HISTONES HAVE HIGH AFFINITY FOR THE GLOMERULAR BASEMENT MEMBRANE

Relevance for Immune Complex Formation in Lupus Nephritis

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Histones are a set of highly cationic proteins essentially involved in the binding and compaction of DNA in the cell nucleus chromatin (1–3). In mammals, five subclasses of histones exist: H1 (f1), H2a (f2a2), H2b (f2b), H3 (f3), and H4 (f2a1) (4,5), whereby four histones form an octameric core protein aggregate (H2a, H2b, H3, H4) around which a 145-bp DNA molecule is wrapped, forming the core particle followed by a variable (~55 bp) DNA region, complexed with H1 (6). In the cell nucleus, histones and DNA are present in similar quantities.

These components of the cell nucleus are two of the most important antigens for autoantibody formation in SLE (7–12), and it has been reported that the titers of both groups of autoantibodies correlate well with disease activity (13–16).

A prominent manifestation of organ involvement in SLE patients is the occurrence of immune complex (IC)[1] glomerulonephritis. After the original report by Koffler et al. (17), attention has been focussed on the role of DNA-anti-DNA IC for >20 yr. Two proposals have been widely discussed in connection with the glomerular deposition of DNA-anti-DNA complexes: the now unfashionable notion that preformed soluble ICs can locate in the glomerular filter (18); and second, an affinity of DNA for the glomerular basement membrane (GBM) in vivo has been suggested (19–23), which could then initiate in situ IC formation. Extensive studies in animals on the kinetics and clearance of circulating free or immune complexed DNA (24–27) in regard to size and strandedness of DNA, however, revealed rapid nonrenal uptake and clearance. Prolonged DNA persistence is difficult to explain by accepted concepts; new proposals are needed (28). Contradictory findings on the occurrence of free or IC-bound DNA in sera from normal individuals and SLE patients have been reported (for review see reference 29). There are a number of technical problems involved here and it is now accepted that DNA-anti-DNA immune complexes represent only a minor part of the ICs found in lupus patients, and that increased levels of circulating DNA are found in a variety of conditions in which lysis of cells occurs. Preliminary studies by Fournié (29) suggest that nucleosomes, which consist of DNA

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[1] *Abbreviations used in this paper:* GBM, glomerular basement membrane; I, ionic strength; IC, immune complex; KLH, keyhole limpet hemocyanin.

and histones, but not free DNA, are circulating in blood and warrant more attention. In fact, a further possibility, not previously considered, is that a cationic autoantigen is involved; histones as a group of highly cationic nuclear antigens and as integral components of the nucleosome suggest themselves. Experimentally cationized protein antigens are excellent nephritogens. Such an antigen shows high affinity for the anionic sites of the GBM and can act as a planted, target antigen for subsequently injected antibody, leading to IC glomerulonephritis with heavy proteinuria (30–33). The molecular mass (>40 kD) and the isoelectric point (>8.5) of the antigen determines its affinity for the GBM (34).

Since histones not only possess regions of dense positive charge (35) but also aggregate spontaneously to form stable complexes (36–42), they are excellent candidates for inducing IC formation in the renal glomerulus.

Additionally, polycations can very effectively promote the deposition of anionic antigens (43) or ICs (44) in the GBM, which could be the key event in the pathogenesis of lupus nephritis. Histones could induce the deposition of the highly negatively charged DNA in the GBM, due to either local or systemic release of DNA and histone during cell lysis. This combined deposition of DNA and histones could, especially if antinuclear antibodies and complement then bind to the complex, prolong DNA deposition in the glomerular capillary wall, leading to glomerulonephritis.

Materials and Methods

Animals. Male Wistar rats (150 g) (Zentraltierzüchterei Hannover, Hannover, FRG) were used throughout. All injections and surgical procedures were carried out under ether narcosis.

Histone Preparation. Histones were prepared from calf thymus tissue, which had been frozen in liquid nitrogen immediately after slaughtering. Nucleohistone was purified as described by Johns (4) using 50 mM sodium hydrogensulfit, pH 7.4, in 0.1 M sodium chloride as a proteolysis inhibitor (45). Total histone was extracted from nucleohistone with 0.2 M sulfuric acid, precipitated in ethanol, and lyophilized. Histone subfractions were extracted from nucleohistone by the method of Johns (4) and lyophylized. Histone concentration was estimated photometrically (40), using total histones or histone subfractions at 1 mg/ml as standard. Histones were analyzed by SDS-PAGE as described by Thomas and Kornberg (38), except that the total acrylamide concentration was 15%; histone solutions (see below) were diluted in sample buffer without mercaptoethanol and were not boiled. For autoradiography of ^{125}I-labeled proteins (see below), the gels were fixed in 40% (vol/vol) ethanol, 10% (vol/vol) acetic acid, and dried in a slab gel dryer (Bio Rad, München, FRG). The dried gel was overlaid with an X-ray film (Eastman Kodak Co., Rochester, NY) using two titan plates as enhancer (Siemens, Freiburg, FRG).

Preparation of Histone Solutions. All steps were performed at 4°C. Histone subfractions f1 (H1), f2a (H4 + H2a), f2b (H2b), and f3 (H3) were dissolved in 0.01 M hydrochloric acid at 12 mg/ml and dialyzed for 1 h against 10 mM sodium phosphate buffer, pH 7.4, in dialysis tubing (Serva, Heidelberg, FRG) that had been previously boiled. The concentration of sodium chloride was increased stepwise by adding PBS to the buffer until, finally, the sample was dialyzed against PBS (I = 0.15). The histone solutions were then diluted with PBS to final concentrations of 12 mg/ml, 1 mg/ml, and 400 μg/ml and stored frozen at −20°C.

