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Identification of New Pathogenic Players in Lupus: Autoantibody-Secreting Cells Are Present in Nephritic Kidneys of (NZBxNZW)F1 Mice

Stéphanie Lacotte, Hélène Dumortier, Marion Décossas, Jean-Paul Briand, and Sylviane Muller

An important hallmark of systemic lupus erythematosus is the production of autoantibodies specific for nuclear Ags, among which nucleosomes and their constituents, DNA and histones. It is widely admitted that some of these autoantibodies contribute largely in lupus pathogenesis because of their nephritogenic potential. However, the underlying mechanisms are still debated. In this study, we analyzed the autoimmune response against histone H2B during the course of the disease in lupus-prone (NZBxNZW)F1 mice, both in lymphoid organs and kidneys, and we assessed its potential involvement in lupus pathogenicity. We found that the N-terminal region of histone H2B represents a preferential target for circulating autoantibodies, which kinetics of appearance positively correlates with disease development. Furthermore, immunization of preautoimmune (NZBxNZW)F1 mice with H2B peptide 1–25 accelerates the disease. Kidney eluates from diseased (NZBxNZW)F1 mice do contain IgG Abs reacting with this peptide, and this H2B sequence was found to be accessible to specific Ab probes in Ag-containing deposits detected in nephritic kidneys. Finally, compared with control normal mice and to young preautoimmune (NZBxNZW)F1 animals, the frequency of cells secreting autoantibodies reacting with peptide 1–25 was significantly raised in the spleen and bone marrow and most importantly on a pathophysiological point of view, locally, in nephritic kidneys of diseased (NZBxNZW)F1 mice. Altogether our results demonstrate the existence in (NZBxNZW)F1 mice of both a systemic and local B cell response targeting the N-terminal region of histone H2B, and highlight the potential implication of this nuclear domain in lupus pathology. The Journal of Immunology, 2010, 184: 3937–3945.

A nti-histone Abs (AHA) are part of the so-called antinuclear Ab family. Certain AHA subsets recognize individual histones, whereas others preferentially react with histone complexes, associated or not with DNA (e.g., H2A-H2B complexes, H3-H4 complexes, [H2A-H2B]-DNA complexes, or with nucleosomes) (1). In contrast to antinucleosome Abs that almost exclusively occur in systemic lupus erythematosus (SLE), AHA are present with significant frequencies in several systemic and organ-specific autoimmune diseases, including SLE, as well as in infectious and neurologic diseases.

A number of studies designed to decipher the possible involvement of AHA in the development of lupus have been performed in humans and animal models such as (NZBxNZW) F1 (NZB/W) and MRL/lpr mice. It has been described that serum AHA levels significantly decrease just before the appearance of glomerular injury and proteinuria, suggesting that AHA may bind to unsaturated epitopes of the autoantigen in target organs (2). Using rabbit Abs to H2A and H3 histone peptides and mAbs, immune deposits containing histones have been detected in both glomerular and epidermal basement membranes of lupus patients (3, 4) as well as in the kidneys from NZB/W mice and mice with graft-versus-host disease (5). Furthermore, it was reported that patients with both initial anti-DNA Ab and AHA reactivity have a greater risk of developing proliferative glomerulonephritis (6).

How lupus autoantibodies to nucleosome (or dsDNA) act as pathogenic factors still remain a matter of controversies. Diverse pathways have been proposed, among which one possibility involves a direct interaction of circulating autoantibodies with “planted” Ags accessible in the glomerular membrane. These Ags could be nucleosomal structures bound via histone positively charged N termini to intrinsic glomerular components [e.g., heparan sulfate, laminin, or collagen IV (7–10)]. Recent studies suggest that autoantibodies rather react with apoptotic chromatin fragments deposited in the glomeruli of nephritic NZB/W mice and lupus patients, this apoptotic material resulting from a reduced fragmentation and clearance of chromatin (11–13). Another possible pathway involves cross-reactions of lupus anti-DNA/nucleosome autoantibodies with glomerular constituents, such as α-actinin, laminin, mesangial cells, or mesangial matrix structures (14–16). It has also been proposed that already formed circulating immune complexes bind to components of the skin and glomerular basement membrane (GBM). Raised concentrations of circulating nucleosomes are effectively found in SLE patients (17–20) and during the evolution of lupus disease, immune complexes might readily form in the peripheral circulation (21).

In this context, we focused our study on histone H2B, a core histone that shows important properties in lupus. H2B is effectively targeted by lupus autoantibodies, and mAbs were generated from unprimed lupus mice with a higher prevalence compared with the other core histones (22–24). It contains a number of potential sites for post-translational modifications, including apoptosis-specific ones (1). Using overlapping peptides covering the whole sequence of H2B, CD4+ T cell epitopes have been identified in lupus mice and patients.

