Detection of Nucleosome Particles in Serum and Plasma from Patients with Systemic Lupus Erythematosus Using Monoclonal Antibody 4H7

RALPH C. WILLIAMS Jr, CHRISTINE C. MALONE, CHRISTOPHER MEYERS, PATRICE DECKER, and SYLVIANE MULLER

ABSTRACT. ***Objective.*** To develop a monoclonal antibody reagent that would react with nucleosomes but not directly with constituent double stranded DNA (dsDNA) or with histones.

Methods. Mice were immunized with highly purified chicken mononucleosomes and hybridomas employed to produce Mab that did not react with dsDNA or histones but still showed reactivity with nucleosomes.

Results. Murine monoclonal IgG antibody 4H7, generated from a mouse immunized with highly purified chicken erythrocyte nucleosomes, showed no direct ELISA reactivity with either dsDNA or isolated histones or with Sm and RNP antigens or combinations of any of these components. Mab 4H7 did show strong ELISA reactivity for chicken erythrocyte and calf thymus nucleosomes as well as for human leukocyte nucleosomes. The Mab did show strong ELISA reactions with peptides 1–25 of histone H2B and 1–21 of H3, which correspond to sequences known to be located at the surface of nucleosomes. We then measured relative serum levels of 4H7 reactive nucleosome antigen in 140 patients with systemic lupus erythematosus (SLE) in parallel with 50 non-SLE patients with other types of connective tissue disease and 92 healthy subjects. Occasional low levels of serum nucleosomal antigen were seen in 4 of 92 controls, but many patients with SLE (66/140) showed marked elevations of serum nucleosomal antigen. No difference was observed when serum or plasma samples were studied. A marked correlation ($R = 0.401$, $p < 0.0001$) was noted when disease activity score (SLEDAI) was plotted against optical density value measured with 4H7 in ELISA. Further, the levels of circulating nucleosomes were raised in SLE patients with very active central nervous system and renal involvement.

Conclusion. Presence of nucleosome related antigen in sera from patients with SLE may provide insight into the sequence of disease related antigenic stimuli in active SLE. (J Rheumatol 2001;28:81–94)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS NUCLEOSOMES DISEASE ACTIVITY

The pathways by which antibodies with various antinuclear specificities are stimulated during generalized connective tissue disorders such as systemic lupus erythematosus (SLE) are not clearly understood. Although most patients with SLE show the presence of serum antinuclear antibodies with anti-DNA or other nuclear specificities, how these antibodies are induced or actually arise during evolution of clinical disease is not accurately defined. Attempts to produce anti-DNA or autoantibodies with other types of antinuclear reactivities by immunization with DNA from various mammalian sources were unsuccessful[1,2]. More success in producing anti-DNA antibodies in nonautoimmune mice was achieved using immunization with various bacterial DNA preparations[3,4]. Moreover, viral infection with the BK virus, but not immunization with noninfectious BK DNA, induced autoantibodies to autologous native DNA[5]. In addition, *in vivo* expression of a single viral BK DNA-binding protein, the polyoma virus T antigen, was sufficient to initiate the production of anti-double stranded (ds) DNA and histone antibodies, thus providing an interesting experimental model for SLE[6].

Recently, attention has been redirected to nucleosomes as a potential major vehicle for antigenic stimuli that may actually induce SLE. Fournier[7] and Rumore and Steinman[8] first identified endogenous circulating DNA in plasma from patients with SLE as multimeric complexes of DNA bound to histone

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called oligonucleosome-like structures. Moreover, recent studies by Mohan, Datta and coworkers provide strong support for the concept that nucleosomes may be directly involved in production of lupus nephritis, particularly through induction via histone components of T cell help for pathogenic autoantibody production in this disease manifestation[9-11]. In addition, other lines of investigation indicate that constituents of nucleosomes are involved in the fundamental antigenic stimulus in SLE. Nucleosomal autoantigens have been identified as important in the genesis of anti-chromatin autoantibodies in murine lupus[12]. Work by several groups identified nucleosome restricted antibodies as an important feature in lupus pathogenesis[13-15]. A recent report by Amoura, *et al*[16] described detection of what appear to be whole nucleosomes in plasma from patients with SLE at various stages of their disorder. These authors found that levels of plasma nucleosomes were significantly increased in patients with SLE compared to the controls, and were apparently highest when the clinical SLE was least active.

We describe the production and specificity of a mouse monoclonal antibody (Mab) called 4H7 that was generated from a normal mouse by immunization with chicken erythrocyte nucleosomes. This antibody reacts with nucleosomes but not with the nucleosomal subcomponents DNA and histones or with high mobility group (HMG) proteins, Sm, and RNP antigens, or various combinations of the latter. We used this Mab to measure the relative levels of nucleosomes in serum, plasma, and glomerular eluates of patients with SLE and found that serum and plasma levels of circulating nucleosomes were highest in patients with very active central nervous system (CNS) or renal involvement.

