

Immunisation with histones or nucleosomes triggers lupus disease in susceptible mice

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Abstract

Background: Nucleo-protein instead of DNA could be a major antigenic system at work in the pathogenesis of lupus disease with T cells sensitised against proteins associated to DNA in chromatin, playing a pivotal role. **Objective:** To investigate in young lupus-prone mice that share a genetic background for lupus but do not develop early anti-chromatin autoimmunity and rapid glomerulonephritis, the effects of immunisations with DNA-free histones or nucleosomes on the development of autoimmunity to chromatin. **Methods:** To address this question, Male and female NZB mice, female MRL-Mp^{+/+} mice, female BXSB mice and female Balb/c mice were immunised with histones or nucleosomes at 9, 11 and 13 weeks of age. Autoantibodies were investigated in plasma and the development of albuminuria was looked for from 14 to 21 weeks of age. Immunofluorescent studies were done on kidney at 21 weeks of age. **Results:** We observed in the three lupus-prone strains of mice but not in control Balb/c mice: (1) an auto-immune responses against nucleosomes, histones and DNA after immunisation with nucleosomes; (2) an auto-immune response not only against histones, but also against DNA after immunisation with histones. The induction of anti-chromatin autoimmunity after immunisation with nucleosomes or histones was followed by the appearance of albuminuria, and by the deposition of immunoglobulins and complement in a granular pattern in glomeruli. Conclusion: Lupus-prone mice but not normal mice can develop autoimmunity against proteins associated to DNA in chromatin that triggers anti-DNA antibodies and subsequent development of immune complex nephritis.

Key words: Lupus mice, autoimmunity, autoantibodies, chromatin, glomerulonephritis, apoptosis

Introduction

A hallmark of autoimmune diseases such as systemic lupus erythematosus (SLE¹), sclero-derma, rheumatoid arthritis, type I (insulin-dependent) diabetes mellitus, and dermatomyositis is the production of highly specific autoantibodies that recognize evolutionarily conserved molecules. The mechanisms by which these largely intracellular molecules are recognized as

foreign are poorly understood.

Thanks to the murine models, the lupus is the model more studied of the autoimmune diseases. Among these models: NZB mice at sex mouse develops lupus-like autoimmune disease characterized by production of pathogenic IgG and an immune-complex-induced glomerulonephritis. MRL-lpr/lpr mice carrying the lymphoproliferation (lpr) mutation, have defects in the fas gene they develop a systemic lupus erythematosus-like autoimmune, however MRL-Mp ^{+/+} develop the typical signs of disease late in life (Sex mounths).

¹The abbreviations used are: SLE: Systemic Lupus Erythematosus; ELISA: Enzyme-Like Immunosorbent Assay, DsDNA: double-stranded DNA; NZB: New Zealand Black, Nuc: nucleosome, Hst: Histone, Adj: Adjuvant, CHO: cells of ovary of Chinese hamster. OD: Optical density.

BXSB mice spontaneously develop a human lupus-like autoimmune disease and die from immune complex-mediated glomerulonephritis. A mutant gene, Yaa, located in the Y chromosome of BXSB mice, was found to profoundly enhance autoimmune abnormalities in this strain.

Autoantibodies to DNA and histones are frequently found in the sera of lupus patients and of lupus mice (Hardin, 1986 and Plaué et al., 1989) and are thought to play a pathogenic role in the development of lupus glomerulonephritis (Elouaai et al., 1994; Koffler et al., 1971 and Lambert and Dixon, 1968). In recent years, substantial experimental data have been accumulated concerning possible involvement of nucleoprotein component as antigens at work in lupus autoimmunity (Fournié, 1988, Fournié, 1996 and Rumore and Steinman, 1990). In vivo, DNA does not exist as isolated molecule but is associated to histones in chromatin: In blood, extracellular DNA circulates mainly under the form of mononucleosomes (Carson, 1991 and Fournié et al., 1992). In lupus, nucleosome might well be the autoantigen at work in the induction of anti-DNA antibodies (Mohan et al., 1993). There is now increasing evidence that the nucleosome is the auto-antigen that drives the auto-immune response in SLE. Indeed on the one hand T helper clones specific for nucleosomal antigens have been described in NZBxSWR lupus-prone mice (Tan, 1994), and on the other hand, the possible association of apoptosis and autoimmunity has been highlighted (Casciola-Rosen et al., 1994). Taken together these facts support the hypothesis that T-cell sensitised by structural protein (s) of chromatin can trigger an autoimmune response from resting autoreactive anti-DNA B-cell.