Radioisotopic Labeling of Proteins. Lysozyme (Sigma Chemical Co., Deisenhofen, FRG) and histone solutions at concentrations of 1 mg/ml in PBS were labeled with ^{125}I (Amersham, Frankfurt, FRG) by the chloramine T method. The separation of free and protein-bound iodine was done by gel filtration with an Enzymplex column (E-Y-labs, San Mateo, CA), using PBS as an eluent. The ^{125}I-labeled histone subfractions were mixed with nonlabeled fractions, dissolved in PBS as described above.

Determination of DNA Content of Different Histone Preparations. The DNA content was estimated

by comparing the absorbance of histone solutions at 260 and 280 nm (A_{280}/A_{260} DNA = 0.5; histone = 1.68) (16).

Analysis of Aggregation Behavior of Histones. Under physiological conditions (ionic strength [I] = 0.15, pH 7.4) gel matrices like Sephadex G100 or Superose 6 or 12 (Pharmacia Fine Chemicals, Freiburg, FRG) completely absorb histones, which can then only be desorbed by acidic or high molar salt solutions. This binding could be prevented by equilibration of the column with a solution of the polycation polyethylenimine (PEI) (1 mg/ml, mol wt 1,800) (Polysciences, St. Goar, FRG) in PBS, pH 7.4. We used an FPLC system with a Superose 6 column (total vol, 25 ml) and equilibrated the column with 4 bed vol of polycation polyethylenimine, followed by washing with 4 vol of PBS. The column was calibrated using naturally occurring cationic as well as chemically cationized proteins. The degree of histone aggregation was analyzed at different concentrations (12 mg/ml, 1 mg/ml, or 400 μg/ml) of nonlabeled and ^{125}I-labeled histone. The resulting peaks were analyzed by SDS-PAGE to reveal their histone composition (Fig. 2).

Affinity of Histone Subfractions and Lysozyme to Heparin-Sepharose. Affinity chromatography was done using a column with a diameter of 0.5 cm and a gel length of 1 cm containing 200 μl of packed Heparin-Sepharose CL-6B (Pharmacia Fine Chemicals) in 0.15 M sodium chloride, 10 mM sodium phosphate, pH 7.4, using a sodium chloride gradient of 0.15–2 M, followed by elution with 6 M guanidine-HCl, pH 7.4. The ^{125}I-labeled samples were applied at a concentration of 400 μg/ml in PBS in a total vol of 0.1 ml, containing 13,000–40,000 cpm/μl. The flow rate was 0.2 ml/min. Fractions (500 μl) were collected and the ^{125}I content was analyzed using a gamma counter (MAG 312; Berthold, Bad Wildbad, FRG).

Antihistone Antibody. Rabbits were immunized either with histone-RNA complexes, as described by Stollar and Ward (46) or with keyhole limpet hemocyanin (KLH) (Calbiochem, Frankfurt, FRG) histone complexes, both in CFA (Sigma Chemical Co.). In the latter case, 300 μl of histone (12 mg/ml in PBS) was mixed with 100 μl of KLH (5 mg/ml in PBS) at 4°C, incubated for 30 min at 20°C, mixed with 400 μl CFA, and emulsified. Rabbits were injected subcutaneously three times at monthly intervals and bled 2 wk after the last injection. Sera were tested for antihistone antibodies by Western blotting (see below) using sera obtained before immunization as well as sera from rabbits immunized with either OVA, human serum albumin, or horse spleen ferritin as controls.

Western Blot Procedure. Total histone was loaded onto a SDS-PAGE gel (C = 15%; T = 2.7%) with a 9.5-cm long sample pouch (12.6 μg histone/cm gel) using a Havana electrophoresis system (Desaga, Heidelberg, FRG) with a gel height of 11 cm and thickness of 1.5 mm. Histones were transferred to nitrocellulose with a pore size of 0.2 μm, (Schleicher & Schüll, Inc., Dassel, FRG) by electrotransfer (47). A 1-cm wide strip of the membrane was cut and stained with amidoblack. The remaining nitrocellulose was incubated in 10% (wt/vol) skimmed, powdered milk/PBS solution and cut into 0.5–1-cm wide strips with a noodle machine, and strips were incubated overnight with a 1:1,000 dilution of the appropriate rabbit sera in milk/PBS plus 0.1% Tween 80 (Serva) followed by several washings in PBS/Tween. Then the strips were incubated with peroxidase-labeled goat anti-rabbit IgG (Dianova, Hamburg, FRG) (1:5,000 dilution) for 1 h, followed by washing in PBS/Tween. The strips were developed with diaminobenzidine (Sigma Chemical Co.) (40 mg diaminobenzidine + 7.5 μl H_2O_2 in 100 ml PBS).

Immunofluorescence. 5-μm cryosections of snap-frozen renal tissue were incubated with specific rabbit antihistone antisera (dilution 1:40–1:80) for 30 min. For the second-step, fluorescein-labeled goat anti-rabbit antibody (Nordic, Tilburg, Netherlands) at a dilution of 1:80 was used. Controls were as described for Western blotting.

Coded sections were always analyzed by the same observer and graded from negative (ϕ) to + + + +. Selected sera from SLE patients with antibodies to ssDNA (tested by a Farr assay with ^{125}I-labeled ssDNA) but without antibodies to f3 (tested by Western blotting) were used to detect ssDNA by indirect immunofluorescence.

Kidney Binding of Intravenously Injected Histone. Histone f2a was injected intravenously (2 mg/100 g body weight) into each of four rats, a control group of four rats received the same dose of the highly cationic, but not spontaneously aggregating protein lysozyme. Kidneys were removed 15 min after injection and analyzed by immunofluorescence for histone or lysozyme deposition, respectively.