MATERIALS AND METHODS

Nucleosomes, histones, and histone peptides. Nucleosomes containing H1/H5 and ~ 180–210 bp of DNA were prepared from chicken erythrocyte nuclei using methods outlined by Muller, *et al*[17]. The nucleosome fractions were characterized by 2% agarose gel electrophoresis and their histone content by sodium dodecyl sulfate-18% polyacrylamide gel (SDS-PAGE). We also prepared calf thymus nucleosomes as described[18]. An example of purification is shown in Figure 1. In addition, nucleosomes from human leukocytes were prepared as described by Suenaga and Abdou[19]. Five × 10^6 peripheral blood mononuclear cells obtained from heparinized blood of healthy donors by Ficoll gradient were cultured in 10% fetal calf serum (FCS) RPMI 1640 for 7 days. Culture supernatants and cells were then harvested and stored at –20˚C. To estimate apoptosis in the culture, DNA was extracted from the cell supernatants and the cultured cells were lysed and centrifuged at 9000 g for 20 min. Ten percent to 20% of the cultured cells were apoptotic by FACS analysis. Supernatants and culture supernatants were digested with 20 µg/ml of RNase (Sigma Chemical Co., St. Louis, MO, USA) for 1 h at 37˚C, followed by proteinase K digestion as used for the chicken erythrocyte nuclei preparation. Phenol extraction and ethanol precipitation followed standard procedures[20]. OD 260/280 ratios showed that the dsDNA preparation contained less than 3% protein. ELISA reactivity of SLE serum IgG with nucleosomes from chicken erythrocyte nuclei and cultured human leukocytes showed very similar results; however, higher yields of nucleosomes were generally obtained when chicken erythrocytes were used. Histones were either purchased from Sigma or purified from chicken erythrocytes and calf thymus, as described[21]. The purity of histone fractions was assessed by SDS-18%-PAGE. Purification of calf thymus histone peptides has been described[18].

Production of Mab 4H7. Balb/c mice were injected subcutaneously with 10 µg (as expressed in DNA concentration) whole chicken erythrocyte mononucleosomes using complete Freund's adjuvant for the initial injection and incomplete adjuvant for subsequent immunizations. After 3 series of injections at 2 to 3 week intervals, when tail vein bleedings indicated production of antibody reacting in ELISA with whole nucleosomes but not with dsDNA, mice were sacrificed and fusions made for hybridoma production using our standard mouse SP-2/0 B cell line fusion partner (ATCC).

Hybridomas were screened for ELISA reactivity against whole mononucleosomes as well as dsDNA. Antibody-secreting cells were selected that gave strong reactivity with nucleosomes but not with DNA or individual histones, and these cell lines were subcloned several times. One Mab called 4H7 (IgG_1,K) was selected that gave strong ELISA reactions with our standard human and chicken nucleosomal preparations, but none with individual nucleosomal components such as dsDNA, H1, H2A, H2B, H3, H4, or total histones, or Sm/RNP antigens. The dsDNA employed in the studies was obtained from Sigma. This dsDNA preparation was agarose gel isolated for consistent size, and any residual protein contaminants removed by phenol/chloroform extraction followed by ethanol precipitation before use. The Mab 4H7 was isolated from cell culture supernatants by protein G chromatography. This procedure produced 2 bands (heavy and light chains) on SDS gel analysis indicating its purity and no trace of histones.

ELISA for anti-nucleosome, anti-DNA, Sm, and RNP activity. In the direct ELISA used to measure the reactivity of Mab in culture supernatants with chicken and human nucleosomes, ELISA plates were coated with mononucleosome preparations in pH 7.5 Tris buffer at a concentration of 10 µg (expressed as DNA) per ml and blocked with 2% bovine serum albumin (BSA). After culture supernatants were incubated with nucleosome coated plates for 1 h, plates were washed 3 times with phosphate buffered saline (PBS) and developed with 1:10,000 dilution of peroxidase conjugated $F(ab')_2$ fragment of goat anti-mouse IgG followed by completion of color development and reading in an automated ELISA reader at 492 nm. The same procedure was used with Mab 4H7 using isolated Mab at 5 µg/ml. All samples were run in triplicate and averaged. Negative controls included PBS in place of serum and/or Mab and were subtracted from antigen coated plates. The ELISA format used with calf thymus nucleosomes was as described[18]. A competition assay where histone peptides were used as coated antigens and free nucleosomes as inhibitors in the fluid phase was also employed, as described[18]. Briefly, nucleosomes were used as competitors to inhibit the reaction between coated peptides 1–21 of H3 or 1–25 of H2B at 2 µM each and 4H7 (0.5 µg/ml) reacting with the adsorbed peptides. This approach allowed us to test nucleosomes as free antigens in solution; as well, only the antibody specifically reacting with the peptide on the ELISA plate was examined for inhibition by free nucleosomes in solution. The results were expressed as the inhibition percentage of the antibody binding. This allowed testing of mono-, di-, and tri- as well as polynucleosomes, the concentration of which was expressed as ng DNA/ml.

Reactivity of Mab 4H7 with dsDNA, Sm, and Sm/RNP antigens was tested by ELISA as described[22]. The dsDNA was obtained from Sigma and purified as described above. Commercial histones H1, H2A, H2B, H3, and H4 (Sigma) were coated on polystyrene ELISA plates at 5 µg/ml and employed as antigens in ELISA with increasing dilutions of Mab 4H7. The reactivity of 4H7 with individual calf and chicken histones (400 ng/ml) coated on polyvinyl plates and overlapping histone peptides (2 µM each) was also tested as described[18]. HMG proteins 1+2, 14, and 17 (kindly provided by Dr. A. Mazen, CNRS, Strasbourg, France, and Dr. M. Bustin, NIH, Bethesda, MD, USA) were also assayed using 200 ng HMG/ml for coating polyvinyl plates[23,24].