The aim of this study was to test such a possibility by immunising mice sharing a genetic background of lupus mice and normal mice with histones or nucleosomes.

Materials and Methods

Mice: Inbreeding nuclei from, NZB and BXSB mice were purchased from Jackson

Laboratory, Bar Harbor, Maine, USA. Mice were raised under conventional conditions. MRL-Mp^{+/+} and Balb/c female mice were purchased from Harlan, France.

Experimental approach: Groups of 5 (2 males, 3 females) NZB mice, 6 female BXSB, 6 female MRL-Mp^{+/+} and 6 female Balb/c mice were injected three times at biweekly intervals starting at 9 weeks of age with histone (55 µg), nucleosomes (10 µg DNA and 11 µg histones) or PBS. Preparations diluted in PBS and emulsified in complete Freund's adjuvant (1st injection at 9 weeks of age) or in incomplete Freund's adjuvant (2nd and 3rd injection at 11 and 13 weeks of age) were injected intraperitoneally and sub-cutaneously. Plasma and urine samples were collected before immunisation at 9 weeks of age and then at 1, 2, 4 and 8 weeks after the last immunisation i.e. at 14, 15, 17, and 21 weeks of age.

Sample collection: Blood was collected under ether anaesthesia by retroorbital sinus puncture, on EDTA (10-20 mM final concentration, pH 8.0). After centrifugation at 10000 g during 3 minutes, plasma was withdrawn. Urine was collected by vesical pressure. Plasma and urine were stored at -20°C until used.

Biochemicals: Histone preparation of calf thymus containing a mixture of the histones H1, H2A, H3 and H4 was from Boehringer Mannheim, Meylan, France. Histone preparations were assayed from contaminating DNA: after treatment for DNA extraction (Le Lann et al., 1994) no DNA can be found in histone (<20 pg of DNA per mg of histone).

Nucleosomes preparation: Mononucleosomes were obtained from CHO cells (cells of ovary of Chinese hamster) using digestion of chromatin by S7 nuclease and purification of nucleosomal fractions by ultracentrifugation on sucrose gradients as described (Le Lann et al., 1994). DNA size was controlled by agarose gel electrophoresis and the presence of the five histones was controlled by polyacrylamide gel electrophoresis. Nucleosome

concentration was established using spectrophotometry.

Biological determinations: Anti-double stranded DNA antibodies were measured by enzymatic immunocapture assay using Protein A sensitised microtiter plates as described (Elouaai et al., 1994). Anti-histone and anti-nucleosome antibodies were assayed by ELISA as described (Elouaai et al., 1994). Albuminuria was determined by single radial immunodiffusion, while using a rabbit anti-mouse albumin antiserum (ICN Biomedical, Orsay, France).

Kidney studies: Kidneys obtained at autopsy were studied by immunofluorescence on snap-frozen 4 μ m sections coloured with fluorescein-conjugated antisera to mouse IgG (MelyLaboratories) and C3 (Bulk Cappel Immunoreagents). The intensity of staining was scored on a scale from 0 to 2 (0: none or rare; 1: mild; 2 intense lesions) by an observer who had no knowledge of the protocol.

Statistical analysis: Mann and Whitney's U-test was used. P values <0.05 were considered as significant.

Results

Mice survey: Four out of the 15 NZB mice (2 immunised with nucleosome, 2 control mice) died between 15 and 22 weeks of age and one out of the 24 MRL-Mp^{+/+} mouse died between 15 and 17 weeks of age from unknown reasons.