Organ Binding of ^{125}I-histone Subfractions. Groups of five to six rats were analyzed for each histone, control experiments were done with lysozyme. After opening the peritoneal cavity, a number 20 needle was inserted into the aorta and tied in place so that the tip was level with the left renal artery. The aorta was ligated above the left and below the right renal artery and the left kidney was injected with 0.3 ml of PBS to remove blood, followed by 0.5 ml of the ^{125}I-histone fraction (200 μg protein) at a flow rate of 1 ml/min, followed again by 0.3 ml of PBS; the ligature was then removed to reestablish normal blood flow. 15 min later, 1ml of blood was taken from the right jugular vein and then both kidneys were perfused with 5 ml of PBS via the aorta after clamping the aorta above the arteries and severing both renal veins. Both kidneys were removed and the radioactivity was counted, then the upper fifth part of each kidney was taken for immunofluorescence; the glomeruli were isolated from the remainder of the left kidney only, and the radioactivity was measured. The number of glomeruli isolated was estimated by direct counting of aliquots; it was assumed that a single kidney contained 38,000 glomeruli (48). In addition, liver, spleen, lung, and heart were removed, and the radioactivity was counted. When kinetic studies were performed, perfusion procedures were done as above, then the abdominal wall was closed in the normal fashion and the skin was joined with clamps. At selected time points, organs were removed and treated as detailed above.

In Situ Formation of Histone-Antihistone IC in the GBM. In a group of three rats, 500 μg histone f2a was injected intraaortally into the left kidneys. 10 min later, 0.6 ml/100 g body weight of rabbit antihistone antiserum was injected intravenously into the tail vein. Animals were killed 1 h later. Both kidneys were analyzed by direct immunofluorescence for rabbit Ig deposition. A control group of three rats received only rabbit antihistone antiserum.

Binding of ssDNA Fragments after Histone Injection. Highly polymerized calf thymus DNA (Worthington/Cooper Biomedical, Frankfurt, FRG) was dissolved in PBS at 2 mg/ml containing 5 mM $MnCl_2$ and digested with DNAse I (10 μg/ml) (Sigma Chemical Co.) at 37°C. Digestion was stopped after intervals of between 15 s and 3 min by adding 0.2 M Na-EDTA, pH 7.4, to a final concentration of 10 mM. DNA was denatured by the procedure of Doty et al. (49). Each digestion batch was analyzed by FPLC-gel filtration chromatography on a Superose 12 column (Pharmacia Fine Chemicals). Different batches were mixed to get a polydisperse ssDNA pool with uniform DNA distribution throughout ranging from the exclusion volume of the column (Einstein-Stokes Radius > 100 Å) to an ESR of ~17 Å. The DNA pool was treated with proteinase K and purified by phenol-chloroform extraction and alcohol precipitation as described by Maniatis et al. (50). The precipitated DNA was dissolved and dialyzed against PBS, denatured again, and reanalyzed by FPLC-gel filtration. The final concentration of ssDNA was 820 μg/ml, measured photometrically at 260 nm. 500 μg histone f3 was injected intraaortally into the left kidneys of a group of five rats, kidneys were perfused afterwards with 300 μl of PBS, and subsequently, 30 s later with 320 μg ssDNA fragments.

In control group 1, five rats received PBS instead of histone f3 followed by ssDNA fragments; in control group 2, two animals received only histone f3. Kidneys were excised 15 min after the injection and studied by immunofluorescence using rabbit anti-f3 and human anti-ssDNA for the detection of f3 and ssDNA, respectively.

Statistical Analysis. Statistical analysis was done using a KWIKSTAT Software program (Mission Technologies, Cedar Hill, TX). Independent groups were compared by the *t* test, if the distribution of the individual values was consistent with the normal distribution.

Results

Composition of Nonlabeled and ^{125}I-labeled Histone Subfractions. The histone subfractions prepared according to Johns (4) were composed of the corresponding histone proteins at good purity (Fig. 1). f3 was composed not only of H3 and its dimer, but also of H2A and H4. Autoradiographic analysis of ^{125}I-labeled samples showed that the protein profile was nearly identical to that seen with Coomassie blue staining. In the case of f3 the contaminating H4 component was labeled to nearly the

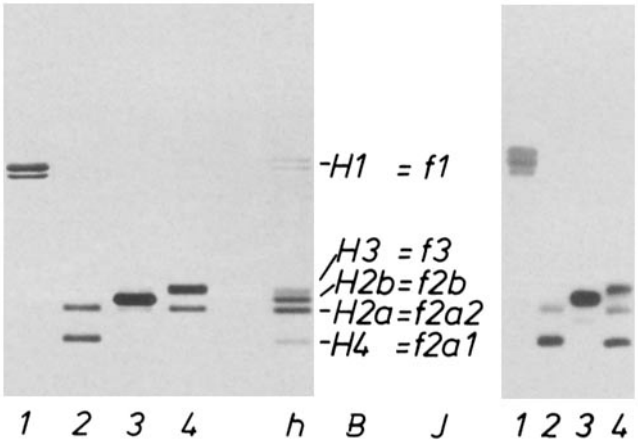

FIGURE 1. SDS-PAGE (C = 15%, T = 2.7%) of histone subfractions, prepared according to Johns (4) and solubilized as described in Materials and Methods. (*Left side*) Coomassie blue staining (lanes *1–4* and *h*); (*right side*) autoradiographic analysis of ^{125}I-labeled histone subfractions (lanes *1–4*). Lane *1*, f1; lane *2*, f2a; lane *3*, f2b; lane *4*, f3; *h*, total histone. Classification of Bradbury (*B*) (5) and Johns (*J*) (4).

same extent as H3, indicating that tyrosine might be more accessible in H4 than in H3 (51). In spite of the lack of protease inhibitors during preparation of histone solutions used for injection, the extent of proteolysis was very low and was not evident in the case of f2a and f3. Only f1 showed initial proteolytic degradation after ^{125}I labeling.

Analysis of DNA Content of Different Histone Preparations. The histone subfractions contained only low quantities of contaminating DNA: f1, 1%, f2a, 0.3%; f2b, 1%; and f3, 0.4%.