Immunofluorescence studies. Mab 4H7 was studied using indirect immunofluorescence and HEp-2 cells. The Mab at 5 µg was overlaid on HEp-2 cells prepared on a microslide by a commercial supplier. After 1 h incubation, the HEp-2 cells were washed 3 times with 0.1 M PBS (pH 7.0) and counterstained with 1:1000 dilution of fluorescein conjugated $F(ab')_2$ IgG fragment of goat anti-mouse IgG. After 30 min the HEp-2 cells were washed twice with PBS and examined under a Zeiss fluorescence microscope. Controls includ-

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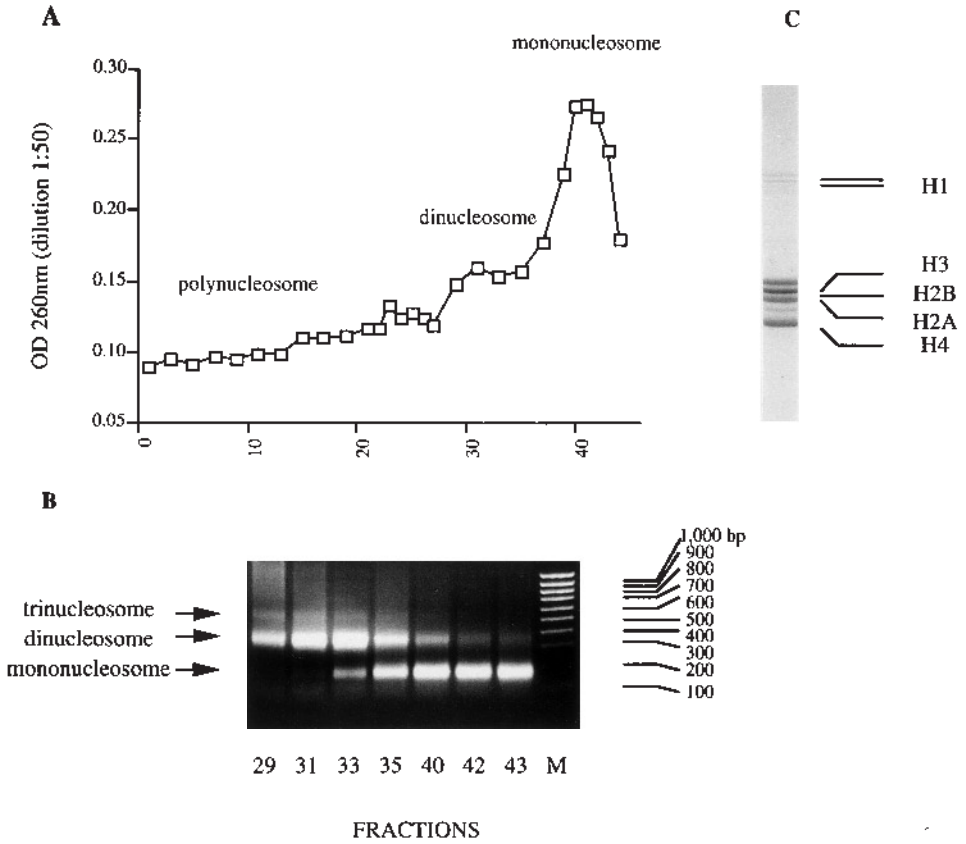

Figure 1. Preparation and composition of nucleosome fractions extracted from calf thymus. Nucleosomes prepared as described[29] were purified on a 5–29% sucrose gradient (A). Analysis for DNA content of these fractions was by electrophoresis using 2% agarose gel (B); analysis for histone content (C: fraction 40) was by SDS-18%-PAGE. M: size markers (100–1000 bp of DNA).

ed HEp-2 cells and the FITC antibody alone, and the sections without Mab and without FITC conjugate. A nonrelevant Mab of the same isotype was also used as a control.

Patient sera and plasma. We studied serum samples from 140 patients with SLE being followed at the University of Florida Health Sciences Center. In many patients (n = 50), serum and plasma samples collected at the same time were studied together, and in these instances no significant difference in ELISA values was recorded. All serum samples were obtained from individual blood samples by prompt centrifugation using a serum/cell separator gel pledget, which does not allow contact between serum and centrifuged cells even during the centrifugation process. The serum samples were collected in a vacutainer containing SST gel and clot activator (Becton-Dickinson, Franklin Lakes, NJ, USA). Sera at the top were carefully aspirated from the pledget and cells beneath the latter within 20 min of centrifugation and stored frozen at –20˚C until assays were performed. Plasmas were collected immediately after EDTA tubes were centrifuged; they were frozen and kept at –20˚C until assay. All patients had a definite diagnosis of SLE confirmed by American College of Rheumatology 1982 diagnostic criteria. In some patients, serial serum samples were available over an 8–9 year period between 1988 and 1997. All serum samples were correlated with a concurrent global assessment of SLE disease activity at the time of serum collection using the SLE Disease Activity Index (SLEDAI) as described[25]. The SLEDAI score was determined independently by an investigator who had no knowledge of the ELISA readings with Mab 4H7. Ninety-two healthy control subjects from medical center personnel, ages 21–69 and of both sexes, were also studied after giving informed consent. In addition, 50 disease controls were studied, including 20 patients with active rheumatoid arthritis (RA), 2 with fibromyalgia, 15 with osteoarthritis (OA), 8 with mixed connective tissue disease (MCTD), 2 with polymyositis, and 3 with active scleroderma. All control patients showed marked elevations of sedimentation rate or C-reactive protein and clinical signs of active disease.