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among which one that is disease-relevant present in residues 10–33 (25, 26), and a second one encompassing residues 31–50 (27). Strikingly, however, although a few B cell epitopes recognized by circulating autoantibodies have been identified (28), a systematic identification of H2B C cell epitopes is not available in lupus, neither in mice nor in humans. With the aim of unraveling the possible involvement of the autoimmune response targeting H2B in the pathogenic lupus processes, we undertook a detailed analysis of the anti-H2B C cell response that develops in NZB/W mice, both in lymphoid organs and in the main affected target organs, (i.e., kidneys). Our findings fill one of the missing gaps existing in the pathophysiological picture of lupus nephritis by showing that both autoantibody-secreting cells and their cognate autoantigen corresponding to the N-terminal region of histone H2B, are present in the kidneys of diseased NZB/W mice.

Materials and Methods

Synthetic peptides and nucleosomes

Eight peptides covering the entire sequence of histone H2B (Table I) were synthesized using Fmoc solid-phase synthesis, and analyzed for purity and integrity by HPLC and mass spectrometry, respectively. One additional H2B peptide encompassing residues 1–24 to which a cysteine residue was introduced at its C terminus (peptide 1–24(C)) was synthesized, purified, and conjugated to Injekt maleimide-activated BSA (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s recommendations. Mononucleosomes were prepared from LiCl10 m urine cell line as described (29). They were characterized by 1.5% agarose gel electrophoresis (DNA) and 18% SDS-PAGE (histone content).

Mice

Female BALB/c and NZB/W mice were purchased from Harlan (Gannat, France) and maintained in our animal facilities. Animal experiments were approved by our Regional Ethics Committee (No. AL/08/11/03/07). Proteinuria was measured in fresh urine using Albustix (Bayer Diagnostics, Basingstoke, U.K.) and was semiquantitatively estimated according to a 0–5 scale recommended by the manufacturer (no proteinuria = 0; traces = 1; 1+ = 2; 2+ = 3; 3+ = 4; 4+ = 5).

Immunization of NZB/W mice

Fourteen 10-wk-old NZB/W female mice received s.c. 100 μg H2B peptide 1–25 emulsified in CFA (Difco, Detroit, MI) (group 1). In parallel, 14 female mice of the same age received CFA alone (group 2), and 14 other mice were left untreated (group 3). The same treatment was repeated three times, 3 wk apart, except that incomplete FA (Difco) was used instead of CFA.

Cell preparation

Bone marrow (BM) and spleen cells were isolated using standard procedures. Cell suspensions were passed through a 100 μm-cell strainer (Falcon, BD Biosciences, San Jose, CA). RBCs were lyzed using a hypotonic solution. Mononuclear cells were isolated from the kidneys of female NZB/W and BALB/c mice. Briefly, kidneys were finely minced with a razor blade and incubated for 40 min at 37°C with 1 mg/ml collagenase D (Sigma-Aldrich, St. Louis, MO) in HBSS (Lonza, Verviers, Belgium). Fragments were then crushed, and cell suspensions were filtered through a 100 μm- and a 70 μm-cell strainer, successively. The crude renal cell suspensions were washed (150 g, 10 min) with RPMI 1640 culture medium and layered on a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) as described (30).

Detection of Ab-secreting cells by ELISPOT

The 96-well multiscreen plates (Millipore, Billerica, MA) were coated overnight at 4°C with either BSA-conjugated 1–24(C) peptide or BSA alone (40 μg/ml PBS in both cases) or anti-mouse IgG (H+L) (2 μg/ml) (Jackson ImmunoResearch, West Groves, PA) in PBS. After washings with PBS, membranes were saturated for 1 h with RPMI 1640 medium supplemented with 10% (v/v) FCS (Dutscher, Brumath, France), 10 μg/ml gentamycin (Lonza), 10 mM HEPES (Lonza), and 5 × 10–5 M β-mercaptoethanol (Sigma-Aldrich). Serial dilutions of spleen, BM, and kidney mononuclear cell suspensions were then incubated for 3–7 h at 37°C. After washing plates successively with PBS and PBS containing 0.05% (v/v) Tween20 (PBS-T), anti-mouse IgG (Fcγ-specific) biotin-labeled Ig (1:10,000 in PBS-T, Jackson ImmunoResearch) was added for 12 h at 4°C, and subsequently revealed with alkaline phosphatase-labeled extravidin (Sigma-Aldrich) for 1 h at 37°C. Final reaction was detected by adding 5-bromo-4-chloro-indolyl phosphate and nitro-blue tetrazolium chloride substrate (Sigma-Aldrich) and reaction was stopped with water when spots were clearly visible. Spots were counted with a Biorad 4000 (BioSysGmbH, Karben, Germany).

