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Glomerular immune deposits in murine lupus models may contain histones

T. SCHMIEDEKE, F. STOECKL, S. MULLER*, Y. SUGISAKI†, S. BATSFORD, R. WOITAS & A. VOGT
*Institute of Medical Microbiology, Freiburg, Germany, *Institut de Biologie Moleculaire et Cellulaire, CNRS, Strasbourg, France, and †Nippon Medical School, Tokyo, Japan*

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SUMMARY

Two types of lupus mice, NZB/NZW F_1 female hybrids and mice with graft-*versus*-host disease (GVHD), were studied. Histones H3 and H2A were detected by immunofluorescence in glomeruli of 22/22 proteinuric GVHD and 8/12 proteinuric NZB/W F_1 female mice; in non-proteinuric animals, 3/5 GVHD and 2/27 NZB/W F_1 female were positive. Using antibodies to histone peptides it was shown that mainly the N-terminal regions of histones H3 and H2A were exposed in glomerular deposits. Western blot analysis revealed antibodies to histone subfractions in sera of 33/34 lupus mice that developed proteinuria. This study provides evidence that histones are involved in the pathogenesis of lupus nephritis.

Keywords murine lupus nephritis histones histone peptide antibodies ubiquitin

INTRODUCTION

In systemic lupus erythematosus (SLE) the role of DNA and anti-DNA antibodies in the induction of glomerular injury has been extensively investigated. Several lines of evidence now point to an involvement of histones and/or DNA–histone complexes at an early stage of the disease: nucleosome-like particles (DNA–histone complexes) may circulate in patients with SLE [1,2]; eluates from kidneys of lupus mice contain both anti-DNA and anti-histone antibodies [3]; histones have high affinity for the rat glomerular basement membrane (GBM) *in vivo* [4]; and histone H3 was shown to very efficiently mediate *in vivo* deposition of DNA in the GBM [5]. Another idea that has received attention in recent years is that anti-DNA antibody may cross-react with and bind directly to tissue structures in the GBM, for example heparan sulphate [6]. However, histones can also mediate binding of these latter antibodies, primarily via immune complexes (IC) consisting of anti-DNA antibody and DNA-histones [7,8], true cross-reacting antibodies playing only a minor role [9].

Direct evidence for participation of histones in human lupus nephritis was presented recently, as glomerular deposits of histone could be detected in kidney biopsies of SLE patients [10]. We have now looked for evidence of a similar involvement of histones in two lupus-like murine models, namely NZB/W F_1 female hybrids and mice with chronic graft-*versus*-host disease (GVHD).

Correspondence: Dr S. Batsford, Department of Immunology, Institute of Medical Microbiology, Hermann-Herder Str. 11, W-7800 Freiburg, Germany.

MATERIALS AND METHODS

Mice

Male and female DBA/2 (H-2d/d), male C57B10S (H-2b/b) mice, and female F_1 hybrids of New Zealand black (female) and white (male) mice were purchased from Harlan/Olac Ltd (Bicester, UK).

*Induction of graft-*versus*-host disease*

Chronic GVH reaction was established as described previously [11]. Lymph node and spleen cells were prepared from 6 to 8 week old female DBA/2 donor mice and resuspended to a concentration of 200×10^6/ml vital cells in PBS. Thirty female, 9 to 10 week old (C57B10S/DBA/2)F_1 hybrids (recipients), were each injected (designated day 0) intravenously with a total number of 60×10^6 vital cells (one part lymph node and two parts spleen cells, total volume 0·3 ml); the procedure was repeated once (day 7).