Anti-nucleosome antibodies: In the three strains of lupus-prone mice but not in Balb/c mice, immunisation with nucleosomes triggered the induction of anti-nucleosomes antibodies (figure 1). A slight increase in anti-nucleosome antibodies was also found in mice immunised with histones particularly at the end of the experiment, this increase was only significant in MRL-Mp^{+/+} mice at 21 weeks of age ($p < 0.01$).

Anti-histone antibodies (Table 1): No anti-histone antibody response was observed in Balb/c mice. Significant increase in anti-histone titres following immunisation was observed in the three lupus-prone strains- of

mice. In most cases this increase was transitory with a peak response 2 or 4 weeks after the last immunisation. Different patterns of anti-histone antibody responses to immunisation with histones or nucleosomes were observed according to the strain and to the stimulus (Table 1).

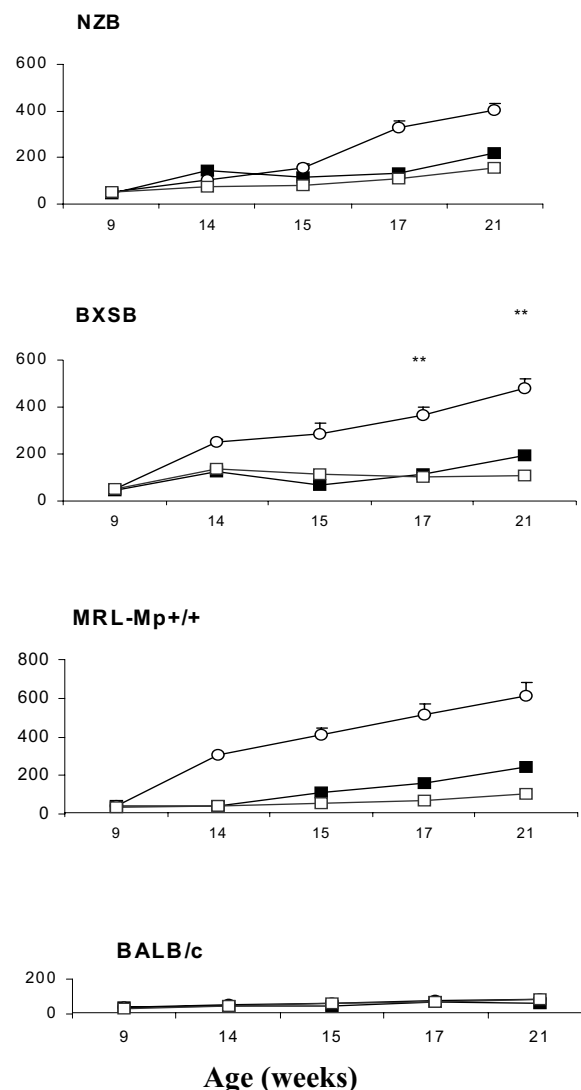


Figure 1. Anti-nucleosomes antibody in NZB, BXSB, MRL-Ipr/lpr and Balb/c mice immunised at 9 (in CFA), 11 and 13 (in IFA) weeks of age with nucleosomes (O), histones (■) and in control mice injected with Freund's adjuvant (□). Symbols represent the mean of OD values obtained in each group in ELISA assay. Antibody levels are expressed as the mean absorbance values (450 nm x 1000) $\pm$ one standard error of the mean (S.E.M; vertical bars). When not seen, S.E.M. are included inside the symbols.

Particularly, anti-H2A histone antibodies were not found in immunised BXSB mice immunised with histones, while they were observed in BXSB and MRL-Mp^{+/+} mice immunised with histone or nucleosome.

Table 1. Patterns of anti-histone reactivity in mice immunized with histones or with nucleosome¹.