The results were expressed as the number of Ab-secreting cells (ASCs)/106 total cells using the following formula: (number of spots/well – background measured in control wells coated with PBS or BSA) × dilution factor of the cell suspension.

ELISA

Polyethylene plates (MaxiSorb, Nunc, Rochester, NY) were coated overnight at 37°C with the following Ags: histone H2B (200 ng/ml; Roche Applied Science, Meylan, France) or H2B peptides (2 μM) in 0.05 M carbonate buffer (pH 9.6), dsDNA (Sigma-Aldrich; 100 ng/ml in 25 mM citrate buffer, pH 5.4), and mouse mononucleosomes (1 μg/ml expressed as dsDNA concentration in PBS). For the test of Ig in eluates, polystyrene plates (Falcon, Oxnard, CA) were coated with goat anti-mouse Ig Abs (Sigma-Aldrich; 0.5 μg/ml in carbonate buffer). Mouse sera (1:500 in PBS-T), kidney eluates (1:10), or pure cell culture supernatants were added for 1 h, followed by goat anti-mouse IgG1 (1:20,000 in PBS; Jackson ImmunoResearch) supplemented with goat anti-mouse IgG3 (1:7500; Nordic Immunology, Tilburg, The Netherlands) conjugated to HRP. The final steps were as described (31).

Immunoblotting inhibition experiments

Abs to histone H2B were detected by Western immunoblotting using purified calf thymus H2B. The latter (100 μg) was resolved on an 18%–polyacrylamide-SDS gel and transferred to a 0.2 μM nitrocellulose membrane (BioRad, Marnes-la-Coquette, France). The membrane was saturated for 1 h in TBS

FIGURE 1. NZB/W autoantibodies predominantly recognize the N-terminal domain of H2B. A. Sera from 36-wk-old NZB/W female mice (n = 16) tested in ELISA to detect IgG Abs reacting with eight overlapping H2B peptides. Each bar corresponds to one mouse (results expressed as the mean OD of two independent experiments). The cutoff points for positivity were determined with a series of sera from nonimmunized BALB/c mice. Results were considered positive when OD values were higher than the mean OD +2 SD (i.e., OD ≥ 0.15 for all Ags, except for peptides 21–38 and 36–50 [0.3 and 0.9 OD unit, respectively]). B. Western blotting analysis of H2B recognition by Abs present in the serum of a proteinuria-positive NZB/W mouse (left band) and inhibition of this interaction by each of the eight H2B peptides. C. Follow-up of the lifespan of NZB/W mice (% survival, black line), proteinuria (0–5 scale; dotted line) and serum reactivity (% positive sera) toward H2B peptide 1–25 (hatched bars), dsDNA (gray bars), and nucleosomes (black bars). The number of animals alive is indicated at the top of the bars for each time point.
containing 0.05% Tween 20 and 5% (w/v) milk, and subsequently incubated for 2 h at room temperature with NZB/W sera diluted 1:1000 in blocking buffer. For inhibition experiments, sera were preincubated with individual H2B peptides (20 μM) for 1 h at 37˚C and then overnight at 4˚C. After washing, H2B-bound Abs were detected by incubating membranes with alkaline phosphatase-conjugated goat anti-mouse IgG and IgG3. Finally, immunoblots were developed using enhanced chemiluminescent reagent (Amersham Pharmacia Biotech).

Ig elution from kidneys

Kidney eluates from 35-wk-old NZB/W (harboring high proteinuria levels) and BALB/c mice were prepared as described previously (11). A glycine/HCl elution buffer, pH 2.9, was used to eluate bound Ig.

Histology

Kidney specimens were fixed with 4% (v/v) paraformaldehyde (PFA) and embedded in paraffin. Sections (4 μm) were stained with periodic acid-Schiff reagent. Glomerular areas of the kidneys (150 glomeruli/mouse) were observed and scored semiquantitatively using an Image J plug-in, which allows scoring in a blinded manner (scale from 0 to 3; grade 0, no visible lesion; grade 1, mild cell and/or mesangial proliferation; grade 2, marked mesangial proliferation, hyaline droplet plus capillary wall thickening; grade 3, the same as grade 2 plus sclerosis or wire loop lesions).

Immunohistochemistry

Frozen kidney sections (6 μm) were acetone-fixed and blocked with saturation buffer (TBS containing 4% [w/v] BSA, 0.2% [w/v] water fish skin gelatin, and 0.1% Tween 20) for 30 min. To detect glomerular deposits, sections were stained with FITC-conjugated Abs to C3 (1:100; Cappel, West Chester, PA) or IgG (1:10; Jackson ImmunoResearch). Ten glomeruli per mouse were observed and semiquantitated using the Image J plug-in, which allows scoring in a blinded manner (scale from 0 to 3; grade 0, no visible lesion; grade 1, mild cell and/or mesangial proliferation; grade 2, marked mesangial proliferation, hyaline droplet plus capillary wall thickening; grade 3, the same as grade 2 plus sclerosis or wire loop lesions).

