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The MHC Haplotype H2b Converts Two Pure Nonlupus Mouse Strains to Producers of Antinuclear Antibodies

Kristian Hannestad2* and Helge Scott†

Studies of mouse lupus models have linked the MHC H2b haplotype with the earlier appearance of antinuclear autoantibodies and the worsening of nephritis. However, it is unknown whether H2b by itself, in the context of pure nonlupus strains, is “silent” or sufficient with regard to loss of tolerance to chromatin (nucleosomes). In this study we show that, beginning 6–9 mo of age, H2b-congenic BALB/c (denoted BALB.B) mice, unlike BALB/c (H2b) and H2b-congenic BALB/c (denoted BALB.K) mice, develop strikingly increased serum levels of anti-chromatin Ab dominated by the IgG2a subclass, along with minor increase of Abs to DNA and moderately increased total serum IgG2a. The BALB.B mice did not have glomerulonephritis or an increased mortality rate. H2b-congenic C3H/He mice (designated C3.5SW mice), unlike C3H/He (H2b) mice, showed low but measurable serum levels of chromatin-reactive IgG2a Abs and minor but significant hypergammaglobulinemia. By immunofluorescence, IgG2a of sera from H2b-congenic strains stained HEp-2 cell nuclei, confirming the presence of antinuclear autoantibodies. Thus, in the context of two pure nonlupus genomes, the MHC H2b haplotype in homozygous form is sufficient to induce loss of tolerance to chromatin. The Journal of Immunology, 2009, 183: 3542–3550.

The classical spontaneous mouse lupus models are the New Zealand Black (NZB), New Zealand White (NZW), and their inbred recombinant NZM2410 strains, males of the BXSB strain, and the MRL/lpr strain. Usually, Abs to chromatin (nucleosomes) appear first (1, 2), followed by hypergammaglobulinemia, splenomegaly, and fatal glomerulonephritis mediated at least in part by the deposition of harmful immune complexes (IC) containing antinuclear Abs (ANA) typically directed to intact chromatin, dsDNA, and ssDNA (3). By activating the Cpg DNA-binding TLR9 in B cells, DNA of chromatin acts as a built-in autoadjuvant that, in combination with BCR signals, recruits and expands chromatin-specific B cells during the autoimmune response (reviewed in Ref. 4). This dual engagement of BCR and TLR9 likely plays a critical, but not necessarily sufficient, role for the initiation and predominance of chromatin-reactive Abs in the early stage of mouse lupus.

Many genes acting together cause mouse lupus, complicating the task of sorting out those genes that have a major impact on autoimmunity to chromatin. The complexity can be reduced by breeding small chromosomal intervals, identified in advance by genetic mapping of lupus traits, onto the nonlupus-prone C57BL/6 (B6) background. In this way it was shown that intervals located on distal chromosome 1 such as Sle1 and its high penetrance sublocus Sle1b from NZW, Nba2 from NZB, and Bsx1–4 from BXSB mice are sufficient to induce production of ANA (reviewed in Refs. 5 and 6). The Sle1b interval contains a number of polymorphic candidate genes (7), among which the Ly108 gene has been considered a strong candidate for mediating the Sle1b phenotype (8).

Another lupus susceptibility locus is the MHC of both mice (known as H2) and humans (HLA) that contains numerous genes including the polymorphic MHC class I and II genes (reviewed in Refs. 9–11). The H2 region of the NZW strain also contains the Sle1 locus, lupus-resistance locus, which is located in an interval that also contains the class II genes (12). From the standpoint of the current report, relevant past studies of B6/lpr, BXSB, and NZB.H2m12 lupus mice have shown that the MHC H2b and H2b-congenic strains revealed evidence that the Eα gene of the H2b locus is defective, expression of the I-E α-chain (Eα-chain) and functional I-E heterodimers are absent in H2b mice (18). Furthermore, a peptide derived from the Eα-chain, Eα52–68, binds to I-Aβ molecules with high affinity, so that in mice expressing both I-Aα and I-Eβ this self-peptide is present in ~12% of I-Aβ molecules (19). Analyses of Eα-transgenic BXSB and related lupus mice have provided evidence that the Eα-chain inhibits lupus autoimmunity but not immune responses against several foreign Ags. Thus, transgene-encoded Eα-chains suppress serological and renal manifestations of lupus and prolong survival, most pronounced for mice that carry an unphysiologically high number (50–100) of transgene copies (20). These data have led to the proposal that the Eα52–68 self-peptide (which is missing in H2b mice) partially blocks I-A molecules of B cells from presenting putative pathogenic self-peptides to Th cells, resulting in the lowering of autoantibody levels (10, 20). According to this hypothesis, production of ANA depends on Th cells, and Eα is a lupus resistance gene.