Strain	Immunization	Antibody against				
		H1	H2a	H2b	H3	H4
BXSB	Histone	++	-	++	++	++
	Nucleosome	++	+	+	+	+
NZB	Histone	-	++	+	-	++
	Nucleosome	-	++	+	-	++
MRL-Mp^{+/+}	Histone	-	++	-	-	+
	Nucleosome	++	++	+	++	+
Balb/c	Histone	-	-	-	-	-
	Nucleosome	-	-	-	-	-

¹: Results are presented according to the following classification:

-: No significant response

+: Significant response ($p < 0.05$ or 0.01) with less than a two fold increase in antibody titers as compared to levels found in the control group immunized only with Freund's adjuvant.

Anti-DNA antibodies: Immunisations with nucleosome but also with histones trigger the induction of anti-DNA antibodies in the three lupus-prone strains of mice as illustrated on figure 2 that shows the anti-DNA antibody titres expressed as mean of absorbance values. Increase in anti-DNA antibodies in immunised mice as compared to mice receiving the Freund's adjuvant alone reached statistical significance at least in two subsequent occasions (BXSB mice: immunised with histones: $p < 0.01$ at 14 and 15 weeks; immunised with nucleosomes: $p < 0.01$ at 14, 15 and 17 week). NZB mice immunised with histones: $p < 0.05$ at 15, 17 and 21 weeks; immunised with nucleosomes: $p < 0.01$ at 14 and 15 $p < 0.05$ at 17 weeks. MRI-Mp^{+/+} mice immunised with histones: $p < 0.01$ at 14, 17 and 21 weeks; $p < 0.05$ at 15 weeks immunised with nucleosomes ($p < 0.01$ at 14, 15, 17 and 21

weeks). The kinetics of anti-DNA antibodies are increased until the end of the experiment. In Balb/c mice immunised with a slight but not significant increase in anti-DNA antibodies was observed at 21 weeks of age.

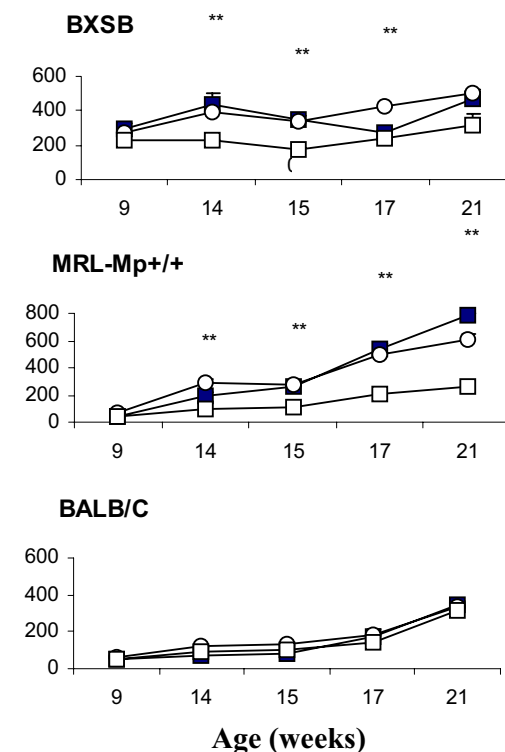


Figure 2: Kinetics of anti-DNA in NZB, BXSB, MRL-Mp^{+/+} and Balb/c mice immunised with histones (■) or nucleosomes (○) and in control mice injected with Freund's adjuvant (□). Symbols represent the mean of OD values obtained in each group in ELISA assay

Kidney involvement. Figure 3 shows that, after immunisation with nucleosome and histones, albuminuria increase in lupus-prone strains of mice but not in Balb/c mice. Increase in albuminuria concentration in BXSB and MRL-Mp^{+/+} mice immunised with nucleosomes or histones as compared to control groups was significant ($p < 0.05$ or $p < 0.01$) from 15 to 21 weeks of age. This increase was also significant in 14 weeks old MRL-Mp^{+/+} immunised with nucleosomes. In NZB mice, due to a lower number of mice, increase in albuminuria concentrations observed in immunised mice was not statistically significant as compared to control mice.