Analysis of Aggregation Behavior of Histones. The results of gel filtration chromatography and peak composition analysis in SDS-PAGE are shown in Fig. 2. All histone subfractions, including f1, revealed at least two peaks, one of low molecular weight and the other of high molecular weight, due to aggregates of varying size. f2a and f3 showed marked size heterogeneity and peak composition was variable. H4 was enriched in the high molecular weight fraction of f2a and H3 in the high molecular weight fraction of f3, respectively. Reanalysis of the highly aggregated fraction revealed two peaks, a major one corresponding to the original material and a minor one of lower molecular weight resulting from dissociation. Analysis of histone solutions at low concentrations showed a reduction in the degree and extent of aggregation; the proportion having a high molecular weight (aggregate) and the maximum size decreasing. These data are consistent with the assumption of a reversible aggregation process, as has already been described in ultracentrifugation studies by Sperling and Bustin (40).

In our studies, f3 showed the highest degree of aggregation and f1 and f2b showed the lowest. Further studies showed that the elution profile of radioactivity of the ^{125}I-labeled histone preparations was identical with the optical absorption profile at 280 nm, so we could assume that these fractions were representative of the whole histone fractions, which were later used for in vivo studies.

Affinity of Histone Subfractions and Lysozyme to Heparin-Sepharose. A Heparin-Sepharose column was used to study the affinity of histones and lysosome to a GBM heparan-sulfate-like matrix. This in vitro test correlates well with our in vivo findings. Fig. 3 shows the elution profiles of histone subfractions f1, f2a, and lysozyme using a

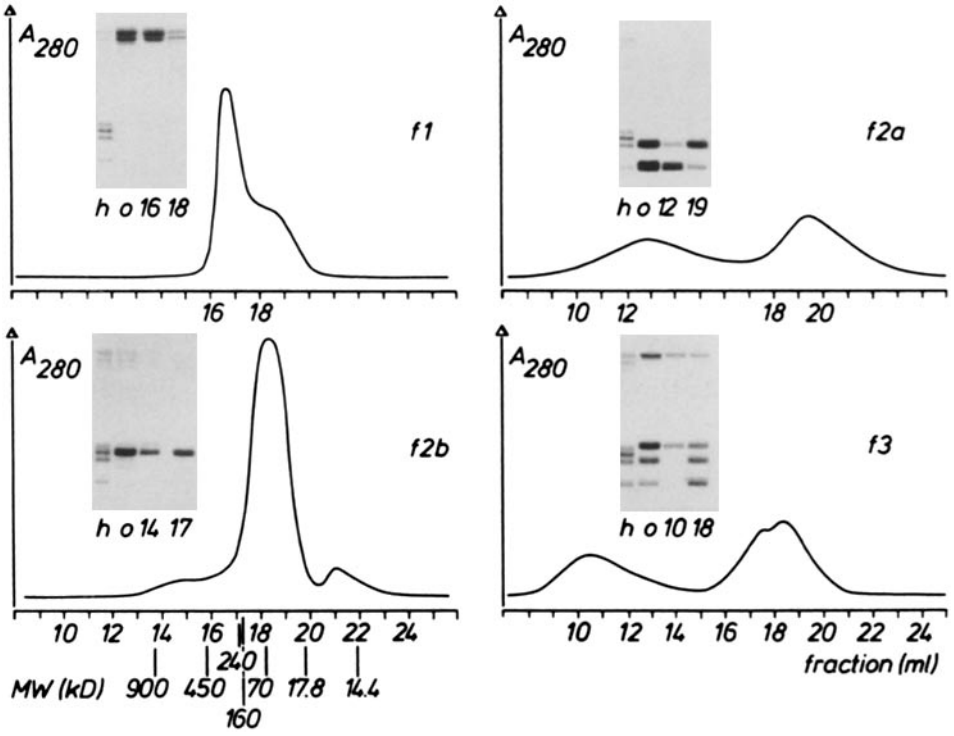

FIGURE 2. Aggregation behavior of histone subfractions, concentration 12 mg/ml, sample volume 0.2 ml, flow 0.5 ml/min, analyzed by FPLC-gel filtration chromatography on a Superose 6 HR 10/30 column (total gel bed vol, 25 ml). Protein profiles of peak fraction analysis in SDS-PAGE are shown in inserts. *h*, total histone; *o*, analyzed material. Markers for molecular masses were: 900 kD/450 kD, dimeric/monomeric cationized ferritin; 240 kD, cationized catalase from bovine liver; 160 kD, cationized human IgG; 70 kD, cationized human serum albumin; 17.8 kD, myoglobin from horse; 14.4 kD, ovine lysozyme.

salt gradient of 0.15–2 M NaCl. Lysozyme elutes at very low salt concentrations, indicating a low affinity, whereas histone f1 has a medium affinity, the main portion eluting at 1 M NaCl. Histone f2a and histone f3, with a high affinity, could not be desorbed even with 2 M NaCl; an even higher salt concentration, 6 M guanidine-HCl, had to be used. The elution profile of f3 was nearly identical to f2a. In

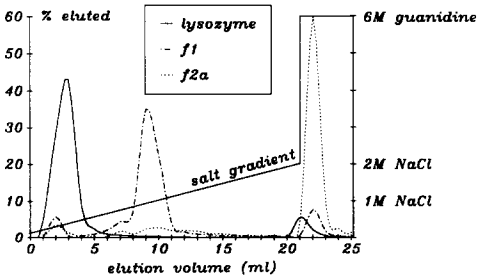

FIGURE 3. Affinity of histone subfractions f1, f2a, and lysozyme to Heparin-Sepharose tested by salt elution studies. Running conditions were: Total bed volume, 200 μl; sample volume, 100 μl (400 μg/ml in PBS). Elution buffers: buffer 1 (b1), 0.15 M NaCl, 10 mM sodium phosphate; buffer 2 (b2), 2 M NaCl, 10 mM sodium phosphate; buffer 3 (b3), 6 M guanidine-chloride, pH 7.4. Gradient: 0–20 ml, linear gradient start 100% b1, 0% b2, end 0% b1, 100% b2; 20–25 ml, 100% b3. Fraction vol, 1 ml.

the case of f2b, ~50% of the radioactivity eluted with the sodium chloride gradient. The data for f2b and f3 are not shown in Fig. 3 for improved clarity.