Electron microscopy

Mice were perfused with a PFA (4%/glutaraldehyde (0.2% v/v) solution. Kidneys were taken out and cut using a vibrating microtome. Sections (50 μm) were processed for pre-embedding, dehydration, and embedding as previously described (32). Staining was performed using 5 μg/ml LG11-2 mAb directly conjugated to ultrasmall gold particles (Aurion, Wageningen, The Netherlands).

Statistical analysis

Statistical significance was assessed using either a Student t test or a Mann-Whitney U test (for small sample numbers). Survival data were analyzed using Kaplan-Meier plots, and significance was determined using a log-rank test. All p values, 0.05 were considered significant.

Results

The N-terminal region of histone H2B is a preferential target of circulating autoantibodies in NZB/W mice

With the aim of identifying one or several major B cell epitopes in histone H2B, eight overlapping 15–25-mer synthetic peptides spanning the entire protein sequence (Table I) were tested in ELISA using the serum samples from sixteen 36-wk-old NZB/W female mice (Fig. 1A). Strikingly, >70% of the sera (71.5%) contained IgG Abs reacting with the peptide encompassing the N-terminal residues 1–25, whereas <5% of these sera showed IgG reactivity with the other H2B peptides (cutoff values calculated as described in Fig. 1). Weakly

FIGURE 2. Quantification of IgG ASCs and ASCs reacting with the H2B peptide 1–25 in the spleen and BM of lupus mice. The frequency of ASCs (total IgG) and anti-1–25 H2B IgG specific ASCs in the spleen (A) and BM (B) of 32–42-wk-old (proteinuria-positive) and 8-wk-old (proteinuria-negative) NZB/W mice was measured by ELISPOT. The results are expressed as the number of ASCs/×10⁶ mononuclear cells. Control age-matched naive BALB/c mice were used for comparison. Each symbol (●, ●, and ▲) corresponds to one individual mouse, and mean values are shown (horizontal line). Photographs of representative wells are shown. *p < 0.05, Student t test.
positive reactions were recorded on peptides 21–38 and 36–50 with both BALB/c (not shown) and NZB/W sera. In contrast peptide 1–25 was recognized by IgG Abs contained in lupus NZB/W sera only, which reinforces its potential functional relevance. The levels of IgM Abs were weak with all H2B peptides except with peptide 21–38 in a few cases (data not shown).

To assess the relative significance of the N-terminal part of H2B in the cognate antiprotein response, we performed Western blotting experiments in which the eight H2B synthetic peptides were used as competitors of the H2B protein recognition by Abs present in the serum of diseased NZB/W mice. As shown in Fig. 1B (four individual sera were tested and one is shown as an example), the H2B peptide 1–25 was the only one inducing a clear decrease of the signal. This indicates that, as far as continuous/linear epitopes are concerned, a major part of the NZB/W anti-H2B response, if not all, effectively targets the N-terminal region of the protein.

A longitudinal analysis of 16 NZB/W mice starting from the age of 12 wk showed that serum autoantibodies recognizing the H2B peptide 1–25 can be detectable as early as 15 wk (i.e., much before the first clinical signs occur; Fig. 1C). They progressively increase and are found in ~35% of 30-wk-old mice when the disease becomes clinically more acute, as reflected by proteinuria scores and survival characteristics; they are frequent (55–75%) in 35-wk-old mice. Although in a few sera we observed that Abs reacting with H2B peptide 1–25 were not concomitantly detected with Abs reacting with dsDNA and nucleosomes, the overall kinetics of production of 1–25 peptide-reacting Abs positively correlated with that of the latter.

The ELISPOT technique was used to detect and quantify IgG ASCs, and especially ASCs reacting with the H2B peptide 1–25 (Fig. 2). This experiment was carried out using total cells isolated from the spleen and BM of NZB/W mice, either diseased (i.e., proteinuria-positive; 32–42-wk-old) or not (proteinuria-negative; 8-wk-old) and of control BALB/c mice. IgG ASCs were detected much more frequently (15 ± 3 on average; \( p = 0.001 \)) in the spleen of diseased NZB/W mice than in healthy BALB/c mice (Fig. 2A). On the contrary, young NZB/W mice, which have not yet developed the lupus disease, harbored similar IgG-secreting cell numbers than BALB/c mice. More importantly, raised numbers of IgG ASCs specific for H2B peptide 1–25 was the only one inducing a clear decrease of the signal. This indicates that, as far as continuous/linear epitopes are concerned, a major part of the NZB/W anti-H2B response, if not all, effectively targets the N-terminal region of the protein.