Production and analysis of antisera to total histone, histone subfractions, histone synthetic peptides and ubiquitin

Rabbits were immunized with total histone (Worthington Corp., Freehold, NJ) or histone subfractions (H1, H2A/H4, H2B and H3) prepared from calf thymus tissue [12] or chicken erythrocytes. For immunization histone fractions were either complexed to RNA as described by Stollar & Ward [13] or complexed to keyhole limpet haemocyanin (KLH) (Calbiochem., Frankfurt, Germany) as described previously [4]. Antisera were analysed by Western blot [4] and by ELISA [14,15] for antibodies against the individual histones as well as against dsDNA and ssDNA. They were tested against nucleic acids by the Farr-assay using preparations of dsDNA, ssDNA and

ribosomal RNA (Sigma, Deisenhofen, Germany), radiolabelled with ^{125}I [16]. The antisera to calf core histones were subjected to a more detailed analysis with synthetic peptides in order to better define their fine specificity [15].

Antisera to synthetic histone peptides were prepared as described previously [17] by immunizing rabbits with ovalbumin-conjugated peptides for fragments of less than 15 residues or with unconjugated peptides for the others. Eleven anti-peptide antisera were used in this study; they were directed to peptides covering residues 1–9, 1–15, 28–42, 65–85, 85–100 and 90–105 of H2A and to peptides 1–21, 18–32, 30–45, 40–55 and 130–135 of H3. The characteristics of these antisera were described previously [17–19]. Rabbit antisera to ubiquitin and to the branched region of ubiquitinated histone H2A were prepared and tested as described previously [20,21].

Selected antisera (see Results) were tested for anti-immunoglobulin activity by ELISA [22]. Inhibition experiments using synthetic peptides were also performed in the ELISA system. For this purpose diluted antisera were preincubated with the appropriate peptide in up to 100 molar excess and then the mixture was added to peptide precoated microtitre plate wells, the rest of the procedure being as before [15]. In the case of the parent proteins the high molar excess required for inhibition could not be achieved due to solubility problems.

Rabbit antibody directed against lysozyme, a highly cationic 14-kD protein, served as a control for Western blot analysis and for immunofluorescence (IF) studies.

Analysis of mouse sera

Sequential serum samples from lupus mice and control mice were tested against calf thymus histones by Western blotting at dilutions of 1:200 (GVHD and controls) or 1:100 (NZB/W F_1 female).

Immunofluorescence studies

Acetone-fixed, 4-μm frozen kidney sections were incubated with rabbit antiserum raised against calf thymus or erythrocyte histone fractions (1:40), synthetic histone peptides (1:20) or ubiquitin fractions (1:20), followed by FITC-labelled mouse anti-rabbit IgG serum, previously absorbed with mouse, human and goat serum proteins (Dianova, Hamburg, Germany); sections were mounted using a glycerol/dithiothreitol anti-fade mixture (pH 8·2) [23]. Sections were also stained with FITC-labelled rabbit anti-mouse IgG and C3 (Dianova).

Inhibition of specific fluorescence

Dilutions (usually 1:20) of the appropriate anti-histone antiserum were mixed with either equal volumes of dilutions (100 μg/ml to 1 mg/ml) of histone H2A/H4, H3, total histone, nucleohistone (Worthington Corp.), highly polymerized DNA (Worthington Corp.), ribosomal RNA (Sigma), or with PBS as control, incubated overnight at 4°C and centrifuged, and the supernatant was then used for immunofluorescence studies.

Experimental design

NZB/W F_1 female hybrids. Urine and blood samples were collected at 2-week intervals from 28 mice (until sacrifice at 9–38 weeks); in 11 additional mice urine and blood samples were collected only near the time of sacrifice (34–41 weeks). Total urinary protein excretion was estimated by the biuret method [24], following trichloroacetic acid precipitation. Mice were killed at ages between 9 and 12 weeks ($n=7$), 13 and 18 weeks ($n=7$), 22 and 26 weeks ($n=7$) and 34 and 41 weeks ($n=18$). The kidneys were flushed free of blood with PBS before being shock frozen.

GVHD mice. Urine and blood samples were collected at 2-week intervals from 15 mice; in 12 other GVHD mice only urine was regularly collected, blood being taken at sacrifice. Mice were killed when the urinary protein excretion exceeded 10 mg/24 h or, in non-proteinuric mice, when signs of advanced chronic GVH reaction (e.g. ascites and enlarged lymph nodes) appeared. Renal tissue was treated as described above for NZB/W F_1 female hybrids. Times of sacrifice were: 6–9 weeks ($n=5$), 10–16 weeks ($n=16$) and 20–29 weeks ($n=6$).