Antisera to Histone Subfractions. The antihistone reactivity of various rabbit antisera raised against complexes of histones with either hemocyanin (KLH) or RNA are shown in Fig. 4. Rabbits immunized with histone KLH developed antibodies to histones more frequently and in higher titers than animals treated with histone RNA. Some sera showed broad reactivity against nearly all histone subfractions, whereas others were specific for single subfractions, such as H3 or H2a/H4. All control sera showed essentially no antihistone reactivity.

Kidney Binding of Histone f2a and Lysozyme after Intravenous Injection. Histone f2a binds to the GBM as effectively when given intravenously as when it is given intraaortally (see below). We used higher doses for intravenous injection, because of the ability of histones to bind to various other tissues throughout the body. Histone f2a showed a linear to fine granular pattern of deposition along the GBM and peritubular capillaries (Fig. 5 *A*). It was also seen in reabsorption droplets in the proximal tubules, whereas lysozyme, injected in control rats, was found only in the latter location (Fig. 5 *B*). Preliminary studies with ^{125}I-labeled histone fractions showed increasing affinities in the order of f1, f2a, and f3 (data not shown).

Organ Distribution of Histone Subfractions after Injection via the Aorta. After injection of ^{125}I-labeled histones, all subfractions tested showed a high affinity to glomerular structures in comparison with lysozyme. The ratio of glomerular bound histone to bound lysozyme ranged from a factor of 33 (f1) to a factor of 79 (f3). The organ distribution of ^{125}I-histone and ^{125}I-lysozyme is shown in Table I. Renal histone binding was seen almost exclusively in the left (perfused) kidney, whereas lysozyme was deposited in the right kidney as well. Virtually no lysozyme was found in iso-

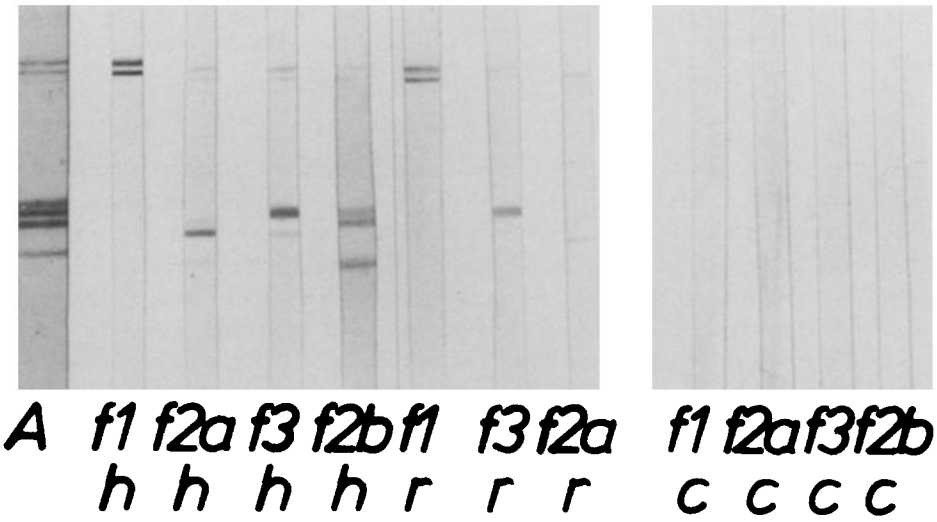

FIGURE 4. Western blots of sera from rabbits immunized with histone-RNA (*r*) or histone-KLH (*h*) complexes. Control sera (before immunization) of the animals receiving histone-KLH are shown on the right side (*c*). All sera were used at a dilution of 1:1,000. Amido black stain of histone antigen fixed on the nitrocellulose strip (*A*).