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were only detected in the spleen of diseased NZB/W mice (p = 0.01 compared with both BALB/c and young NZB/W mice). These autoantigen-specific cells represent on average 0.25% of the total spleen cell population in diseased mice (2500 specific cells per 1 × 10⁶ spleen cells). It should be kept in mind that direct comparison between tests quantifying ASCs specific for total IgG or for IgG to H2B peptide 1–25 is not possible as the coated Ags are different. IgG ASCs were also detected in the BM of diseased NZB/W mice, although at a lower frequency compared with the spleen (Fig. 2B). Their frequency was only slightly increased compared with the BM of BALB/c mice (2× on average; p = 0.07) or of young NZB/W mice (5× on average; p = 0.02). As in the spleen, IgG ASCs specific for H2B peptide 1–25 could be detected in the BM and were present in higher numbers in diseased NZB/W mice compared with young animals (3× on average; p = 0.02) and healthy BALB/c mice (3× on average; p = 0.02). Altogether our results unambiguously identify the N-terminal region of H2B as a key target of the autoantibody response developing in NZB/W mice.

Development of lupus disease is accelerated in NZB/W mice immunized with H2B peptide 1–25

To assess the potential pathogenic impact of the autoimmune response targeting the N-terminal part of H2B, we immunized 10-wk-old prelupus NZB/W mice with H2B peptide 1–25 in FA and monitored the progression of the disease (Fig. 3A). Mice that received the H2B peptide had a clearly apparent (although nonstatistically significant) shortened lifespan compared with untreated mice or mice that received FA only (Fig. 3B). Their degraded health status was underscored by an important weight loss, which was statistically significant compared with controls (p < 0.05; Fig. 3B). These mice also developed a severe proteinuria, which was significantly more frequent at weeks 23, 27, and 31 (p < 0.05) and much more rapid than in control mice (25 versus 35 wk, when they reached the highest proteinuria score).

A direct evaluation of tissue injury was performed in another set of three groups of NZB/W mice, which were treated as described previously and sacrificed when they were 23 wk old. Glomerular areas of the kidneys were observed and scored semiquantitatively using an Image J plug-in, which allows scoring in a blinded manner. Histological examination of kidneys of the peptide-immunized mice (group 1) revealed an overall deterioration compared with control groups 2 and 3 (Fig. 4). The tissue sections obtained from mice of group 1 showed a dramatic change in the glomeruli morphology, including enlargement, marked mesangial expansion, capillary wall thickening and inflammation. In contrast, lesions in adjuvant-treated NZB/W mice (control group 2) were characterized by mesangial proliferation with lower pathological scores (Fig. 4, lower panel). At the same age (23 wk old), nonimmunized mice of the control group 3 harbored almost intact glomeruli. Kidney sections were also stained for IgG and for the C3 complement component. As shown in Fig. 4, deposits of IgG and C3 into glomeruli were clearly detected in mice of group 1, when they were weakly or not visible in the two control groups 2 and 3. Semiquantitative scoring of the deposit-containing surfaces confirmed this tendency (Fig. 4, lower panel). Taken together, these results strongly suggest that immunizing NZB/W mice with H2B peptide 1–25 leads to an acceleration of kidney damage with increased frequencies of immune complex deposits.

Finally, we performed a sequential measurement of autoantibodies reacting with nucleosomes in NZB/W mice (Fig. 3C). Nucleosome Abs were detected as early as 23 wk of age in the sera of mice immunized with H2B peptide 1–25, whereas the first bleedings showing significant antinucleosome reactivity were collected at weeks 27 and 31 in the mice of groups 2 and 3, respectively. Of note, in mice of group 1, antinucleosome reactivity generally appeared later and persisted longer than Abs to H2B peptide 1–25 induced on immunization (not shown).

Both Abs reacting with H2B peptide 1–25 and the cognate Ag are detectable in NZB/W kidneys

To our knowledge, no cross-reaction between AHA and structural kidney Ags has been described. Therefore, we subsequently addressed two interconnected questions. The first one concerned the possible