The H2b haplotype by itself, in the context of pure nonautoimmune backgrounds, has to date not been associated with loss of tolerance to chromatin (21). In this study we demonstrate that, upon aging, H2b-congenic (called H2b here) haplotype-congenic BALB/c and C3H mice spontaneously produce anti-chromatin Abs dominated by the IgG2a isotype. These findings indicate that the H2b haplotype...
Materials and Methods

Mice

BALB/cOlaHsd \((H^2^b)\) mice and the \(H^2^h\) haplotype-congenic BALB.B/OlaHsd (also known as C.B10-H^2^h) and \(H^2^h\) haplotype-congenic BALB.K/OlaHsd mice were obtained from Harlan. The \(H^2^h\) congenic interval of the BALB.B strain is externally delimited by the microsatellite markers D17Mit80 (at 12.9 centimorgans (cM)) and D17Mit232.1 (at 20.37 cM), implying that the size of the congenic interval is 7.47 cM (Ref. 22 and correction from C. Penha-Goncalves, unpublished observations). This interval includes the MHC class I, II, and III genes. Endotoxin-sensitive C3H/HeSnJ \((H^2^h)\) mice and \(H^2^h\)-congenic C3.SW-H2b/SJ mice were purchased from Jackson ImmunoResearch Laboratories. Sera from 9-mo-old B6.Sle1b mice were kindly provided by Dr. E. Wakeland (University of Texas Southwestern Medical Center, Dallas, TX). Unless indicated, all mice were female. Sentinel mice were examined according to recommendations from the Federation of European Laboratory Animal Science Associations and tested negative for parasitical, bacterial, and viral agents. An in-house expanded virus test program revealed that approximately one-third of the sentinel mice tested positive for Abs to mouse norovirus. Animal care was in accordance with national legislation and institutional guidelines.

Preparation of soluble chromatin

To prepare nucleosome core particles, chicken erythrocytes were lysed and the nuclei were washed and digested with micrococcocal nuclease (Worthington Biochemical) as described by Ausio et al. (23). We used 100 U of enzyme per 100 OD_260_ U of DNA for 15 min at 37°C. The digestion was terminated by adding EDTA to a final concentration of 10 mM, the suspension was spun down at 12,000 \(g\) for 10 min, and the supernatant was collected, aliquoted, and stored at \(-70^\circ\text{C}\). The preparation contained a single broad DNA band of \(\sim 150 \text{ bp}\), and electrophoresis on 15% polyacrylamide gel (SDS-PAGE) revealed the characteristic band pattern of the four core histones H2A, H2B, H3, and H4 (data not shown). The same preparation was used throughout the study.

ELISA for specific Abs

Anti-chromatin. Ninety-six-well Nunc Maxisorp plates (Nalge Nunc) were coated overnight with 5 \(\mu\text{g/ml}\) nucleosome core particles in PBS. After washing with PBS, the wells were blocked with 100 \(\mu\text{l}\) of PBS with 1.8% BSA for 1 h at 37°C. Sera typically diluted 1/200, 1/1000, and 1/5000 in PBS, Tween 20, and 0.5% BSA were added to the wells for 1 h. Then, 1/2000 diluted affinity-purified biotinylated goat Abs against mouse Fcγ (Jackson ImmunoResearch Laboratories) or the relevant IgG isotype (SouthernBiotech) were added for 30 min. Finally, bound secondary Abs were detected with 1/3000 diluted streptavidin-alkaline phosphatase (GE Healthcare catalog no. RPN1234V1) followed by \(p\)-nitrophenyl phosphate (p-NPP) substrate. After 30 min, plates were analyzed at 405 nm in a Victor3 V MultiLabel plate reader (PerkinElmer). The wells were washed four times with PBS with 0.05% Tween 20 between each step.

Anti-DNA. Plates precoated with methylated BSA (5 \(\mu\text{g/ml}\)) were coated with 10 \(\mu\text{g/ml}\) dsDNA or boiled ssDNA from salmon sperm. Serum from a patient with active systemic lupus erythematosus served as positive control for coating.

Anti-total histones. Wells were coated with calf thymus histones (Roche Applied Science catalog no. 10 223 565) at 2.5 \(\mu\text{g/ml}\) in 0.1 M carbonate buffer (pH 9.6). An in-house IgG2a mAb (3F7-A10) derived from a patient with active systemic lupus erythematosus served as positive control for coating.

Anti-cardiolipin. Cardiolipin-precoated wells (Varelisca kit) were purchased from Phadia.

Anti-U1RNP, -Sm, -Ro, and -La. Precoated wells (Varelisca Recombi ANA Screen; Phadebas) were purchased from Pharmacia. Other Ag-specific ELISAs were performed by coating with 1 \(\mu\text{g/ml}\) heat-aggregated human IgG (AHGG), 10 \(\mu\text{g/ml}\) boiled LPS, and 5 \(\mu\text{g/ml}\) chicken gizzard actin. Unless specified, all reagents were purchased from Sigma-Aldrich.