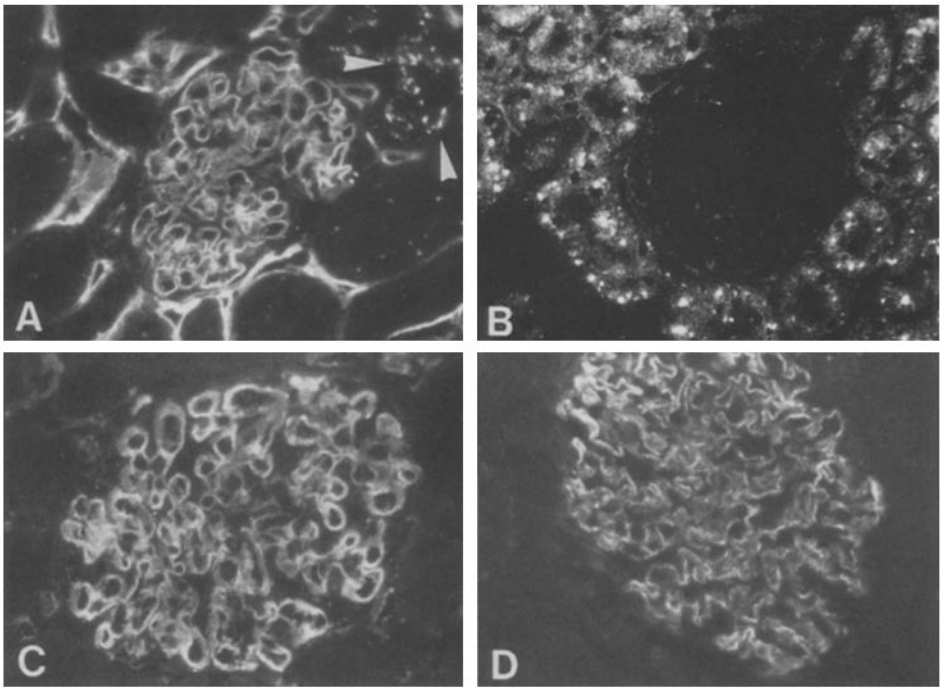

FIGURE 5. Histone as a planted antigen. Indirect (*A–C*) or direct immunofluorescence (*D*). (*A*)After intravenous injection of histone f2a (2 mg/100 g body weight), the antigen is deposited in a linear to fine granular fashion along the GBM and the peritubular capillaries. Only a small amount is detectable in reabsorption droplets in the cytoplasm of tubular cells (*arrows*). Note absence of nuclear staining. Compacted f2a is not accessible for the antibody. (*B*) In contrast, lysozyme used as control is found only in tubular cells. (*C*) Localization of histone f2a after intraaortal injection of 200 μg/100 g body weight. Kidneys were excised 15 min after injection. f2a is seen in a linear to fine granular pattern along the capillary wall. (*D*) Deposition of rabbit IgG after injecting 500 μg histone f2a into the left kidney via the aorta followed by subsequent intravenous injection of 0.6 ml/100 g body weight rabbit anti-f2a-serum. Antibody is deposited along the glomerular capillary wall. Direct immunofluorescence using FITC-labeled anti-rabbit IgG. ×640.

lated glomeruli, by immunofluorescence it was located in the tubular cells. Histone f1 shows the lowest, f2a a medium, and f3 the highest amount of deposition in glomeruli. The affinity of the various histone subfractions correlated well with their affinity to Heparin-Sepharose in vitro (see above). The pattern of glomerular-bound histone f2a after injection via the aorta is shown in Fig. 5 *C*; the distribution as well as the intensity was similar to that of intravenously injected histone f2a. In the case of f3, which was contaminated with H2a and H4, we found a very strong staining of the GBM with specific anti-H3 serum, whereas staining with specific anti-H4/H2a serum was much weaker. The opposite was found in protein reabsorption droplets in tubular cells, which stained strongly with anti-H4/H2a and with reduced intensity with anti-H3. This is in agreement with the assumption that the highly aggregated fraction has a greater affinity for the GBM than the less-aggregated fraction, the latter passing the glomerular filter to reach the urinary space.

Kinetic Studies of Renal ^{125}I-histone-f2a and ^{125}I-f1 Binding after Injection via the Aorta. The results of the kinetic studies are shown in Table II. These show that, as mea-

TABLE I

Organ Distribution of ^{125}I-histone Subfractions and ^{125}I-lysozyme after Intraaortal Injection

Organ	Lysozyme (n = 5)	f1 (n = 5)	f2a (n = 6)	f2b (n = 5)	f3 (n = 5)
			μg		
Left kidney	43.0 ± 10.0*	113.0 ± 13.0	50.0 ± 15.0	60.0 ± 16.0	74.0 ± 20.0
Right kidney	20.0 ± 10.2	5.0 ± 1.7	1.7 ± 0.7	2.2 ± 0.8	3.3 ± 2.1
Liver	14.4 ± 1.4	70.0 ± 20.0	93.0 ± 40.0	36.0 ± 17.0	106.0 ± 55.0
Spleen	1.3 ± 2.8	3.6 ± 2.8	5.3 ± 2.1	1.9 ± 1.0	5.0 ± 1.8
Lung	2.0 ± 0.3	3.5 ± 0.9	4.2 ± 2.2	1.9 ± 0.7	6.8 ± 4.1
Heart	0.9 ± 0.2	1.1 ± 0.9	1.0 ± 0.4	0.3 ± 0.1	0.8 ± 0.4
Blood (1 ml)	4.1 ± 1.5	1.5 ± 0.3	6.3 ± 2.3	2.0 ± 0.4	4.7 ± 1.2
Isolated glomeruli from left kidney	0.4 ± 0.2	13.3 ± 5.3	17.0 ± 2.3	17.0 ± 5.9	31.7 ± 9.6
p vs. lysozyme		<0.0005	<0.0005	<0.0005	<0.0005
p vs. f1			<0.07		<0.005
p vs. f2a					<0.005
p vs. f2b					<0.05

200 μg of ^{125}I-labeled histone f1, f2a, f2b, f3, or lysozyme was injected into the left renal artery via the aorta. Animals were killed 15 min after injection and radioactivity was counted.

* Mean ± SD.

sured in isolated glomeruli, f1 is more rapidly cleared than f2a, in spite of the greater quantity of f1 found in the whole kidney at all times tested. The latter is mainly located in tubular cells, resulting from reabsorption of histones, which have penetrated the GBM to reach the urinary space.

In Situ Immunecomplex Formation. After injection into the left kidney via the aorta, glomerular-bound histone f2a was accessible for a subsequently intravenously injected antibody. IC formation occurred in situ in the GBM, as only the left kidney showed linear deposition of rabbit Ig by immunofluorescence (Fig. 5 *D*), whereas the right kidney showed no Ig deposits. Additionally, there were some reabsorption droplets containing rabbit Ig in the left kidney only, indicating that histone could have disturbed normal glomerular protein restriction, leading to enhanced protein

TABLE II

Disappearance of ^{125}I-f1 and ^{125}I-f2a from Left Kidney and Glomeruli after Intraaortal Injection

Time interval after injection	f1		f2a	
	Left kidney	Isolated glomeruli	Left kidney	Isolated glomeruli
		μg		
15 min	113.0 ± 13.0*‡	13.3 ± 5.3*	50.0 ± 15.0	17.0 ± 5.9
1 h	62.9 ± 3.2*	4.9 ± 0.5*	48.3 ± 8.5	21.5 ± 5.8
4 h	44.0 ± 4.6*	1.7 ± 0.4*	21.5 ± 6.4	9.4 ± 3.4
8 h	ND	ND	9.5 ± 2.4	4.1 ± 0.9

Five animals were examined for each group.

* p (vs. f2a) < 0.001.

‡ Mean ± SD.