Figure 4 shows the semiquantitative evaluation of IgG deposits in glomeruli. In the majority of lupus-prone mice immunised with nucleosome or histones, intense deposits of IgG were found in glomeruli. These deposits were granular, and located in mesangium and along glomerular capillary walls. In contrast, deposits were less intense in all groups of Balb/c mice as well as in the control groups of lupus-prone mice injected with only the Freund's adjuvant. Similar results were found for C3 deposits (not shown).

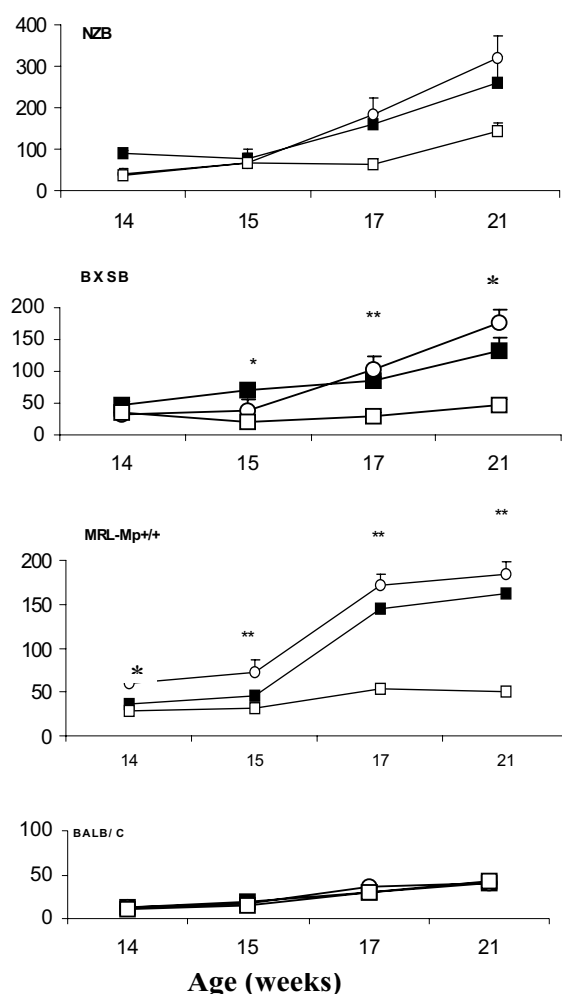


Figure 3. Kinetics of albuminuria ($\mu\text{g} / \text{ml}$) in NZB, BXSB, MRL-Mp $^{+/+}$ and Balb/c mice immunised with histones (■) or nucleosomes (○) and in control mice injected with Freund's adjuvant (□).

*: $p < 0.05$; **: $p < 0.01$ (Significance of results according to Mann and Whitney in immunised mice as compared to mice receiving the Freund's adjuvant alone).

Discussion

This study confirms the role of genetic factors in the development of lupus diseases. In fact it shows that mice with a genetic background of lupus developed serological and pathohistological features of lupus disease under immunisation with nucleosomes or histones while normal Balb/c mice did not.

In the three lupus-prone strains of mice, auto-immune responses were observed against nucleosomes, histones and DNA after immunisation with nucleosomes and against histones after immunisation with histones or nucleosomes. Differences in the pattern of anti-histones antibody induced by histones and nucleosomes could be dependent on several factors including genetic and quantitative factors, as well as factors dependent on the structure of the immunogen. It has been previously reported that carrier molecules modulate the induction of anti-histone antibodies (Muller et al., 1991).