Detection of circulating nucleosomes in SLE serum and plasma using Mab 4H7. Microtiter plates (Dynatech Laboratories Inc., Chantilly, VA, USA) were precoated at 4˚C with 200 µl/well of poly-L-lysine (Sigma) at 50 µg/ml in carbonate-bicarbonate buffer, pH 9.6, for 4 h. Plates were washed 3 times with H_20 before addition of serum. Serum or plasma samples were diluted 1:10, 1:50, and 1:1000 in PBS, pH 7.4, then added to the precoated plates (50 µl/well) and incubated overnight at 4˚C. The plates were washed with H_20 and blocked using 1% BSA in PBS, pH 7.4. After washing, Mab 4H7, purified as described above, was added at 5 µ/ml and incubated 1.5 h at room temperature. The plates were washed again and 50 µ/well of horseradish peroxidase conjugated $F(ab')_2$ fragment of goat anti-mouse IgG, Fc-specific (Jackson Immuno-Research Laboratories Inc., West Grove, PA, USA) diluted 1:10,000 in PBS was added. After 1 h incubation, plates were washed, developed with o-phenylenediamine (Sigma), and read in an automated ELISA reader at 492 nm.

Studies of renal biopsy eluates. Renal biopsy eluates were prepared as described[26] from previously frozen biopsy cores that were thawed and carefully separated from excess OCT embedding medium. One hundred microliters of 0.2 M glycine, 0.4 M NaCl, pH 2.0, were added to each tissue sample in Eppendorf tubes and incubated 5 min at room temperature and 15 min at 37˚C. The tissue was then sonicated for 2 min in the low pH Tris-glycine buffer, neutralized to pH 8.0 by addition of 20 µl of 0.1 mM Tris buffer (pH 9.0) and 280 µl of 0.1 mM Tris, and tissues were pelleted in Spin X tubes using a microfuge. Tissue biopsy eluates were then studied for protein concentration using the Pierce BCA protein assay and tested by ELISA for presence of reactivity with Mab 4H7.

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RESULTS

As shown in Figure 2, a strong ELISA reactivity with chicken and human nucleosomes was identified with Mab 4H7, but no significant reactivity was found with individual histones or a mixture of histones, or with histone complexes associated to dsDNA such as H2A-H2B-DNA complexes, H1-DNA, H3-H4-DNA complexes or total histone mixture plus DNA. No reaction was observed with mixture containing Sm and RNP antigens.

Addition of known amounts of chicken or human mononucleosomes to normal human serum for 4 h at 4°C showed that Mab 4H7 could also detect the nucleosome related antigen when added to serum over a broad concentration range. Construction of a standard curve using an extended range of concentrations of chicken or human nucleosomes in PBS showed a reasonably straight curve between concentrations of 0.625 and 10.0 µg/ml for quantitation of human nucleosomes by ELISA (Figure 3). The curve for quantitation of chicken

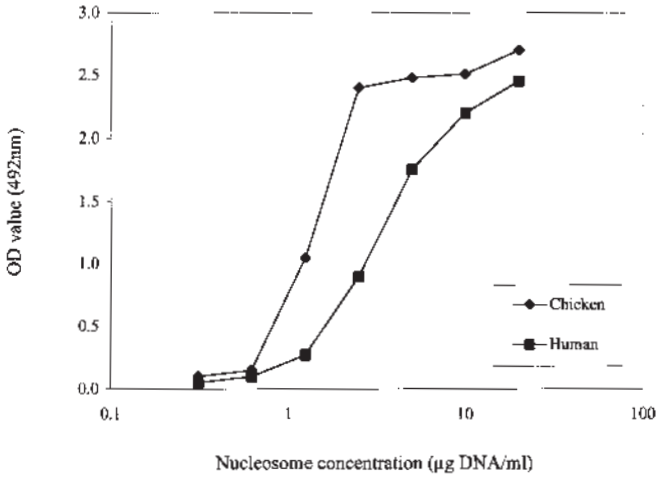

Figure 3. ELISA titration curves using 4H7 diluted 1:100 and normal human serum containing increasing concentrations of nucleosomes from chicken erythrocytes and apoptotic human leukocytes. Individual data points are indicated.