Control mice. For each GVHD mouse a non-injected sex- and age-matched (C57B10S/DBA/2)F_1 female hybrid mouse was studied in parallel.

RESULTS

Development of lupus-like disease

Twenty-two GVHD mice were killed after onset of proteinuria (10–55 mg/24 h). The remaining five were not proteinuric (three had ascites and other signs of disease). Twelve of the 39 NZB/W F_1 female mice studied had proteinuria (> 10 mg/24 h) when killed; the remaining 27 showed signs of systemic disease (enlarged spleens and lymph nodes, weight loss). A further four NZB/W F_1 female and three GVHD mice that died spontaneously were excluded.

Immunofluorescence studies on kidney sections

Initial screening of proteinuric NZB/W F_1 female and GVHD mice was made with a set of rabbit antisera to the full range of core histones. One antiserum in particular (no. 496), directed mainly against H2A and H3 (see footnote to Table 1), regularly produced bright staining (Table 1, Fig. 1b). Antisera reacting predominantly with other histones (H1, H2B, H4) produced infrequent, weak positive staining. To confirm that histones H2A and/or H3 were present in glomerular deposits, all kidneys were tested with antisera raised against synthetic peptides in H2A and H3 (see Materials and Methods). Three antisera, raised against the N-terminal domains of histones H2A (fragments 1–9 and 1–15) and H3 (fragment 1–21), produced positive glomerular staining in all proteinuric GVHD ($n=22$) and in eight out of 12 proteinuric NZB/W F_1 female mice (Table 1, Fig. 2b). Granular staining was observed along the glomerular capillary walls and in the mesangial area of nearly all glomeruli examined (Figs 1b, 2b). Kidneys of five proteinuric GVHD mice also showed positive staining for histone and IgG along the peritubular capillaries (Fig. 2a). In addition, kidneys of some non- or not yet proteinuric lupus mice (3/5 GVH and 2/27 NZB/W F_1 female) also showed positive staining for histone that was restricted mainly to the mesangial area of the glomerulus (Table 1). Antisera recognizing other peptide regions of H2A and H3 (i.e. not the N-terminus) were not regularly positive. The anti-histone and anti-histone peptide antisera did not stain glomeruli from normal control mice. Staining with anti-lysozyme antibody was always negative.

Deposits of histones were always accompanied by autologous IgG and C3 (see Table 1), and the staining patterns were similar (Figs 1a, 2a). Kidney sections from a total of 29 mice

Table 1. Immunofluorescent findings in glomeruli of lupus mice

Murine model	Proteinuria	Intensity of glomerular immunofluorescence* (antiserum recognising)					
		H2A/H3†	H3(1-21)‡	H2A(1-15)‡	H2A(1-9)‡	IgG	C3c
GVH	Yes ($n=22$)	+++ ($n=11$)	++ ($n=8$)	++ ($n=7$)	++ ($n=22$)	++++ ($n=16$)	++ ($n=15$)
		++ ($n=11$)	+ ($n=14$)	+ ($n=15$)		+++ ($n=6$)	+ ($n=7$)
	No ($n=5$)	+++ ($n=3$)	+ ($n=3$)	+ ($n=3$)	+ ($n=3$)	+++ ($n=5$)	+ ($n=5$)
NZB/W F_1 female	Yes ($n=12$)	++ ($n=8$)	+ ($n=8$)	+ ($n=8$)	+ ($n=8$)	++++ ($n=12$)	++ ($n=12$)
	No ($n=27$)	++ ($n=2$)	+ ($n=2$)	+ ($n=2$)	+ ($n=2$)	+++ ($n=18$)	+ ($n=18$)
						+ ($n=9$)	

* Staining pattern was capillary and/or mesangial, graded from + to ++++ by two independent investigators.

† Serum 496, major specificity according to ELISA analysis, recognises H2A fragments 1-20, 65-85 and 85-100 and H3 fragments 1-21, 40-55, 53-70 and 130-135.