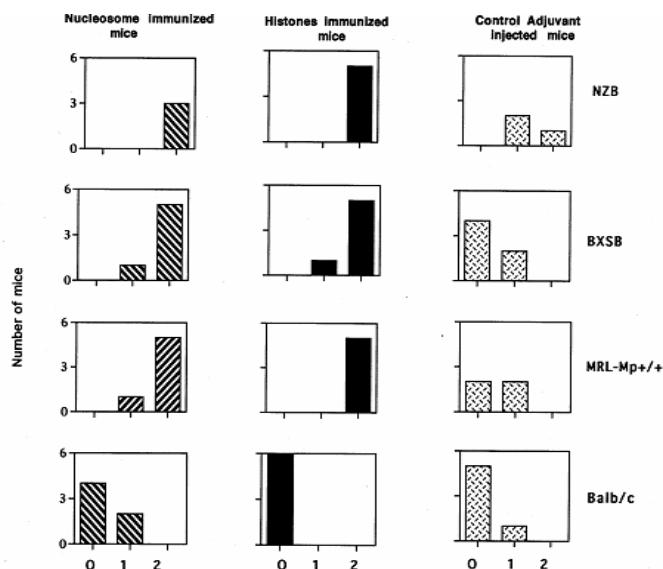


Figure 4. Semiquantitative evaluation of IgG deposits in glomeruli from 21 week-old NZB, BXSB, MRL-Mp $^{+/+}$ and Balb/c mice immunised at 9 (in CFA), 11 and 13 (in IFA) weeks of age with anucleosomes or histones and in control mice injected with Freund's adjuvant. The intensity of lesions was scored on a scale from 0 to 2 (0: none or rare; 1: mild; 2: intense).

The induction of anti-DNA antibodies in response to immunisation with histones does not appear to be driven by a DNA-containing immunogen since we have controlled that histones preparations were devoid of contaminating DNA. Such an anti-DNA response immunisation with histone has already been reported in rabbits with a given genetic background (Atanassov et al., 1991). This previous report and our results support the view that the development of T cells sensitised against proteins associated to DNA in chromatin can trigger the induction of anti-DNA antibody from resting autoreactive anti-DNA B-cells. A study conducted on the specificity of T helper cells in NZBxSWR lupus mice has indicated that 50% of T helper clones selected for their ability to induce the production of pathogenic anti-DNA antibodies were specific for nucleosomal antigens, and that nucleosome-specific T helper cells were present in the spleen of lupus mice as early as 1 month of age (Mohan et al., 1993). T cells sensitised by structural proteins of chromatin could therefore help an anti-DNA or anti-nucleosome B-cell response, and might play a role in lupus pathophysiology.

In our study a relationship exists between the induction of anti-chromatin autoimmunity and the development of kidney lesions. While the pathogenic role of anti-DNA antibodies is recognised, the role of antibodies directed against structural proteins of chromatin is still questioned. Our studies on IgG eluted from glomeruli of lupus mice have indicated that anti-histone and anti-nucleosome antibodies could play, as well as anti-DNA antibodies, a pathogenic role in the development of lupus nephritis (Elouaai et al., 1994 and Van Bruggen 1997). It is therefore likely that the development of kidney lesions is related to the deposition in glomeruli of the autoantibodies reacting with chromatin that have been induced. The mechanism(s) by which the development of these lesions is (are) mediated is not known. Recent studies suggested that anti-DNA could be deposited through a mechanism of local formation in lupus nephritis mediated by planted histones (Schmiedeknecht et al., 1989 and Termaat 1992).

Apoptosis and its role in the development of autoimmunity have been extensively reviewed by several authors (Rosen and Casciola-Rosen, 1999, Tan, 1994 and O'Reilly and Strasser, 1999).

If products of chromatin catabolism are released in situ from dead cells, they can adsorb any circulating autoantibody directed against accessible epitopes in chromatin. Such a mechanism of local formation of immune complexes can trigger further injury of the kidneys in a self-perpetuating mechanism of tissue lesions. Since the results of Marko (Marko et al., 2004), indicate a direct role for nucleosomes in the execution of apoptosis, clearance of apoptotic cells, and regulation of anti-nuclear autoantibody production.

In conclusion, on a pre-disposed genetic background, immunity against nucleosome or histone can trigger the induction of anti-chromatin antibodies including anti-DNA antibodies, and the development of kidney lesions. At a general point of view, these results strengthened the paradigm that chromatin, instead of DNA, is one of the antigenic systems at work in the pathogenesis of lupus diseases.

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