Standard curves established by sandwich ELISA

Wells were coated with 2 \(\mu\text{g/ml}\) unlabelled IgG subclass-specific polyclonal goat anti-mouse capture Abs (SouthernBiotech) that were blocked and the corresponding serially diluted purified mouse IgG subclasses (SouthernBiotech) were added at known concentrations. After washing, the standards were revealed with the corresponding biotinylated isotype-specific secondary Abs (SouthernBiotech) diluted 1/2000, AP-streptavidin diluted 1/5000, and p-NPP substrate. The serum Ab level of a given isotype was calculated from the OD of a serum dilution that fell on the steep slope of the standard curve for that isotype. The background OD was subtracted to obtain the final OD values.

Sandwich ELISA of total serum IgM, IgG1, and IgG2a

To wells coated with unlabelled, isotype-specific, goat capture Abs (2.5 \(\mu\text{g/ml}\)) were added appropriate dilutions (determined by pilot experiments) of sera or known concentrations of purified mouse Ig standards for 1 h. After washing, bound Igs were revealed by incubation with AP-conjugated, isotype-specific, goat anti-mouse Abs (SouthernBiotech) and p-NPP substrate. The amount of bound Ig isotype was calculated from OD values of sera dilutions that fell on the steep slope of the standard curve.

Urine analysis

Proteinuria was measured using urinalysis dipsticks (Multistix 8 SG; Bayer Diagnostics) and a Clinitest 500 reader and was graded as follows: +/−, trace; 1+, 0.3 g/L; 2+, 1 g/L; and 3+, 3 g/L.

Statistics

All statistics were calculated using GraphPad Prism version 4 for Windows. A two-tailed Mann-Whitney rank sum U test was applied to the data to assess statistical significance \((p < 0.05)\) is considered significant.

Results

Serological analysis of BALB/c and \(H^2^h\) congenic BALB/c strains

To investigate the impact of the \(H^2^h\) haplotype in the context of pure nonultrapar antibodies on serum Abs to chromatin, we bled mice of the BALB.B \((H^2^h)\) \((n = 17–18)\), BALB/c \((H^2^h)\) \((n = 12–17)\), and BALB.K \((H^2^h)\) \((n = 18)\) strains at 3, 6, 9, 12, and 14 mo of age. ELISA showed that the serum IgG reactivity of BALB.B mice with immobilized nucleosome core particles increased gradually in an age-dependent fashion, indicating a developing autoimmune response to chromatin (Fig. 1). By contrast, the autoantibody levels of mouse norovirus. Animal care was in accordance with national legislation and institutional guidelines.

For the BALB.B group: significant by 9 and 12 mo of age for BALB.B vs BALB/c mice (Fig. 1). At 9 mo of age, 100% of BALB.B mice were deemed positive for IgG anti-chromatin (OD value 4 SD above the mean level of 18 BALB.K mice of the same age). The average IgM anti-chromatin activity was also higher for the BALB.B group: significant by 9 and 12 mo of age for BALB.B vs BALB.K mice \((p < 0.0001)\), and by 12 mo of age for BALB.B vs BALB/c mice \((p = 0.0003)\) (data not shown). These results indicate that BALB.B mice are susceptible to late-onset augmentation of chromatin-reactive IgM and IgG Abs.

Isotypes, specificity, and age and gender dependence of autoantibodies

We assessed IgG subclass levels of chromatin-specific Abs of 14-mo-old BALB.B mice \((n = 17)\). ELISA showed that IgG2a was the dominant subclass; its average concentration was 138 ± 28 \(\mu\text{g/ml}\) compared with 13 \(\mu\text{g/ml}\) for IgG2b, 5.4 \(\mu\text{g/ml}\) for IgG3, and 1.9 \(\mu\text{g/ml}\) for IgG1 (Fig. 2A). Another cohort of 12-mo-old BALB.B mice \((n = 14)\) had average anti-nucleosome levels of 14.8 ± 5.8 \(\mu\text{g/ml}\) for IgG2a, 7.2 ± 3.8 \(\mu\text{g/ml}\) for IgG2b (not significantly different vs IgG2a), 0.69 ± 0.2 \(\mu\text{g/ml}\) for IgG3 \((p = 0.0006)\) vs IgG2a, and 0.12 ± 0.02 for IgG1 \((p < 0.0001)\) vs IgG2a (data not depicted). Thus, the IgG2a/IgG1 ratio of Abs to chromatin ranged from ~70–120. A third sample of 12-mo-old BALB.B mice \((n = 12)\) had an average serum level of chromatin-specific IgG2a and IgM of 81 ± 23 \(\mu\text{g/ml}\) and 19 ± 7 \(\mu\text{g/ml}\), respectively, i.e., an IgG2a/IgM ratio of ~4. These results demonstrate that the autoantibodies of BALB.B mice have a distinct...
profile with respect to isotype distribution: IgG > IgM, and IgG2a > IgG2b > IgG3 > IgG1. For the remaining analyses we focus mainly on chromatin-specific IgG2a.