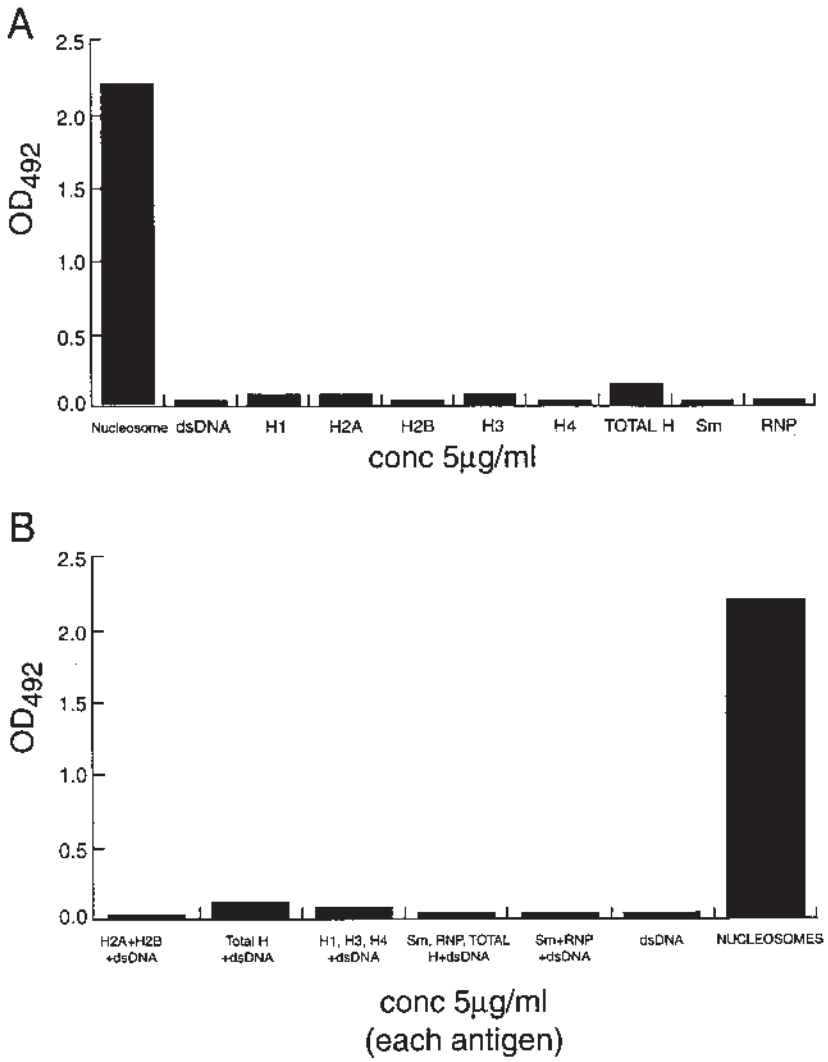

Figure 2. ELISA reactions using Mab 4H7 (diluted 1:100) with nucleosomes, dsDNA, as well as individual histones Hl, H2A, H2B, H3, H4, and total histones (total H), as well as Sm and RNP coated on ELISA plate at 5 µg/ml.

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nucleosomal antigen was displaced to the left and was straight between 0.05 and 2.5 µg/ml. In accord with the fact that the Mab had originally been produced by immunization with chicken nucleosomes, OD absorbance readings were higher with chicken than with human nucleosomes.

Since Mab 4H7 reacted strongly in direct ELISA with whole nucleosomes, whereas no significant reactions were detected with other presumed major individual nucleosome components such as DNA, histones, or combinations of the latter, there was some uncertainty whether the Mab actually reacted with true nucleosome antigens or even nuclear components. Accordingly, immunofluorescence staining of HEp-2 cells was performed by overlaying HEp-2 cells with Mab 4H7. These experiments showed a distinct pattern of staining that was associated with perinuclear immunofluorescence as well as speckled nuclear staining. A representative immunofluorescence photomicrograph is shown in Figure 4. Absorption of 4H7 Mab with purified nucleosomes affixed to an insoluble immunoabsorbent completely removed the immunofluorescence staining.

Whole chicken nucleosomal preparations were also studied by Western immunoblotting, using Mab 4H7 after separation of nucleosomes on SDS-18%-PAGE. These experiments showed that 4H7 reacted with a single well defined component of ~27–28 kDa (data not shown). Additional ELISA studies performed in parallel using highly purified HMG-1[23,24], HMG 1+2 fraction, HMG 14, and HMG 17 proteins indicated that 4H7 did not react with these proteins (data not shown), leading to the conclusion that the reactive 28 kDa component present within our nucleosome preparations was not HMG-1 or another HMG protein.