‡ Sera raised against synthetic peptides of histones H3 (residues 1-21) and H2A (residues 1-15 and 1-9 respectively). (There was agreement between positive reactions with serum 496 and anti-peptide antisera.)

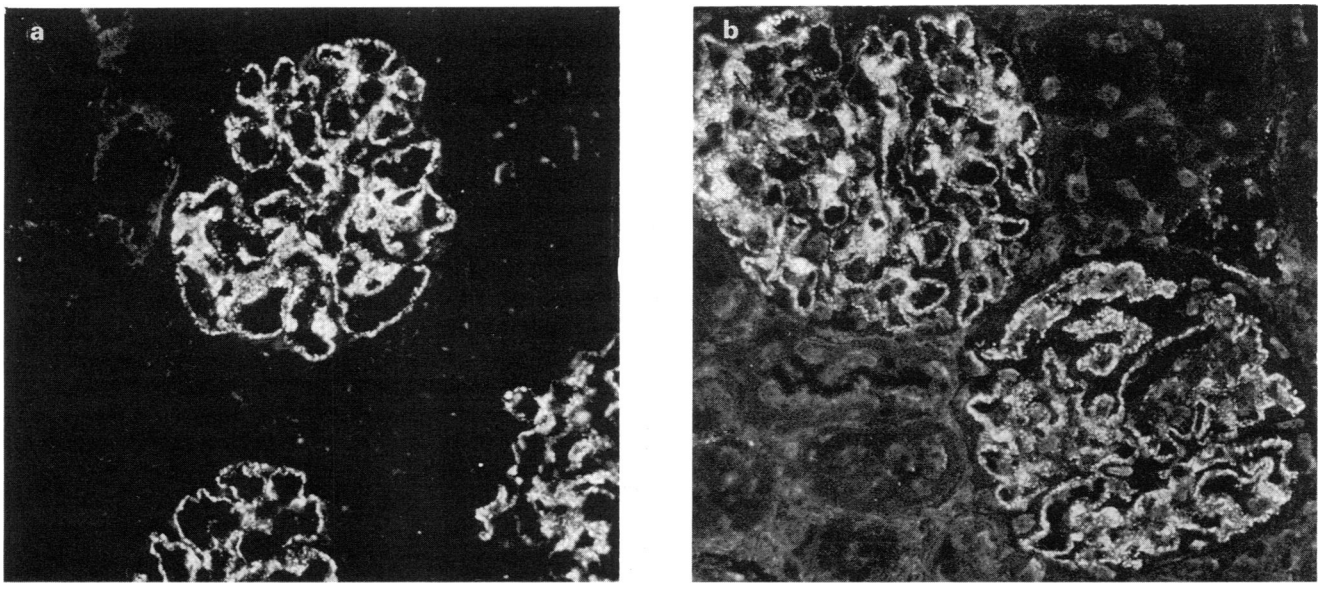

Fig. 1. Immunofluorescence staining of a kidney from a proteinuric NZB/W F_1 female mouse killed at the age of 37 weeks. (a) Staining for mouse IgG. Granular deposits are located along the glomerular capillary walls and in the mesangium. (b) Indirect immunofluorescence with the rabbit antiserum (No. 496) reacting predominantly with histone H2A and H3. Granular glomerular deposits are seen in a similar distribution to mouse IgG. In addition, faint staining of cell nuclei is seen. No reaction was seen with anti-lysozyme antibody, used as a control. (× 320)

(two GVHD and 27 NZB/W F_1) having IgG deposits did not stain with these antisera (Table 1).

Nuclear staining was seen only with sera raised against total histone, histone H1, and H2B; with sera directed against the other histone subfractions or synthetic histone peptides no, or only weak, nuclear staining was seen.

Kidney sections from 15 GVHD and 10 NZB/W F_1 female mice were also screened using rabbit antisera to ubiquitin or to a peptide corresponding to the branched moiety of ubiquitinated histone H2A. Very faint glomerular staining was seen with 5/15 GVHD and 2/10 NZB/W F_1 female mice, the remainder being negative.

