this seems likely to be due to enhanced B cell activation and expansion of autoreactive B cells. Alternatively, this could be due to a defect in central tolerance that results in a greater proportion of self-reactive B cells in the periphery.

Polyclonal B cell activation has been shown to precede the production of autoantibodies and to predict the development of nephritis in lupus-prone BWF1 mice (33). The increased levels of total IgM and IgM anti-ssDNA Abs ~2 mo before the increases in

**FIGURE 5.** Serial analysis of splenic Ig and anti-dsDNA AFCs in Lbw2 congenic and wild-type BWF1 mice. The mean and SE of spontaneous AFCs per 10^6 spleen cells (upper four panels) or the ratio of anti-dsDNA/total Ig AFCs (lower two panels) are shown for BWF1 ( ○ ) and BWF1.NZW-Lbw2 mice ( ⌠ ) at the indicated ages. n = 3–16 mice. *, p < 0.05; **, p < 0.01; ††††, p < 0.001. Similar results, showing reduced numbers of AFCs in BWF1.NZW-Lbw2 mice, were obtained when data were expressed as AFCs per 10^6 B cells (B220^+ cells).

**Genome-wide linkage of the IgM anti-dsDNA Ab response in BWF2 mice**

Because previous studies that mapped the IgM hypergamaglobulinemia of NZB mice to chromosome 4 did not perform a genome-wide search (8), we mapped the IgM anti-chromatin response to chromosome 4 with the same panel of BWF2 mice used previously to identify the Lbw loci (5). Composite interval mapping of the IgM anti-chromatin Ab response revealed significant linkage to the distal half of NZB chromosome 4 (genome-wide significance, p < 0.05; Fig. 7), but no additional QTL that reached suggestive significance. The peak of this QTL overlapped the two markers (D4Mit169 and D4Nds2) with the highest linkage to accelerated mortality (Fig. 7). The data are consistent with a single major NZB genetic defect on chromosome 4 contributing to IgM anti-chromatin Abs and also to accelerated mortality, although the possibility of two or more closely linked genetic defects is not excluded.

**Discussion**

In this study comparison of interval-specific congenic mouse-derived BWF1 hybrids with no, one, or two copies of the NZB-Lbw2 locus chromosome 4 region was used to define the role of Lbw2, the strongest QTL in BWF2 mice, in lupus. Consistent with the original mapping study (5), Lbw2 was dominantly expressed and was found to have a major impact on immune-mediated GN and mortality in BWF1 mice. Additional studies revealed that the absence of the NZB-Lbw2 locus reduced polyclonal IgM and IgG anti-DNA Abs, decreased LPS responsiveness of B cells, particularly the MZ population, reduced numbers of spontaneous IgM and IgG polyclonal and autoantibody AFCs, and led to decreased glomerular IgG autoantibody deposits. Lbw2, however, did not alter
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Table III. Total and anti-dsDNA IgM AFCs in BWF1 mice with no or one copy of the NZB Lbw2 locus

<table>
<thead>
<tr>
<th>Splenic AFCs</th>
<th>1.5 mo</th>
<th>3.5 mo</th>
<th>p Valuea</th>
<th>p Valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NZWc</td>
<td>F1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 mo</td>
<td>3.5 mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total IgM</td>
<td>2.482 ± 299</td>
<td>3.754 ± 85</td>
<td>0.02</td>
<td>2.695 ± 138</td>
</tr>
<tr>
<td>IgM anti-dsDNA</td>
<td>6 ± 3</td>
<td>47 ± 25</td>
<td>0.03</td>
<td>37 ± 9</td>
</tr>
<tr>
<td>LPS stimulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total IgM</td>
<td>24,411 ± 6,433</td>
<td>24,576 ± 3,568</td>
<td>&gt;0.05</td>
<td>11,296 ± 1,392</td>
</tr>
<tr>
<td>IgM anti-dsDNA</td>
<td>121 ± 14</td>
<td>349 ± 72</td>
<td>0.001</td>
<td>116 ± 31</td>
</tr>
</tbody>
</table>

a Lbw2 genotype.
b Comparisons of BWF1.NZW-Lbw2 and BWF1 mice by unpaired t test.
c Mean ± SE of the number of AFCs per 10^6 cells. Number of mice (age: BWF1.NZW-Lbw2, BWF1) = 1.5 mo: 7, 3 and 3.5 mo: 4, 6.