From 3 to 12 mo of age, the average anti-chromatin IgG2a level of BALB.B mice (n = 17–18) increased ~90-fold, from 0.49 ± 0.06 μg/ml at 3 mo, 1.67 ± 0.47 μg/ml at 6 mo (p < 0.0001 vs BALB.K and p < 0.017 vs BALB/c), 22.1 ± 13.9 μg/ml at 9 mo (p < 0.0001 vs BALB.K and BALB/c), and 45.4 ± 11.6 μg/ml at 12 mo (p < 0.0001 vs BALB.K and BALB/c) (Fig. 2B). By comparison, BALB.K and BALB/c mice were typically negative or low for IgG2a Abs to chromatin (Fig. 2B). Thus, in these cohorts the average serum IgG2a anti-chromatin level of 12-mo-old BALB.B mice was ~35- and ~110-fold higher as compared with BALB/c and BALB.K mice, respectively. The same sera were assayed for ANA activity by indirect immunofluorescence with HEp-2 cells. A homogenous nuclear staining pattern was produced by IgG2a of sera from 15 of 16 BALB.B mice (Fig. 2E), two of 16 BALB/c mice (the same two mice that had anti-chromatin IgG by ELISA; see Fig. 1), and none of 18 BALB.K mice. Serum IgG1 produced no nuclear staining, and no IgG2a staining of HEp-2 cytoplasm was observed.

The average anti-dsDNA IgG2a levels of the sera from 12-mo-old mice were very low: 0.62 μg/ml for BALB.B mice; 0.09 μg/ml for BALB/c mice (p < 0.0001 vs BALB.B), and 0.02 μg/ml for BALB.K mice (p < 0.0001 vs BALB.B) (Fig. 2C). Thus, despite the low BALB.B serum levels of anti-dsDNA Abs, they were significantly higher than those of BALB/c and BALB.K mice. Of 17 sera from 14-mo-old BALB.B mice, 5 (~30%) stood out with respect to elevated levels of IgG2a Abs against both ssDNA (mean of 3.2 μg/ml) and dsDNA (mean of 4.5 μg/ml); four of the five sera also contained ~1.5 μg/ml LPS-reactive IgG2a (Fig. 2D). Of note, the abundance of anti-dsDNA IgG2a of the five sera was 8-, 15-, 62-, 83-, and 94-fold lower compared with anti-chromatin IgG2a. No IgG2a reactivity was detected by ELISA against cardiolipin, actinin, human IgG, U1RNP, Sm, SS-A/Ro, and SS-B/La (our unpublished data). Sera from another sample of 12-mo-old BALB.B mice (n = 12) were analyzed for IgG2a Abs to total histones. ELISA showed that the average level was 0.093 μg/ml for BALB.K mice (p < 0.0001 vs BALB.B and BALB/c), and 0.02 μg/ml for BALB/c mice (p < 0.0001 vs BALB.B) (Fig. 2D). By comparison, BALB.K and BALB/c mice were typically negative or were euthanized before 12 mo of age.

Taken together, we conclude that the bulk of IgG2a autoantibodies of BALB.B mice is directed to intact nucleosome core particles and is not polyreactive. The low level IgG2a reactivity with dsDNA, ssDNA, and LPS found in 30% of BALB.B mice could represent a minor polyreactive fraction (25, 26).

Serum levels of total IgM, IgG1, and IgG2a

We also measured total serum concentrations of IgM, IgG1, and IgG2a (IgG2b and IgG3 were not measured). The most striking finding was that the total IgG2a levels of BALB.B mice rose steeply from 3 mo through to 14 mo of age, whereas those of BALB/c and BALB.K mice rose more slowly and hit a lower plateau (Fig. 3). By 14 mo of age, BALB.B mice had ~2.6-fold more total IgG2a (4.01 ± 0.35 mg/ml) compared with BALB.K (1.52 ± 0.11 mg/ml; p < 0.0001) and BALB/c (1.48 ± 0.14 mg/ml; p < 0.0001) mice (Fig. 3). As the average level of chromatin-specific IgG2a in this sample of 14-mo-old mice was ~0.14 mg/ml (Fig. 2A), the autoantibodies represent a small fraction (3.5%) of total IgG2a. The total serum levels of IgM and IgG1 of BALB.B mice did not differ markedly from those of age-matched BALB/c and BALB.K mice (Fig. 3). Thus, the hypergammaglobulinemia of BALB.B mice mainly presents as a moderate elevation of IgG2a. This result is consistent with that of a previous study showing that H2b° mice with B10 or BALB/c backgrounds have higher serum levels of IgG2a than H2b° and H2a° congenic strains (27).