Specificity of antisera employed

The major results are summarized in Table 2. All four antisera reacted with the homologous peptide and the parent protein in

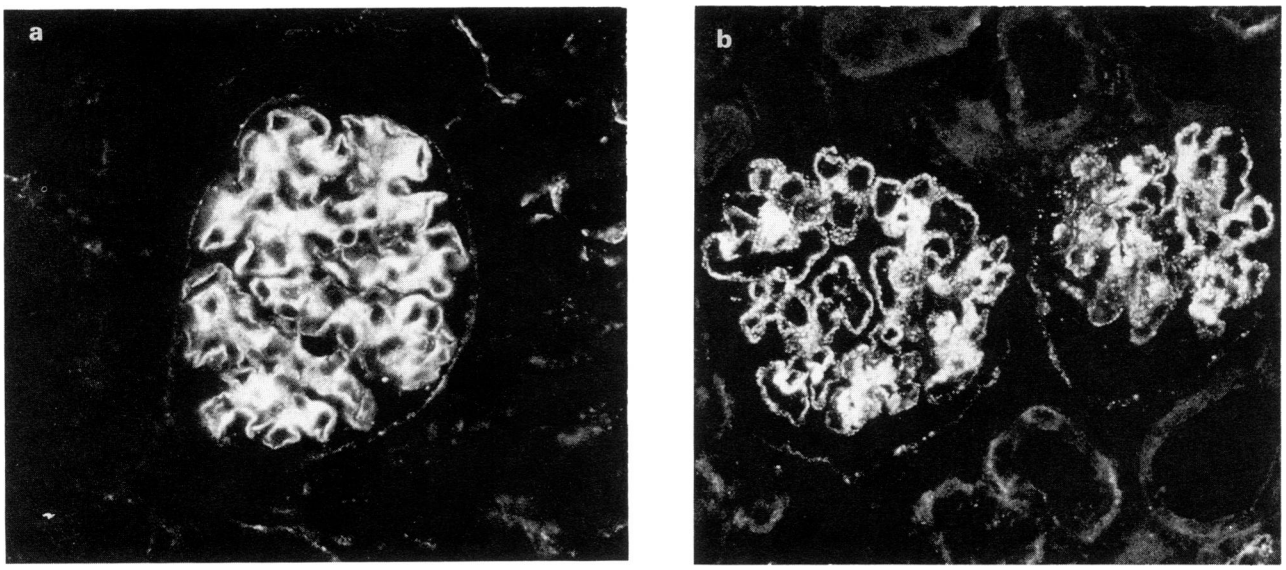

Fig. 2. Immunofluorescence staining of a kidney from a lupus mouse that developed chronic graft-*versus*-host disease with proteinuria and ascites. (a) Staining for mouse IgG shows deposits in the mesangial area and along the glomerular and peritubular capillary walls. (b) Indirect immunofluorescence with a rabbit serum directed against the N-terminal region (residues 1–21) of histone H3. Granular glomerular deposits are seen in the mesangial area and along the capillary walls. (× 320)

Table 2. Specificity of polyclonal synthetic peptide antibodies

Antiserum to	ELISA with synthetic peptides		Reaction with intact parent protein		ELISA with nucleosome/ chromatin
	Homologous peptide	Inhibition by peptide (%)	ELISA	Immune blot	
H3(1–21)	+	90	–	+	–
H2A(1–15)	+	0*	+	–	–
U(22–45)	+	93	+	+	–
U-H2A (branched peptide)	+	20	ND†	+‡	–

* H2A(1–20) used for inhibition.
† ND, Not done, pure parent protein not available.
‡ Tested with complete core histone extract.

at least one test sytem. Inhibition experiments were successful, except in the case of anti-H2A(1–15). These antibodies may react preferentially with conformational domains in the protein (target antigen in free and bound form); it was the only antiserum not recognizing the parent protein in the immune blot (Table 2). As previously reported [17–19], no reactions with synthetic peptides from other histones or from other regions on the same histone were seen. None of the antisera revealed anti-immunoglobulin activity.