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**FIGURE 4.** H2B peptide 1–25 administered in NZB/W mice accelerates the appearance of Ig and complement deposits in their kidneys, as well as the development of glomerulonephritis. The experimental time schedule was as described in Fig. 3, except that mice were killed at 23 wk of age. Staining of frozen kidney sections from untreated mice (group 3, n = 3), mice injected with H2B peptide 1–25 in adjuvant (group 1, n = 7) or mice injected with adjuvant alone (group 2, n = 6) were performed with fluorescent Abs specific either for complement C3 or for IgG (middle and right column, respectively). In parallel, glomerulonephritis was assessed in fixed paraffin sections derived from the same organs upon periodic acid-Schiff reagent staining (left column). Images shown are derived from one representative mouse out of each group (scale bar, 50 μm). Semiquantitative scoring of each parameter (glomerular morphology, C3 and IgG deposits) was performed for the three groups and scores are shown in the lower panel. *Statistically significant difference between values measured in the case of mice that received peptide in adjuvant versus mice that received adjuvant only, p < 0.05, Mann-Whitney U test.
presence of Abs reacting with H2B peptide 1–25 in the kidneys from lupus mice. For that purpose, we eluted Abs bound into the kidneys of 35-wk-old NZB/W mice harboring high proteinuria levels. We found that kidney eluates from these nephritogenic mice contained high amounts of IgG as compared with control eluates from BALB/c mice (Fig. 5A). More interestingly, IgG Abs reacting in ELISA with the H2B peptide 1–25 were present in eluates from five of seven NZB/W mice (71%). Eighty-six and 100% of eluates contained IgG Abs reacting with dsDNA and chromatin, respectively, whereas none of them contained IgG recognizing the H2B peptide 92–110 used as control (Fig. 5A).

The second question concerned the accessibility of residues encompassed in the antigenic H2B region 1–25 in the kidneys from NZB/W mice. First, immunofluorescence staining was performed on frozen kidney slices from proteinuria-positive NZB/W mice and control BALB/c mice, using the LG11-2 mAb, which very specifically recognizes the N-terminal region 6–18 of H2B (24). As shown in Fig. 5B, the labeled LG11-2 mAb reacted with extranuclear Ag deposits present in the glomeruli of diseased NZB/W mice. In contrast, no extranuclear staining was visible in the kidneys of healthy BALB/c mice. A fluorescently labeled isotype control of the LG11-2 Ab showed no staining (data not shown). To get additional information regarding the ultrastructural localization of these H2B-containing deposits, we performed an electron microscopy analysis on kidneys of nephritic NZB/W mice by staining with gold-labeled LG11-2 mAb. Some staining was detected not only in cell nuclei but also in electron dense structures localized within the thick damaged GBM of a diseased NZB/W mouse (Fig. 5C), but not in a healthy age-matched control BALB/c mouse (data not shown). Of note, similar electron dense structures containing apoptotic chromatin particles and representing the binding site of nephritogenic autoantibodies have been described previously (12, 33).

Altogether, our results strongly suggest that histone components encompassing the N-terminal region of H2B are present and accessible in the kidneys of lupus mice. This histone region might thus directly mediate the increase and/or induction of pathogenic effects through the binding of specific autoantibodies and the subsequent cascade of inflammatory events.

**FIGURE 5.** Both H2B peptide 1–25-reacting Abs and their cognate Ag are detectable in NZB/W kidneys. A, Detection of Abs in kidney eluates. *Left panel,* ELISA test of total IgG Abs present in kidney eluates from proteinuria-positive 35-wk-old NZB/W (n = 7) or healthy BALB/c (n = 6) mice. Each symbol corresponds to the result obtained for one individual mouse. *Right panel,* Detection of IgG Abs reacting with H2B peptides 1–25 and 92–110, nucleosomes, and dsDNA, in eluates from the same seven diseased NZB/W mice (gray bars) and one representative BALB/c mouse (black bar). OD values ≥ 0.2 U (horizontal line) were considered positive. B, Detection of the H2B autoantigen in NZB/W kidneys by confocal microscopy using the LG11-2 mAb. Representative fluorescence images of a kidney glomerulus from a control BALB/c mouse and from a nephritic 39-wk-old NZB/W mouse, on staining with AlexaFluor-labeled LG11-2 mAb (green). As expected, some intranuclear labeling is visible. Nuclei were also stained with DAPI (in red for better visualization). In the overlay image, arrows indicate extranuclear H2B-containing Ag deposits. Scale bar, 25 μm. C, Representative electron microscopy images of a kidney glomerulus from a nephritic NZB/W mouse on staining with ultrasmall gold-labeled LG11-2 mAb and silver enhancement. Three regions were magnified to show Ab staining within electron dense structures present in the GBM. Scale bar, 2 μm (low magnification image) or 200 nm (enlarged images). Nuclear staining is also shown (lower right image, scale bar, 500 nm).
Kidneys of diseased NZB/W mice contain cells, which secrete autoantibodies recognizing the N-terminal region of H2B

We have shown above 1) that cells secreting autoantibodies reacting with the N-terminal part of H2B are detectable in the spleen and BM of NZB/W lupus mice, 2) that the N-terminal region of H2B present in immune deposits is accessible to Abs in the kidneys of nephritic lupus mice, and 3) that Abs reacting with this particular region of H2B could be eluted from diseased kidneys. We thus asked whether ASCs could be found at the very same place where the autoantigen is detectable. We isolated mononuclear cells from the kidneys of diseased NZB/W mice and cultured these cells for 5 d in the absence of any stimulus. Culture supernatants were then collected and tested individually in ELISA for the presence of H2B peptide-reacting Abs. Supernatants of cells isolated in the same conditions from the kidneys of BALB/c mice were used as negative controls and the results were expressed as secretion indices. As shown in Fig. 6A, six of nine NZB/W kidney-derived cell supernatants harbored secretion indices above 2 (ranging from 2–11.2), suggesting that they contain autoantibodies reacting with the H2B peptide 1–25. Of note, cell cultures showing the highest secretion levels were those derived from the sickest mice (according to their proteinuria level and their aspect/behavior). Raised Ab levels were also measured in the spleen cell-derived supernatants and the sera of these mice collected concomitantly.