Induced Ag-specific Ab responses of BALB.B mice

To analyze the IgG subclass of the response induced by a foreign protein Ag, 10-wk-old BALB.B (n = 5) and BALB/c (n = 5) mice were injected s.c. with 200 μg of AHGG, and the mice were bled 15 days later. ELISA showed that all mice of both strains produced similar levels of IgG1 anti-AHGG Abs, but only a single BALB/c mouse had a good IgG2a anti-AHGG response (Fig. 4). Thus, young BALB.B mice have normal responsiveness and produce predominantly IgG1 Abs to AHGG.
Increasing NaCl concentrations have been used to gauge the avidity of Abs to dsDNA (28). We applied this method to assess the avidity of serum IgG2a from 14-mo-old BALB.B mice (n = 5) for immobilized nucleosome core particles. The results are shown in Fig. 5. The average concentration of NaCl that gave 50% of maximal absorbance was 0.32M. In the presence of 0.5M NaCl, the amount of polyclonal IgG2a Ab bound to nucleosomes was reduced by 75–90%, whereas that of three lupus IgG2a mAbs derived from (NZB × BXSB)F1, mice with full-blown lupus (29) was reduced by a modest 25–30%. A likely explanation for the high sensitivity of the BALB.B autoantibodies to moderately increased NaCl concentration is that they depend heavily on electrostatic compared with other forces for binding avidity. This interpretation is consistent with the highly cationic IgH CDR3 regions of antinucleosome mAbs derived from NZM2410 lupus mice (30).

**Spleen weight**

Compared with age-matched BALB/c mice (n = 17), the average postmortem spleen weight of 12- to 14-mo-old BALB.B mice (n = 15) was slightly, but significantly, increased (170 mg vs 140 mg; p = 0.0011).

**Renal studies and survival**

The Multistix scores of urine from 12- to 14-mo-old BALB.B mice (n = 12) did not exceed 1+ (0.3 g/L), showing that the mice did not have clinically significant proteinuria. By light microscopic analysis (Fig. 6), histopathological abnormalities were not found in the kidneys of any of 10 14-mo-old BALB.B mice of the cohort shown in Fig. 2A. The survival of BALB.B mice through to 14 mo of age (95%) did not differ significantly from that of BALB/c and BALB.K mice (92% and 100%, respectively).

**Analysis of C3H and C3.SW mice**

The foregoing results prompted us to assess the nonautoimmune C3H/HeSnJ (H2k) strain and its H2k-congenic C3.SW/SnJ strain. ELISA showed that the mean anti-chromatin IgG2a reactivity of 1/200 diluted sera from C3.SW mice (n = 18) was significantly higher at all ages compared with the C3H control mice (n = 12) (Fig. 7A). The same C3.SW mice at 12 mo of age tested negative for chromatin-specific IgG1 (Fig. 6A) and IgG2b Abs (data not shown; IgM and IgG3 were not measured). At 9 and 12 mo of age, 83 and 72%, respectively, of the C3.SW mice were deemed positive for anti-chromatin IgG2a (OD value 4 SD above C3H mean). The same sera were examined at 1/50 dilution for reactivity of IgG2a with nuclei of HEp-2 cells. The sera from 12-mo-old C3.SW mice had low, but highly significant, levels of IgG2a reactivity to total histones (Fig. 7B) and dsDNA (Fig. 7C) compared with sera from the C3H group; 39 and 27% of the C3.SW mice were considered positive for anti-histone and anti-dsDNA, respectively (OD value 4 SD above C3H mean). The average anti-chromatin IgG2a concentration among the eight 12-mo-old C3.SW mice with the highest ODs was 0.56 μg/ml, i.e., ~25–80-fold lower compared with the mean levels of 15 μg/ml and 45 μg/ml found for two independent samples of age-matched BALB.B mice (see above). The sera from 12-mo-old C3.SW mice had low, but highly significant, levels of IgG2a reactivity to total histones (Fig. 7B) and dsDNA (Fig. 7C) compared with sera from the C3H group; 39 and 27% of the C3.SW mice were considered positive for anti-histone and anti-dsDNA, respectively (OD value 4 SD above C3H mean). The same sera were examined at 1/50 dilution for reactivity of IgG2a with nuclei of HEp-2 cells. Whereas all 12 C3H sera were ANA positive, eight of 17 C3.SW sera (47%) were ANA positive. Of these, four exhibited homogenous staining patterns with more bright fluorescence of mitotic chromatin (Fig. 7D, lower panel), indicative of specificity for DNA/histone. The other four sera showed weak speckled nuclear, but not cytoplasmic, staining (Fig. 7D, upper panel). Collectively, these results indicate that the H2k haplotype, when recombined on a C3H background, converts the mice to producers of ANA.