TABLE III

Influence of Histone on ssDNA Binding to Glomerular Capillary Wall

Group	*f3*	*ssDNA*
1 (f3/ssDNA) (n = 5)	+ +/+ + +	+ +/+ + +
2 (ssDNA alone) (n = 5)	∅	∅
3 (f3 alone) (n = 5)	+ + +	∅

Samples were injected directly into the left kidney via the aorta. Rats received f3 alone, f3, and (subsequently) ssDNA or ssDNA alone. Kidneys were removed 15 min after the last injection. Indirect immunofluorescence using rabbit anti-f3 or human anti-ssDNA antibody, respectively.

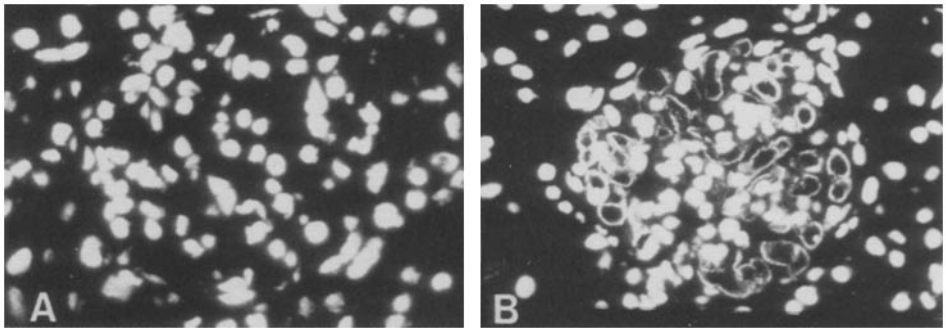

FIGURE 6. ssDNA deposition after injection of 320 μg ssDNA without (*A*) or with (*B*) previous injection of 500 μg f3. Indirect immunofluorescence using an SLE serum, containing antibodies to ssDNA. ssDNA is only seen along the capillary wall when preceded by injection of histone. Nuclear fluorescence is seen in all cases.

leakage through the capillary filter. Control animals receiving only the appropriate antiserum did not show deposition of rabbit Ig.

Binding of ssDNA after Histone f3 Injection. The results are shown in Table III. Polydisperse ssDNA fragments, which we injected into the left kidney via the aorta, did not bind to the GBM as tested by immunofluorescence using human sera from patients with SLE (Fig. 6 *A*). These sera had a high titer of anti-ssDNA antibodies, but no antihistone reactivity. If the same ssDNA material was injected via the aorta after a previous injection of histone f3, we found granular deposits of ssDNA along the GBM and the peritubular capillaries by immunofluorescence (Fig. 6 *B*). The specificity of our anti-ssDNA sera was tested using kidneys from rats injected with histone f3 alone. These were strongly positive with our rabbit antihistone f3 sera, but completely negative with the human anti-ssDNA sera used.

Discussion

In spite of >20 yr of intensive effort to elucidate the immunological basis of renal involvement in SLE, this subject remains somewhat mysterious. The major focus of attention has been on nuclear antigens, principally DNA, and a number of mechanisms have been proposed. These range from the classic concept of glomerular deposition of preformed DNA-anti-DNA complexes (18), in situ formation of immune

complexes initiated by binding of DNA to the GBM (19–23), as well as direct immunological assault mediated by anti-DNA antibodies with crossreactivity for the GBM (52–55). All of these concepts have weaknesses and we have tried to throw some new light onto the mechanisms involved by integrating recently gained insights on charge based glomerular IC formation (30–34) and nuclear antigen involvement in SLE nephritis (56). Positively charged antigens are excellent candidates as "Nephritogens," and the group of nuclear antigens contains an interesting member, the histones.

Our work clearly demonstrates that free, soluble histones show a high affinity for the GBM and other basement membranes, e.g., those in the peritubular capillaries when injected intravenously or intraaortally. For most experiments we preferred intraaortal injection, because it eases analysis of the parameters responsible for binding of histones to the GBM, in contrast to the very complex interactions occurring in the case of intravenous injection. In previous studies from our laboratory (34), two major determinants were shown to be critical for binding of antigen to the GBM: the size (> 40,000) and the charge, the latter roughly estimated from the isoelectric point (pI > 8.5). In spite of a high cationic charge histones would not be expected to persist in the GBM because of their small size (mol wt < 22,000). Histones have the unique function of organizing the chromosomal DNA fiber into a highly condensed chromatin fiber. This is due to some special properties; histones can be considered as being composed of two regions, one positively charged, rich in arginine and lysine residues, and giving the histones a high net positive charge, being responsible for binding to the DNA fiber, the other hydrophobic and interacting with other histones (35). This explains how they are able to aggregate spontaneously, in spite of the repulsion to be expected between molecules of like charge. In fact, aggregation of histones can be promoted by adding counter ions (increase in ionic strength) or by polyanions.

Naturally occurring aggregates, under physiological conditions, are a H2a-H2b dimer and a $H3_2$-$H4_2$ tetramer with association constants of 10^6 M^{-1} and 7×10^{20} M^{-3}, respectively, underlining their stability (37). In chromatin itself the basic histone unit is an octamer, which is only stable in chromatin or in the presence of 2 M NaCl (38). The clustering of positive charges is presumably also responsible for the very avid binding of histones to both Heparin-Sepharose in vitro and to the GBM in vivo as shown here. Variation in the clustering of charges as well as in the tendency to form reversible aggregates explain the differences between the amounts of the various histone subfractions and lysozyme bound to Heparin-Sepharose in vitro or to the GBM in vivo. Variation in the molecular characteristics will also be the reason for the different clearance of f1 and f2a from the glomeruli, as seen in our kinetic studies.