IgM anti-dsDNA and IgM anti-chromatin Abs in BWF1 wild-type compared with BWF1.NZW-Lbw2 congenic mice (Fig. 3B) are consistent with this observation and indicate that Lbw2 promotes autoimmunity by enhancing this early polyclonal activation of B cells. Our findings also suggest that this polyclonal IgM response is not completely nonspecific, but is possibly dependent on both foreign and self-Ag exposure. Interestingly, the absence of the NZB-Lbw2 locus in BWF1 mice, despite reducing total, anti-dsDNA, and anti-Matrigel autoantibodies, resulted in slightly enhanced levels of anti-glomerular Abs. The reason for this is not obvious, particularly because Matrigel and glomerular extracts should have some Ags in common, and Abs to both are clearly highly enriched in the kidney. Nevertheless, these findings indicate significant differences in their fine Ag specificity as well as the effect of Lbw2 locus on autoantibodies to these self-Ags.

The finding that Lbw2 affects primarily IgM, and not IgG, serum levels is also consistent with a previous observation that serum levels of IgM, but not IgG, are predictive of disease in BWF2 mice (43). Nevertheless, it is unlikely that high levels of IgM autoantibodies in BWF1 mice are directly responsible for the observed kidney damage. Previous studies have shown that glomerular eluates from BWF1 are IgG (primarily IgG2a and IgG2b) and predominantly of anti-DNA specificity (53). Furthermore, NZB mice, which uniformly develop high levels of total IgM and IgM anti-dsDNA and anti-chromatin autoantibodies, have only a low incidence of GN (43). Similarly, in the anti-glomerular basement membrane Ab-mediated model, in which GN is produced by the host’s immune response to a single injection of rabbit anti-glomerular basement membrane Ab, CD40-deficient mice, which only produce IgM anti-rabbit Abs, do not develop GN (54). Furthermore, because the FcR γ-chain, but not early complement components, is important for glomerular damage in immune-mediated GN (35), FcR-binding Abs such as IgG2a would be expected to have much greater pathogenic potential than the purely complement-binding IgM. Finally, it has recently been suggested that secreted IgM may protect against GN in lupus-prone MRL-Fas⁻/⁻ mice (55).

The amount and specificity of glomerular IgG eluates from both BWF1 and congenic mice were similar to those in previous studies that showed 25–31 times greater concentrations of anti-dsDNA in BWF1 kidney eluates compared with serum levels (reviewed in Ref. 43). Other studies have suggested that the pathogenicity of anti-dsDNA Abs may be related to cross-reactivity with glomerular membrane proteins (31, 56–58). Our data indicate that glomerular-binding Abs are highly enriched in the glomeruli, but comprise only a minor fraction of the total deposited IgG. Nevertheless, the amounts are significant, and such autoantibodies may be important in initiating glomerular IgG deposition or glomerular damage. Overall, it is likely that combinations of autoantibodies with different specificities and properties contribute to the overall glomerular pathology.

Analysis of subinterval congenic mice identified a common interval between D4Mit203 and D4Mit70 (Fig. 1). This should be considered tentative, because smaller subinterval-specific lines will be required to determine whether this result is due to a single locus or to different combinations of multiple subloci. Nevertheless, the narrowed interval contains 59 potential transcripts, including 38 known genes. Among the potential candidates are CD52 and Map3k6. Recently, an NZB-C1q polymorphism (located at 135 Mb), associated with reduced C1q levels, has been suggested as a possible candidate for the NZB chromosome 4 locus (59). Our findings (Fig. 1, subinterval congenic line C), however, demonstrate that this polymorphism is not required for the reduced mortality in BWF1.NZW-Lbw2 congenic mice.

The Sle2 locus, identified in (C57BL/6 × NZM/Aeg2410) × NZM/Aeg2410 backcrosses and located in the mid to distal chromosome 4, is composed of the NZW genome in the centromeric and the NZB genome in the telomeric parts of the interval, with highest linkage to lupus traits in the NZW region. Sle2 is associated with increases in B1 cells (9, 60) that exhibit increased Ag-presenting capability because of high levels of costimulatory molecules such as B7, LFA-1, and ICAM-1 (61). Because of this, it was hypothesized that the expansion of splenic B1a cells might contribute to disease by augmenting the presentation of autoantigens to pathogenic T cells (61). The Sle2 and Lbw2 intervals overlap, but because of differences in background genome and chromosomal locations, their relationship remains uncertain. Nevertheless, our findings demonstrate that B cell hyperactivity is an NZB trait, and the NZB portion of Sle2 may also contribute to autoimmunity by a mechanism not dependent on increased numbers of B1 cells. Interestingly, the recent observation that MZ, but not follicular, B cells are potent activators of naive CD4⁺ T cells (62) suggests a mechanism by which the increased numbers of activated autoantibody-producing MZ B cells induced by the NZB-Lbw2 locus could enhance autoantibody production.