To provide independent evidence of the production by kidney-derived cells of Abs reacting with the H2B peptide 1–25, we used ELISPOT assays. Fig. 6B shows that IgG ASCs, and even more importantly IgG ASCs specific for H2B peptide 1–25, are systematically present in the kidneys of proteinuria-positive NZB/W mice. Very interestingly, these self-specific cells are present with a much higher occurrence than in healthy BALB/c mice, but also than in young NZB/W mice, which have not yet developed a kidney disease (75×more; \( p = 0.005 \)). The number of cells secreting autoantibodies reacting with the N-terminal region of H2B ranged from 120 to 5770 cells per \( 10^6 \) mononuclear cells in the kidneys of diseased NZB/W mice, as compared with 10–40 cells per \( 10^6 \) mononuclear cells in the kidneys of young NZB/W mice. This is the first demonstration of the existence of autoantigen-specific ASCs in the target inflammatory organs of autoimmune mice, and this finding strongly supports the hypothesis of a pathogenic involvement of the H2B N-terminal-specific B cell response in lupus.

Discussion

In the current study, using a whole panel of overlapping H2B peptides, we showed that the N-terminal region of this protein encompasses a dominant target for autoantibodies produced by NZB/W lupus mice in the course of the disease. Our findings confirm earlier data describing the presence of a major B cell epitope in the N-terminal peptide of H2B recognized by the Abs from patients or mice with lupus (34, 35), juvenile chronic arthritis (36) and graft-versus-host disease murine lupus model; (37)). Using the two large cleavage H2B fragments 1–59 and 63–125 and sera from lupus patients, Hardin and Thomas also drew the same conclusion (22). Interestingly in this context, it has also been shown that the N-terminal region of H2B is readily exposed at the surface of mono- and polynucleosomes (29, 38, 39). The predominant reactivity toward this region of the core histone H2B is very likely to be directly correlated to its structural localization in the nucleosomal particle. The autoantibody we describe in the current study and that react with the N-terminal domain of H2B, are not only AHA but also belong to the large antinucleosome Ab family, although they are not nucleosome-restricted autoantibody.

![FIGURE 6.](Image)

**FIGURE 6.** Cells secreting autoantibodies directed to the N-terminal region of H2B are present in the kidneys of nephritic NZB/W mice. Each symbol corresponds to one individual mouse and mean values are shown (horizontal line). A, Mononuclear cells isolated from the spleen and kidneys of diseased NZB/W (32–42 wk of age; \( n = 9 \)) and control age-matched BALB/c mice (\( n = 5 \)) were cultured for five days (\( 1 \times 10^6 \)cells/ml). Supernatants were collected and tested by ELISA to detect secreted Abs able to recognize the H2B peptide 1–25. Corresponding mouse sera (dilution 1:300) were also tested. Results are expressed as a ”secretion index,” a ratio corresponding to OD values obtained with supernatants from NZB/W cell cultures divided by the mean OD value obtained with supernatants from BALB/c cell cultures. Index values >2 are considered significant. B, Numbers of ASCs (total IgG) and anti-1–25 H2B IgG ASCs per \( 10^6 \) mononuclear cells isolated from kidneys of NZB/W (diseased/old, \( n = 14 \); nondiseased/young, \( n = 5 \)) or control BALB/c mice (\( n = 4 \), as measured by ELISPOT. One representative picture of a culture well is shown for each condition. \( p < 0.05 \), Student \( t \) test.