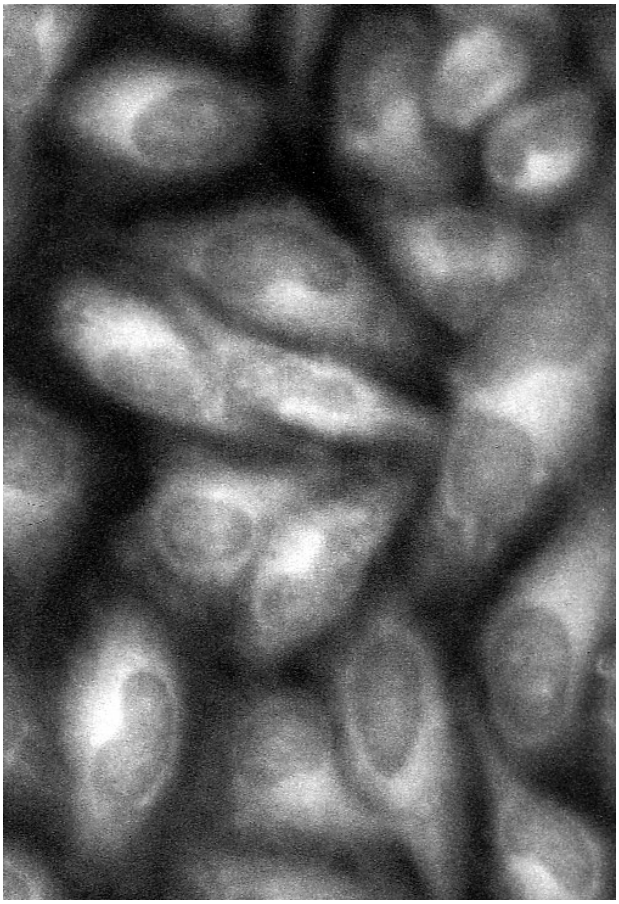

Figure 4. The immunofluorescence pattern of Mab 4H7 staining HEp-2 cells was perinuclear and speckled within the nucleus and often strongest with several cells close together. Cytoplasmic staining in the perinuclear area was also seen (magnification ×450).

To further characterize 4H7, a series of histone peptides known to be accessible at the surface of nucleosomes[18] were tested. Both peptides 1–25 of H2B and 1–21 of H3 showed strong reactivity with Mab 4H7, whereas a number of other histone peptides gave no positive reactions (Table 1). These results were confirmed by showing that mono-, di-, and trinucleosomes as well as long chains of chromatin (20 to 35 nucleosomes) in solution could significantly inhibit the binding of Mab 4H7 to either peptide 1–25 of H2B or peptide 1–21 of H3 coated on the microtiter plate (Figure 5). It was clear from these results that Mab 4H7 reacted with whole nucleosomes and that at least part of the antibody specificity was dependent upon residues 1–25 of histone H2B and 1–21 of histone H3.

Detection of circulating nucleosomes in SLE serum. In the 140 SLE patients, a significant proportion (47%) showed marked elevations of 4H7 Mab reactive nucleosomal antigen in serum samples. Levels of nucleosome related antigen were studied by ELISA with 1:10 and 1:50 serum dilutions. Among the entire group of patients with SLE, mean OD values at 492 nm using 1:10 dilutions of serum were 0.274 ± 0.466, and with 1:50 serum dilution were 0.109 ± 0.202. By contrast, mean values of 1:10 serum dilutions in normal sera were 0.057 ± 0.034, and using 1:50 serum dilution were 0.021 ± 0.025. Studies of 50 other connective tissue disease control sera including many with active RA and OA and a smaller number with MCTD, polymyositis, and scleroderma showed negative or only very low values in all patients studied. Optical density values with these disease controls were 0.043 ± 0.120 using a 1:10 dilution of serum or plasma, and 0.018 ± 0.023 with a 1:50 serum/plasma dilution. Chi-square test comparing all SLE values with healthy controls produced $p < 0.0005$. A similar chi-square test comparing SLE values with disease controls gave $p < 0.0005$. Many SLE patients positive for presence of 4H7 reactive nucleosomal antigen were those with active renal disease or CNS involvement. These findings are summarized in Table 2. It can be seen that virtually all patients with SLE showing serum levels of 4H7 reactive antigen producing an OD ≥ 0.500_{492nm} with 1:10 serum dilution had active CNS lupus or active SLE nephritis. By contrast, elevated levels of Mab reactive nucleosomal antigen producing ELISA OD = 0.100_{492nm} at 1:10 serum dilution were found in only 4 sera from 92 healthy controls, and slight elevations of the same magnitude were recorded in 3 patients in the disease control group, 2 with MCTD and one with very active RA.

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Table 1. Reactivity in ELISA of mouse Mab 4H7 with whole histones and various peptides derived from histones H2B, H2A, H3, and H4.

Histone Tested (100 ng/ml)	Optical Density 450nm mAb 4H7 (3.6 µg/ml)
H1	0.03
H5	0.00
H2A	0.06
H2B	0.04
H3	0.09
H4	0.09
Histone Peptides (2 µM)	
1-13 H2B	0.07
1-25 H2B	0.58
65-85 H2A	0.11
1-21 H3	1.06
11-25 H3	0.01
18-32 H3	0.15
85-102 H4	0.04
72-89 H4	0.05