Another locus, Adnz1, defined using backcrosses of the (C57L/J × NZM2328)F1 to the NZM2328 strain, a NZB/NZW recombinant inbred line, was also mapped to the mid portion of chromosome 4 and has some overlap with the Lbw2 interval (63). In contrast to Lbw2, however, Adnz1 was not linked to GN, but to serum levels of anti-nuclear Abs. This was confirmed by interval-specific congenic NZM2328.C57Lc1 mice, which had markedly reduced antinuclear Abs, but yet GN with similar severity as wild-type NZM2328 (12). The striking differences in autoimmune phenotypes conferred by Lbw2 and Adnz1, despite both BWF1 and NZM2328 being derived from the same NZ strains and examination of virtually the same chromosomal regions, illustrates the
complexity of lupus susceptibility. Further delineation of the relationship of Sle2, Lbw2, and AdncI, should yield important insights into the interactions that determine pathologic B cell activity and the production of autoantibodies.

Interval-specific congenic mice provide the most sensitive means to unequivocally determine the contributions of susceptibility loci (64). In general, two reciprocal types of congenics can be generated, each with certain advantages. Transfer of the susceptible interval to the nonsusceptible background would permit definition of the component phenotype in the absence of other susceptibility loci. This assumes that the nonsusceptible background does not contribute to accelerating or inhibiting the disease phenotype, which may not always be the case (65–67). Another potential disadvantage is that a detectable phenotype may not be expressed in the nonsusceptible background. Contrastingly, the reciprocal subtraction congenic, derived by transferring the nonautoimmune interval to the susceptible strain, permits comparison of autoimmune mice with and without the single predisposing interval, which would provide a direct measure of its impact on overall disease pathogenesis and, importantly, an indication of the potential benefit of therapeutic intervention. In the case of the BWF1 hybrid, however, analysis is complicated by the fact that susceptibility genes from both parental backgrounds are required. Nevertheless, we were able to use crosses of reciprocal interval-specific congenic mice of the NZB and NZW backgrounds to define the role of Lbw2 in BWF1 hybrid mice. Furthermore, the strategy of using (parental × interval-specific congenic)F1 hybrids to cross with the reciprocal NZ strain allowed for control of residual genetic elements. Interestingly, the subtraction model, in which the disease-promoting Lbw2 interval from the NZB strain was substituted with the nonautoimmune NZW interval, provided the best model for defining the contribution of Lbw2.

Finally, susceptibility to spontaneous lupus is polygenic, with each genetic variation contributing to a specific immunopathologic component of the disease process. The finding that removal of a single locus, such as Lbw2, can substantially reduce disease severity provides additional evidence that targeting of one or a few predisposing genes may be beneficial, even in multigenic diseases such as lupus.

FIGURE 6. Clinical manifestations of BWF1 mice with one or two copies of the NZB-Lbw2 interval. A. Cumulative survival. Mice were generated by crossing NZB mice with (NZW × NZW:NBZ-Lbw2)F1 hybrids and selecting those with one (○) or two (■) copies of the NZB-Lbw2 interval. Survivals at 10 mo were 8 of 16 and 8 of 18 mice, respectively, for BWF1 with one or two copies of the NZB-Lbw2 interval (p > 0.05). B. Serum Ig and anti-dsDNA levels. Total serum IgM and IgG (upper panels) and IgM and IgG anti-dsDNA (lower panels) were obtained by ELISA. The mean and SE are shown. n = 5–21, p > 0.05 at all time points.

FIGURE 7. Mapping of survival and IgM anti-chromatin Ab levels for BWF2 mice. Analysis was performed on survival to 12 and 7 mo IgM anti-dsDNA autoantibody levels for 145 BWF2 mice. Chromosome 4 plot of likelihood ratio test (LR) statistics from composite interval mapping for IgM anti-dsDNA levels (solid line) and χ² results for log-rank tests of Kaplan-Meier survival analysis (■). Marker positions, shown below the abscissa (■), are, from left to right, D4Mit1, D4Mit17, D4Mit9, D4Mit205a, D4Mit28, D4Mit119, D4Mit169, D4Nds2, D4Mit16, and D4Mit48. The third and fourth, fifth and sixth, and seventh and eighth markers are closely placed and appear as single triangles. For composite interval mapping, the experimentwise significance levels determined by 1000 permutations of phenotypes and genotypes for α = 0.05 was 16.4 (dashed line above abscissa), and that for α = 0.01 was 14.9. For survival statistics, markers with the strongest linkages were D4Mit169 (χ² = 20.3; p = 4.0 × 10⁻⁵) and D4Nds2 (19.1; p = 7.1 × 10⁻⁴).

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Disclosures
The authors have no financial conflict of interest.

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