![FIGURE 7.](Image)

**FIGURE 7.** Schematic representation of hypotheses leading to autoantibody kidney binding and lupus nephritis. Two general mechanisms have been proposed to explain the development of typical nephritis in lupus (A, B). Centered on nucleosome immunity, these mechanisms involve either direct binding of nucleosome/dsDNA autoantibodies to inherent glomerular constituents, such as membrane structures or \( \alpha \)-actinin or nucleosome-mediated binding of nucleosome/dsDNA autoantibodies to heparan sulfate, collagen IV, laminin, or other constituents of the GBM (10, 14, 16, 43–48). Based on the present findings in NZB/W mice, an additional player has to be included in this scheme (C). Autoreactive IgG-secreting cells specific for nucleosomal Ag are present into the affected kidneys and locally secrete Abs, which may be pathogenic on binding either to their homologous Ags (identified in this study in nephritic kidneys) or to cross-reactive structures.
The importance of the H2B N-terminal domain in lupus was reinforced in the current study by showing that immunization of young NZB/W mice with peptide 1–25 in adjuvant accelerates the disease, increases proteinuria and provokes generation of a subset of Abs reacting with nucleosomes. Characteristic immune deposits containing IgG and C3 were observed in the kidneys of immunized mice. Datta et al. also showed previously that immunization with nucleosomal-derived T cell epitope containing peptides (H2B peptides 10–33 and two H4-derived peptides) accelerates the development of a severe glomerulonephritis in young lupus SNF1 mice (25). A brief therapy with H2B peptide 10–33 in saline solution decreased the incidence of lupus nephritis in treated mice (40). In NZB/W mice, we cannot exclude that the H2B peptide 1–25 does contain a Th cell epitope in addition to one or several B cell epitopes. However, studies by Suen et al. (27) and by us (not shown) using NZB/W-derived CD4+ T cells do not support this hypothesis.

Autoimmune responses in lupus are usually analyzed at the systemic level but very rarely at the level of affected target organs. In this study, we investigated the presence of H2B-specific ASCs not only in the spleen and BM (the regular final destination of long-lived plasma cells) but also in the kidneys of NZB/W mice. We were able to identify these autoreactive IgG-secreting cells both in the spleen and BM of sick proteinuria-negative NZB/W mice in much higher frequencies than in control animals (BALB/c and young proteinuria-negative NZB/W mice). Surprisingly, the total number of ASCs was quite low in the NZB/W BM, suggesting that in autoimmune conditions, these cells may preferentially migrate to other tissues, such as inflammatory sites, in response to specific chemokine signals (41, 42). Rather than the result of migratory processes, ASCs could also be generated locally in inflamed kidneys, within structures resembling secondary lymphoid tissues. Lymphoid neogenesis is indeed strongly associated with autoimmunity (43), and has been described in several autoimmune disorders among which Sjögren’s syndrome (in salivary glands), rheumatoid arthritis (in the synovium), and in the lipogranulomas developing in the tetramethylpentadecane-induced lupus mouse model (44–46). However, it is still a matter of debate whether these ectopic lymphoid structures merely correspond to a site of immune cells accumulation or a real lymphoid tissue where autoimmune responses develop. Both aspects of ASCs homeostasis in lupus (migration and lymphoid neogenesis) are currently under active investigation in our laboratory.

ASCs have been previously described in the kidneys of lupus mice and patients (47, 48). We confirmed these findings here but much more interestingly, we demonstrate that among these cells, cells secreting Ig reacting with the N-terminal region of H2B are present. Whether these autoreactive cells are plasmablasts or short-lived/long-lived plasma cells remains to be determined. To our knowledge, this is the very first time that cells secreting IgG specific for a nuclear autoantigen are described. This demonstration is particularly important because it was made in the affected organs and moreover, it correlated with the development of the disease. We went even further in the elucidation of a potential pathogenic mechanism by evidencing the presence of the cognate autoantigen itself in the kidneys, more precisely within electron dense structures previously described as being the major, if not the unique, glomerular loci for autoantibody binding in vivo (33). This finding strongly suggests that the recognition of the accessible N-terminal H2B region by the locally produced specific autoantibodies participates to the inflammatory cascade of events leading to nephritis. This allows us to add important information in the proposed pathogenic models leading to typical nephritis in lupus (10, 14, 16, 49–54; Fig. 7). Rekvig et al. recently showed that glomerular apoptotic nucleosomes might be the main autoantibody targets in lupus kidneys (12). As we used the LG11-2 mAb for detecting the N-terminal part of H2B in the kidneys, we may speculate that the autoantigen moiety targeted by this autoantibody, which also reacts with nucleosomes (24), is part of a larger, apoptotic or not, nucleosome complex. Of note, the LG11-2 mAb does not react with a H2B peptide containing the apoptotic-specific phosphoserine residue at position 14 (55; unpublished data).

In summary, the results of the current study confirm the existence of a major epitope antigenic area in the nucleosome-exposed N-terminal part of the core histone H2B. Autoantibodies recognizing this autoantigen are secreted by cells located not only in lymphoid organs but also in situ in nephritic kidneys where they can encounter their antigenic partner, possibly as apoptotic nucleosomes. All conditions are therefore gathered for Ab-mediated nephritis to develop. This novel element completes the pathophysiological picture of lupus glomerulonephritis and tends to clear up the complexity of nephritic process(es) in lupus.

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Disclosures
The authors have no financial conflicts of interest.

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