H3 (+) 1_____ 21
(-) 11_____ 25
(-) 18_____ 32

H2B (+) 1_____ 25
(-) 1_____ 13

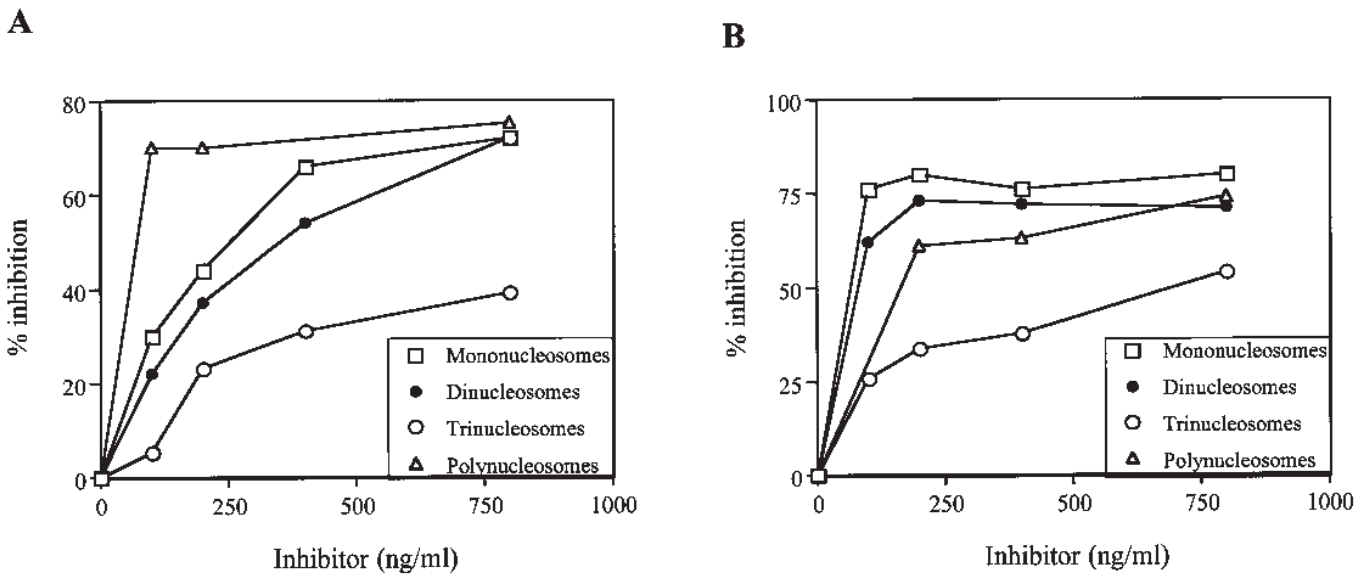

Figure 5. Inhibition of Mab 4H7 binding to 2 µg peptide 1–25 H2B (A) or 2µg peptide 1–21 H3 (B) on the ELISA plate by calf thymus mononucleosomes, dinucleosomes, trinucleosomes, and polynucleosomes expressed as ng DNA/ml. Mab 4H7 was used at a 0.5 µg/ml concentration.

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Table 2. Twenty-six patients with highest levels of 4H7 reactive antigen in serum.

Optical Density of 1:10 Serum Dilution vs Mab 4H7	SLEDAI	Clinical Status at Time of Assay
2.289	9	Recent cerebrovascular accident; dense hemiplegia
2.287	14	Active lupus nephritis
2.255	18	Vasculitis, headache, frequent Raynaud's
2.121	18	Recent cerebrovascular accident; nephrotic syndrome
2.119	22	Lupus nephritis, acute lupus flare with polyarthritis
2.072	9	Chronic hemolytic anemia
2.009	20	Active lupus nephritis
1.904	12	Arthritis, myositis, rash
1.874	19	Active lupus nephritis, rash, arthritis
1.810	12	Active lupus nephritis
1.732	19	Active lupus nephritis
1.702	12	Digital vasculitis; continual Raynaud's
1.676	17	Active nephritis, nephrotic syndrome, arthritis
1.678	18	Active nephritis
1.502	17	Recent cerebrovascular accident, vasculitis
1.481	25	Seizure, active lupus nephritis
1.301	13	Active nephritis, arthritis, Coombs' + hemolytic anemia
1.269	20	Active arthritis, myositis, rash, vasculitis
1.183	22	Organic brain syndrome seizures, proteinuria
1.058	19	Active lupus nephritis, arthritis
1.057	18	Cerebrovascular accident, continuous chorea
1.039	17	Nephritis, previous cerebrovascular accident
1.035	14	Vasculitis, arthritis
1.007	8	Active SLE nephritis
1.004	14	Cerebrovascular accident; active SLE nephritis
1.003	16	Active nephritis, arthritis