Inhibition of specific fluorescence

In mice having positive glomerular fluorescence with anti-histone sera, complete inhibition of glomerular staining was achieved by prior incubation of the antiserum with > 500 μg/ml of nucleohistone, a relatively crude mixture of DNA and total histone. Staining was also reduced by incubation with > 200 μg/ml of histone mixture H2A/H4 or H3. Staining was not reduced by absorption with DNA and RNA alone.

Analysis of lupus mice sera

Western blot analysis revealed anti-histone antibody in sera of all GVHD mice, but in none of the sex- and age-matched control mice. All sera from GVHD mice reacted with histone H1; in addition, most proteinuric and non-proteinuric GVHD mice possessed antibodies binding to either histone H2A ($n = 12$) or H3 ($n = 19$) or both ($n = 10$). GVHD mice also developed antibodies against histone H2B ($n = 5$) and/or H4 ($n = 4$). Histone H3 was the subfraction most strongly recognized by sera of GVHD mice. No subgroup of the mice investigated (e.g. mice showing positive glomerular staining) showed a specific type of antibody profile.

Longitudinal analysis performed with sera from 15 GVHD mice (12 proteinuric and three non-proteinuric) revealed that anti-histone antibody titres rose 2–10 weeks after the second injection of donor cells; this rise always occurred before the onset of proteinuria (appeared 6–10 weeks later) (Fig. 3), declining continuously thereafter ($n = 7$), or falling abruptly 4–6 weeks before the onset of proteinuria ($n = 3$) or remaining

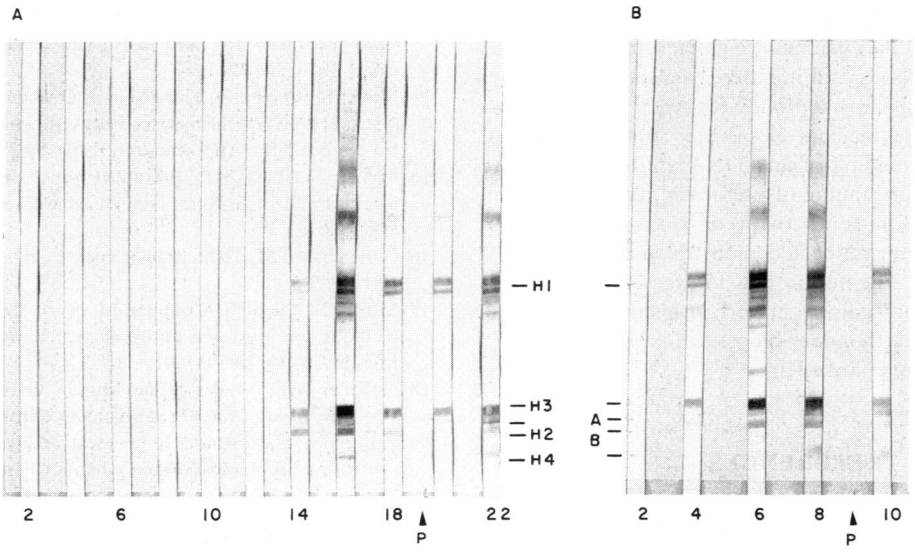

Fig. 3. Analysis of sequential sera from two graft-*versus*-host disease mice. Numbers are weeks after induction of disease. Western blots against total histones show typical antibody responses: the titre of anti-histone antibody rises quickly but drops to a lower level several weeks before (a) or at (b) the onset of proteinuria (marked by arrowhead P). Position of individual histones is shown.

constant till sacrifice, as seen in the remaining two proteinuric and in the three non-proteinuric GVHD mice examined.