**Impact of increased NaCl concentration on autoantibody binding avidity**

FIGURE 2. Analysis of IgG autoantibodies. A, Isotype profile of antichromatin Abs. Sera from 14-mo-old BALB.B mice (n = 17) were diluted 5-fold starting at 1/200 and incubated in wells coated with core particles. Bound Ab was detected with biotin-labeled goat anti-IgG subclass Abs and AP-conjugated streptavidin. Ab levels were calculated by comparison with IgG subclass standard curves generated for each plate by sandwich ELISA. The numbers beside the horizontal bars indicate mean (μg/ml). Each symbol represents one mouse. B, Anti-chromatin IgG2a levels as a function of age and H2 haplotype. See legend to A for details of the assay. Shown are means and SEM (n = 17–18). C, Effect of H2-haplotype on levels of IgG2a anti-dsDNA Abs at 1 year of age. See the legend to A for details of the assay. Each symbol represents one mouse. Horizontal bars represent means. Sera from BALB.B (n = 17), BALB/c (n = 16), and BALB.K (n = 17) mice were analyzed. The groups were compared by Mann-Whitney U test. D, Serum levels of IgG2a Abs to ssDNA, dsDNA, and LPS. The ELISA was conducted with sera from 14-mo-old BALB.B mice (n = 17) as described in the legend to A. Note the low Ab levels compared with IgG2a anti-chromatin Abs of A. Each symbol represents one mouse. E, Typical nuclear staining of HEp-2 cells by IgG2a of BALB.B sera. Serum samples from 1-year-old mice were diluted 1/100 and incubated with HEp-2 cells (Immuno Concepts), followed by biotin-labeled goat anti-mouse IgG2a (2.5 μg/ml), and FITC-labeled streptavidin (10 μg/ml).
The total serum levels of IgM, IgG2a, and IgG1 were moderately (2-fold) elevated in C3.SW compared with C3H mice at all ages (IgG2b and IgG3 were not measured) (Fig. 8). Thus, at 12 mo of age the C3.SW vs C3H levels were mean ± SEM 0.98 ± 0.08 vs 0.73 ± 0.04 mg/ml (p = 0.044) for IgM, 0.8 ± 0.13 vs 0.43 ± 0.05 mg/ml (p = 0.0059) for IgG1, and 2.23 ± 0.24 vs 1.25 ± 0.18 mg/ml (p = 0.0031) for IgG2a. The average spleen weight of C3.SW and C3H mice at 12 mo of age was 178 and 150 mg, respectively (not significantly different). All C3.SW and C3H mice survived through to 1 year of age and appeared healthy.

Anti-chromatin IgG2a/c and IgG1 levels of BALB.B vs B6.Sle1b mice

The spontaneous autoimmuneity to chromatin displayed by BALB.B female mice is not unlike that reported for B6. mice. Anti-chromatin IgG2a/c and IgG1 levels of BALB.B vs B6. mice were compared with those of BALB.B mice by Mann-Whitney U test. ***, p < 0.001, **, p < 0.01; *, p < 0.05.

Discuss the findings and implications

In this work we demonstrated the following: 1) H2b in the context of two nonlupus-prone genomes (BALB/c and C3H) is sufficient to induce production of ANA; 2) the bulk of autoantibodies is specific for intact nucleosome core particles; and 3) the most prominent IgG isotype is IgG2a, with some contribution of IgG2b in BALB.B mice. Abs with similar characteristics are conspicuous among the major spontaneous mouse lupus models (3, 31, 32). However, BALB.B and C3.SW mice differ from these models by minimal representation of the IgG1 isotype, low levels of anti-DNA and anti-total histone reactivity, moderate hypergammaglobulinemia, mild splenomegaly, and no renal disease. The average anti-chromatin Ab levels are 25- to 80-fold higher in BALB.B compared with C3.SW mice, indicating that the penetrance also depends on non-MHC loci.

Unlike the spontaneous anti-chromatin response, IgG1 Abs prevailed over IgG2a in the response of young BALB.B mice to immunization with adjuvant-free AHGG. This dichotomy may reflect different compositions of the Ags driving these responses. Thus,
switching to IgG2a is promoted indirectly by Th1 cells producing INF-γ (33–35) or by direct stimulation of TLR9/MyD88 in B cells by DNA rich in nonmethylated CpG motifs (36, 37). However, CpG DNA can also induce Th1-like responses via TLR9 (38) and may therefore activate both pathways. The direct pathway was responsible for the switching of spontaneous anti-DNA Abs from IgM to IgG2a/2b in B6.