Lysozyme is a cationic, nonagggregating molecule, which shows poor binding to the GBM, although it has been used as a tracer for anionic regions in the glomerular capillary wall before the introduction of superior reagents (57). After chemical crosslinking of lysozyme, leaving charge distribution untouched, the affinity of the polymeric lysozyme to the GBM increases (58). The histone subfractions show different degrees of aggregation and, especially in the case of f1 and f3, the proportion of aggregates and their molecular size correlates well with their affinity for the GBM. When histone subfractions were compared, f1 showed the lowest affinity to the GBM

and the Heparin-Sepharose column, although we are dealing with the most densely charged histone. This apparent contradiction is probably due to the low tendency of histone f1 to form aggregates (35). We do not know which subconstituent of a soluble histone preparation has the highest affinity to the GBM as there will be an equilibrium between the high and low size aggregate forms; in situ aggregation of the histones in the GBM is a possibility as well. In the case of f3, we have some evidence that the high molecular weight fraction has the highest affinity for the GBM. By using specific antisera to H3 or H2a/H4, we could show by immunofluorescence that H3, which was enriched in the high molecular fraction, had superior binding to the GBM and therefore reduced passage through the GBM in comparison with H2a/H4, which were enriched in the low molecular weight fraction.

As has been mentioned above, there is concurrence between charge repulsion and intermolecular adherence. Interaction of the positively charged residues with anionic molecules facilitates adherence between histone molecules. Therefore, interaction of histones with anionic sites of the GBM together with subsequent binding of nucleic acids may enhance aggregation and lead to locally enlarged deposits inside the GBM. This may be comparable with a cristallization process around a cristallization germ.

A difficulty that occurs when working with free histones under physiological conditions is the unavoidable contamination with proteases. Protease inhibitors are toxic and cannot be used in vivo; apart from this, they can interact with protein molecules. In our experiments with ^{125}I-labeled histones, only f1 and f2b showed a slight degree of early stage proteolytic degradation, which may have had some negative influence on their binding.

We also do not know if the low amount of DNA contamination (<1%) in the histone preparations could have had an influence on aggregation behavior and binding of histones to the GBM.

Most of the autoantibodies found in SLE are directed against nuclear antigens, which is the reason that such antigens are suspected to be involved in the pathogenesis of the disease. It has been reported that DNA has an affinity for purified collagen, type I and III, and isolated GBM in vitro; in vivo DNA did not bind to the GBM unless LPS had been previously injected (21). We were also unable to demonstrate an affinity in vivo; as reported here, pure ssDNA did not accumulate along the glomerular capillary wall after intraaortal injection, as tested by immunofluorescence using FITC-labeled anti-ssDNA antibody. ssDNA was only detectable when histone had been previously injected. This could also be confirmed with radiolabeled ssDNA (unpublished data).

It has been clearly demonstrated here that histones have a high affinity for the GBM in vivo. An intriguing aspect is whether histones are liberated during cell breakdown in a free form, or as a complex with DNA, as the principal subunit is the nucleosome structure. It is known that DNA can be liberated from chromatin by treatment with heparin or dextran sulfate, which complex with histones more avidly than DNA (59). This allows the speculation that histones present in circulating nucleosomes, perhaps partially degraded by nucleases, could swap their DNA partner for the negative charges (heparin sulphate) contained in the GBM, due to the higher avidity of the latter interaction.

A similar phenomenon has been described for platelet factor 4 (PF4), which con-

sists of a protein complexed to chondroitin sulphate. After liberation of PF4 from platelets, the protein portion of the molecule can exchange its polysaccharide partner for a molecule having a higher net negative charge, for example, heparan sulfate in the GBM (60).

The binding of histones to the vascular bed could be the key event in the pathogenesis of lupus disease. (*a*) First, the tissue-bound histones can act as planted, target antigen for circulating antibody, leading to immune complex formation. This of course does not exclude the involvement of preformed, circulating antigen (here histone)-antibody complexes. (*b*) Second, the interaction of the positive histone molecules with the negatively charged basement membrane can promote the binding of polyanionic antigens, for example, DNA fragments, normally repulsed, which can themselves act as target antigens. Further studies are necessary to look for histone involvement in murine lupus models and human disease, and they are currently underway in our laboratory.

We are also studying, if a "change of binding partner" of histones from the nucleosome structure (DNA) to the histone-anionic proteoglycan complex occurs in vivo, whereby we have shown, on tissue sections, that this holds true in vitro (unpublished data).

Summary

An effort has been made to integrate insights on charge-based interactions in immune complex glomerulonephritis with nuclear antigen involvement in lupus nephritis. Attention was focussed on the histones, a group of highly cationic nuclear constituents, which could be expected to bind to fixed anionic sites present in the glomerular basement membrane (GBM).

We demonstrated that all histone subfractions, prepared according to Johns (4), have a high affinity for GBM and the basement membrane of peritubular capillaries. Tissue uptake of ^{125}I-labeled histones was measured by injecting 200 μg of each fraction into the left kidney via the aorta and measuring organ uptake after 15 min. In glomeruli isolated from the left kidneys, the following quantities of histones were found: f1, 13 μg; f2a (f2a1 + f2a2), 17 μg; f2b, 17 μg; and f3, 32 μg. Kinetic studies of glomerular binding showed that f1 disappeared much more rapidly than f2a. The high affinity of histones (pI between 10.5 and 11.0; mol wt 10,000–22,000) for the GBM correlates well with their ability to form aggregates (mol wt > 100,000) for comparison lysozyme (pI 11, mol wt 14,000), which does not aggregate spontaneously bound poorly (0.4 μg in isolated glomeruli). The quantity of histones and lysozyme found in the isolated glomeruli paralleled their in vitro affinity for a Heparin-Sepharose column (gradient elution studies). This gel matrix contains the sulfated, highly anionic polysaccharide heparin, which is similar to the negatively charged heparan sulfate present in the GBM. Lysozyme eluted with 0.15 M NaCl, f1 with 1 M NaCl, and f2a, f2b, and f3 could not be fully desorbed even with 2 M NaCl; 6 M guanidine-HCl was necessary.

Two further findings of great relevance for the concept of induction of immune complex glomerulonephritis by histones were: (*a*) glomerular-bound histone was accessible for specific antibody given intravenously; and (*b*) prior binding of histones promoted glomerular deposition of anionic antigens, as could be shown with ssDNA fragments. These data justify the proposal that glomerular deposition of histones

can induce immune complex formation, start an inflammatory process, and produce tissue damage.

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