Serum from 11/12 proteinuric and 18/27 non-proteinuric NZB/W F_1 female mice contained antibodies to at least one histone subfraction, the reactions being consistently weaker than in the case of GVHD mice. Serial analysis was done in 28 mice; circulating anti-histone antibodies appeared in mice subsequently developing nephritis several weeks before the onset of proteinuria. As in GVHD mice, no specific antibody profile was seen in any subgroup. Sera of NZB/W F_1 female mice did not recognize any individual histone subfraction more frequently and antibody titres to each fraction were similar, in contrast to GVHD mice where antibodies to histone H3 were most prominent (see above).

DISCUSSION

Evidence for the presence of histones H3 and H2A in glomerular deposits in two models of SLE was obtained. These deposits were detected in all proteinuric GVHD and a majority of proteinuric NZB/W F_1 female mice, and in some non- (or not yet) proteinuric mice that had glomerular IgG and C3 deposits. Histone deposits were initially detected by immunofluorescence with a rabbit antiserum reacting primarily with a number of epitopes on histones H3 and H2A. The histone nature of the antigen detected was substantiated by inhibition of fluorescent staining after preabsorption of the antisera with nucleohistones and histones H3 and H2A, and by immunofluorescence studies with rabbit antisera to synthetic histone peptides. Positive glomerular staining was found with antibody to peptides 1–21 of H3 and peptides 1–9 and 1–15 of H2A. Antisera recognizing other histones and other regions of H2A and H3 gave negative or very weak reactions. These data suggest that only the N-terminal regions of histones H3 and H2A are exposed in deposits and that, as a corollary, histone deposits are only detectable by immunofluorescence with antisera recognizing epitopes located in these regions. It cannot be excluded that other histones are present or that other domains in H2A and H3 are accessible but were not detectable with the antibody probes used in this study. The complexity of histone–antibody interactions was also demonstrated when antibodies raised against histone subfractions were studied by immunofluorescence on native frozen tissue sections. Although an antiserum may possess high titres of antibodies to histones (all of which are present in the nuclei), only those with antibody against H1 and H2B were shown regularly to produce bright nuclear staining. These latter histones possess the epitopes that are most prominently exposed on nucleosomes [25]. Several recent studies on sera from patients with SLE or from lupus mice demonstrate that there are particular regions in histones recognized by autoantibodies [14,25–28]. In fact these regions correspond to those exposed in chromatin and it has been suggested that autoreactive B cell responses in SLE may be driven by self-antigens, including nucleosomes, the basic unit of chromatin or similar histone–DNA complexes [14,25–30]. The current findings permit the speculation that histones in complexed form, presumably originating from chromatin, may not only be involved in autoimmune responses, but might also have a pathogenic effect in tissue, for example the renal glomerulus.

In the present study analysis of sera of lupus mice by Western blot revealed that histone subfractions are frequently recognized and that anti-histone antibody regularly appears several weeks before the onset of glomerulonephritis, providing further circumstantial evidence that histones and anti-histone antibodies participate in glomerular injury. The fine specificity of these antibodies, i.e. reaction with distinct regions of histone subfractions, is under study in our laboratories.

Attention has been drawn to the role of the stress protein ubiquitin as well as ubiquitinated histone H2A as auto-antigens in SLE [20,21]. In fact we were also able to detect the presence of these antigens in glomerular deposits in some renal biopsies from patients with SLE nephritis [10]. For this reason mouse kidneys were also screened with antisera to ubiquitin and ubiquitinated H2A. In this case the results were largely negative.

The current and other recent studies suggest that histones might play an important role in the pathogenesis of lupus

nephritis [1–5,7–10]. Due to their high affinity for the glomerular basement membrane they can bind to glomeruli, act as a target for anti-histone antibody and initiate *in situ* immune complex formation with anti-histone antibody [4]. Another mechanism, where histones could mediate glomerular binding of DNA, followed by *in situ* binding of anti-DNA antibody [5] is also plausible. Glomerular binding of preformed, circulating complexes consisting of histones (with or without DNA) and autoantibodies has not yet been demonstrated experimentally, but is also possible. The notion that histones are involved in lupus nephritis recently gained support, as histones were found in glomerular deposits in kidney biopsies of patients with SLE-associated glomerulonephritis [10].

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