FcγRIIB−/− mice transgenic for the high affinity IgH anti-DNA gene 3H9/56R (denoted B6.56R.FcγRIIB−/− mice) (39) and for the induced IgG2a response against a T-dependent nonself Ag mixed with CpG (40). Taken together with the chromatin specificity, these findings suggest that the Ag driving the IgG2a class switch of autoantibodies of BALB.B and C3.SW mice contains CpG-rich DNA, likely in the form of nucleosomes.

The benign humoral autoimmunity of BALB/c and C3.SW mice is not unlike that reported for B6.Sle1 and B6.Sle1b congenic mice that produce IgG autoantibodies preferentially targeted to H2A/H2B/DNA subnucleosomes, with little “spreading” of the specificity to histone-free DNA (41, 42). In this study we show that two cohorts of BALB.B and B6.Sle1b female mice aged 12 and 9 mo, respectively, had similar levels of anti-chromatin (nucleosome) IgG2a/2c and minimal expression of IgG1 autoantibodies. One difference between the strains appears to be the age of onset and the progression of autoimmunity. Thus, for BALB.B mice this type of ANAs is usually clearly detectable by 9 mo of age, and the levels continue to increase through to 14 mo of age. B6.Sle1 female mice, by contrast, have detectable ANA as early as 5 mo of age but the levels peak at 7–9 mo of age and wane thereafter (41).

The lupus susceptibility alleles at the Sle1b locus of NZW mice are present in many standard mouse strains, including BALB/c, C3H/He, and 129, but not B6, mice (7). Are these alleles also involved in the autoimmunity of H2b haplotype-congenic BALB.B and C3.SW mice? Because the vast majority of strains that carry the Sle1b susceptibility alleles are not autoimmune (7), it seems likely that these alleles must interact with polymorphic genes of the normal B6 genome to break tolerance to chromatin. This notion is supported by the finding that, unlike nonautoimmune 129 mice, B6 mice congenic for a 129-derived interval on distal chromosome 1 (denoted 129chr1b) encompassing the Sle1b locus also produce ANA (43). Hence, the genetic backgrounds of BALB.B and C3.SW mice may not be compatible with a role for Sle1 alleles in the ANA responses of these mice. Another question raised by our data, whether the H2b interval of the B6 background is needed for the loss of tolerance to chromatin occurring in B6.Sle1b mice, has not yet been studied.

The lupus of another model, that of FcγRIIB-null B6 mice, does depend on the H2b haplotype. The null mutation of these mice was generated in 129 embryonic stem cells (44) and then backcrossed to B6. The fact that FcγRIIB is tightly linked to Sle1b (45) and that a Sle1b-containing chromosome 1 segment derived from the 129

![FIGURE 6. Representative photomicrograph of kidney sections. Kidneys from 14-mo-old BALB.B mice were fixed in 4% formaldehyde, embedded in paraffin, sections were cut, and slides were stained with H&E plus safran. The image was taken at ×400 total magnification.](image)

![FIGURE 7. Analysis of autoantibodies of C3.SW and C3H mice. Serum samples diluted 1/200 were incubated in ELISA wells containing immobilized Ag. Bound Ab was detected using biotinylated goat Abs to IgG2a or IgG1 and AP-conjugated streptavidin. Shown are OD405 values. Horizontal bars indicate means. Each symbol represents one mouse. A, Reactivity of IgG2a and IgG1 with nucleosome core particles; B and C, Reactivity of IgG2a with total histone (B) and dsDNA (C) at 1 year of age. The values of C3.SW mice (n = 18) and C3H mice (n = 12) were compared by the Mann-Whitney U test. ***, p = 0.0025; ***, p < 0.0001; ns, not significant. D, Representative immunofluorescence staining of HEp-2 cells by IgG2a of 1/50-diluted sera from two C3.SW mice at 1 year of age. For details, see legend to Fig. 2D. Upper panel shows speckled nuclear staining. Lower panel shows homogenous staining with bright fluorescence of mitotic chromatin.](image)
within secondary lymphoid organs to capture chromatin-containing IC and present the autoantigen of the IC in a multivalent, membrane-bound form to BCR, leading to immunological synapse formation with B cells (48). The ensuing strong activation of BCR could overcome the inhibitory signals mediated by FcγRIIB and result in highly efficient delivery of DNA-containing IC to endosomes. In this way, DNA may reach threshold concentrations required for TLR 9 activation (49). This idea is supported by the following: 1) the observation that IC-loaded dendritic cells can recycle native Ag to the cell surface in an FcγRIIB-dependent manner and present it to B cells (50); 2) our previous work showing that FcγRIIB of viable splenic B cells binds IC containing IgG2a (IgG2c) anti-nucleosome mAbs in vivo (29); and 3) a recent report indicating that follicular dendritic cells depend on FcγRIIB for IC loading and strong B cell stimulation (51).

Another explanation for why BALB/c.FcγRIIB−/−.H2b mice remain tolerant may be that they carry a recently discovered allele on BALB/c chromosome 12 (designated sbb22b) that eliminates antinuclear IgG2 production in an otherwise B6.FcγRIIB−/− background (52). Our own data showing that FcγRIIB-sufficient BALB.B mice produce ANA despite carrying the sbb22b suppressor locus suggest that the mechanism underlying their autoimmunity is distinct from that operating in B6.FcγRIIB−/−.H2b mice.

The 7.47 cM H2b-congenic interval of BALB.B mice (see the paragraph titled “Mice” in Materials and Methods) contains numerous genes, including genes encoding the polymorphic Ag-presenting MHC class I and II proteins, complements C2, C4, and B, and the cytokines TNF-α, LTα, and LTβ (where LT is lymphotoxin). Thus, the gene(s) in this interval responsible for triggering ANA is unknown. Inasmuch as the present study has revealed a critical impact of the H2b haplotype on an autoantibody response, and given the normal (nonlupus) genetic background of the mice as well as the role of class II H2 alleles in regulating Ab responses to protein Ag (53), it is tempting to consider I-Ab molecules as critical mediators of the BALB.B and C3.SW phenotypes. In this scenario, the role of the I-Ab allotype is visualized as dependent on its peptide-binding properties, i.e., the higher affinity of the I-Ab molecules of B cells for certain peptides (as yet uncharacterized) when compared with the MHC II molecules encoded by H2d and H2k, leading Th cells specific for those peptides to be strongly activated and aid ANA-producing B cells. This hypothesis, like that advanced by Iwamoto et al. to explain the protective effect of transgene-encoded Eor chains in BXSB and related lupus mice (see Introduction) (20), assumes that the anti-chromatin Ab responses depend on Th cells and autoantigen presentation on I-Ab. In this context, it has been proposed that genetic defects of lupus-prone mice lower TCR activation thresholds for autoantigen (20) (54, 55). However, the normal genetic backgrounds of BALB.B and C3.SW mice suggest that this putative mechanism does not apply to these mice.

With respect to the need for Th cells, a recent study of B6 mice transgenic for the high affinity IgH anti-DNA gene 56R reported that, except in young mice, T cells made little difference for the spontaneous development and isotype switch of anti-dsDNA Abs (56). In contrast, as cited by Tsao et al. (56), earlier studies of mouse lupus models with more normal B cell repertoires, such as MRL/lpr, indicated that T cells play important roles in the production of anti-DNA Abs. In line with this conclusion, clinically healthy MHC II-deficient B6.Sle1 and B6.Nha2 mice showed minimal levels of IgG Abs to chromatin, histones, and dsDNA, indicating the need for Ag presentation to Th cells (57). Furthermore,
although TCRαβ-deficient B6.Sle1 mice produced a high abundance of IgM Abs to ssDNA and chromatin, the amount of chromatin-reactive IgG was minimal, in sharp contrast to TCRαβ-sufficient B6.Sle1 mice (58). Thus, despite the importance of TLR9 signals for eliciting production of anti-chromatin IgG in vivo (59–61), it seems that in the presence of a normal B cell repertoire with low-avidity anti-DNA BCRs, Th cells are also required.

Previous work has shown that inoculation of 3- to 4- wk-old mice with the Moloney leukemia virus induced a higher proportion of BALB.B mice to become ANA positive by indirect immuno-fluorescence and to develop higher ANA titers when compared with 5-mo-old BALB/c mice. Because this difference was not observed in nonviremic mice, it was concluded that high viral production was required for ANA triggering (62). In the present study, spontaneous anti-chromatin IgG became prominent beyond 6 mo, which likely explains why 5-mo-old virus-nonreplicating BALB.B mice tested negative for ANA. Nonetheless, the findings by Pietter et al. highlight the link between environmental provocation, the H2b haplotype, and ANA responses (62). In this context it has been reported that, after LPS stimulation, B cells from BALB.B mice display higher cell activation, cell proliferation, and IgM secretion when compared with B cells from BALB/c mice, suggesting that genetic variation at the MHC region contributes to the control of B cell responsiveness to LPS stimulation (22).

Autoimmunity to chromatin has been suggested to involve several mechanisms, including altered apoptosis, reduced clearance of apoptotic material or IC, altered cytokine environment, disruption of the balance between activation and inhibitory signaling pathways, inefficient BCR editing, and TLR9 activation. Our observation that the H2b haplotype triggers ANA even in the context of genetic backgrounds not prone to lupus indicates that the H2b haplotype contains one or more major lupus susceptibility genes or is lacking genes that confer resistance to ANA responses. Thus, BALB.B and C3.SW mouse strains may be important tools for the dissection of yet other mechanisms involved in loss of tolerance to chromatin.

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Disclosures

The authors have no financial conflict of interest.

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