Serial studies of individual patients with SLE often showed elevations of 4H7 reactive nucleosome antigen in conjunction with ongoing flares of SLE CNS or renal disease activity. Representative results of these serial studies are shown in Table 3. The serial determinations of serum nucleosome levels shown in Table 3 in conjunction with parallel descriptions of disease activity illustrate why we have the impression that nucleosome levels correlated with increased SLE disease intensity. Patient 1 showed low, barely detectable serum nucleosome levels over a 9 year period until February, 1998, when for the first time during a followup of 12 years she showed proteinuria, polyarthritis, and interstitial pneumonia and required home oxygen. Nucleosome levels continued to be elevated, with interstitial lung disease and subsequent cardiomyopathy over next 6 weeks. Patient 2 also showed relatively low levels of serum nucleosomes over a 5 year period (1989–94) until onset of seizures and evidence on a magnetic resonance image of multiple cerebral lesions in November 1994. The serum nucleosome levels remained elevated for 2 months and then returned to normal/low levels for the next 2 years. Patient 3 initially showed low levels of serum nucleosomes, but had diffuse proliferative glomerulonephritis confirmed by renal biopsy 3 years previously. At that time the histologic appearance of the renal lesions was considered active but minimal. When she was admitted with pericardial effusion and increasing renal failure in 1994, some elevation of serum nucleosomes was recorded; one year later, although still undergoing dialysis for her renal failure, she was experiencing bouts of polyarthritis and C4 was low, with high levels of serum nucleosome (OD = 0.770). Two years later, when she was still on dialysis without apparent SLE activity, the serum nucleosome level was normal. Patient 4 showed a normal value for serum nucleosomes in 1989 while her disease was quiescent, but a marked elevation 4 years later with a recurrence of her transverse myelitis. Patient 5 showed elevations of serum nucleosome levels with initial onset of diffuse proliferative SLE nephritis, which persisted over the next 5 months despite high dose corticosteroids. One year after repetitive cyclophosphamide treatments in 1994, the serum nucleosomal level was normal. Patient 6 showed modest and then marked elevations of serum nucleosome levels in June and July of 1994 with active SLE nephritis. She received 2 intravenous cyclophosphamide treatments, with resolution of disease activity 2 months later, but then showed a progressive rise in serum nucleosomal antigen levels with worsening SLE nephritis, eventually resulting in dialysis. Even during the dialysis period, serum nucleosome levels remained high. Patient 7 initially showed no serum nucleosome elevation, but later developed increasing proteinuria and progressive renal failure, with increasing serum levels of nucleosomes. Patient

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Table 3. Serial results of serum or plasma nucleosome determination using a 1:120 dilution of test serum/plasma and ELISA with Mab 4H7 correlated with clinical status of patients over an extended period of months or years.

Patient	Date of Sample	ELISA Optical Density of Assay	Disease Activity
1. BF 48*. Deforming polyarthritis; interstitial pulm. fibrosis; leukopenia, progressive intermittent proteinuria	07-14-89	0.055	Polyarthritis, anemia, fatigue, negative proteinuria, ANA 1:1280, Crithidia + 1:40
	05-14-91	0.025	Arthritis activity low grade, some proptosis, no skin lesions
	10-21-94	0.032	Low grade arthritis, ANA 1:640, Crithidia 0
	10-31-95	0.039	Arthritis better, no skin lesions, early interstitial lung disease on chest radiograph
	06-11-96	0.072	Increasing cough; frequent upper respiratory infections, ANA 1:1280; Crithidia (+) 1:80
	02-07-98	1.028	Generalized polyarthritis with severe flare, urine 2+ protein; pneumonia; ANA 1:2560 anti-DNA. Crithidia (+) 1:80, now requiring home O_2, for lung disease
	02-09-98	0.252	Arthritis still active, ANA 1:1280, Crithidia (+) 1:40
	03-13-98	0.864	Hospitalized with interstitial lung infiltrates and congestive heart failure
2. BF 54. Extensive skin involvement, no renal disease. Later developed seizures and cerebral infarctions	09-19-89	0.037	Extensive facial scarring from previous SLE, no joint symptoms, ANA (+) 1:1280, Crithidia (+) 1:160, C3 80
	08-21-91	0.026	Some new skin lesions, no joint or kidney involvement, ANA 1:640, C3 80, C4 48 Crithidia 1:80
	08-21-91	0.026	Skin lesions completely healed, no joint symptoms; ANA 1:160, Crithidia 1:40, C3 82, C4 40
	05-07-93	0.039	Skin lesions healed; occasional back pain, ANA 1:160, Crithidia 0; C3 84, C4 45
	04-22-94	0.046	Skin lesions quiescent, no joint complaints, ANA 1:320, Crithidia 0
	11-02-94	1.874	Sudden onset of seizures, MRI shows multiple small cerebral infarctions, ANA 1:640, Crithidia + 1:160
	02-02-95	0.803	Still having nocturnal seizures, ANA 1:320, Crithidia 1:40
	03-03-95	0.025	No skin lesions, no seizures, ANA 1:160, Crithidia 0
	03-19-96	0.012	No skin lesions
	02-04-97	0.011	No seizures, disease inactive
3. BF 32. Lupus nephritis (DPGN) documented by renal biopsy 1989	10-22-92	0.031	No symptoms of active SLE, prednisone 7.5 mg, 3+ proteinuria, ANA 1:320, Crithidia 0, C3 84, C4 34
	10-16-94	0.237	Admitted with large pericardial effusion, 24 h urine protein 4 g, ANA 1:2560, Crithidia 1:160, urine 8–10, RBC + granular casts, C3 54, C4 13, serum creatinine 3.7 mg%
	01-31-95	0.770	On dialysis, ANA 1:320, C3 62, C4 10, experiencing bouts of polyarthritis
	04-17-97	0.066	Still on dialysis 3×/week, ANA 1:80 oliguric, no clinical signs of SLE activity
4. BF 41. Recurrent spinal cord transverse myelitis, no renal involvement	11-24-89	0.023	Episode 2 yrs previously of transverse myelitis, anti-DNA (–), ANA (+) 1:160, clinically quiescent at this time
	08-17-93	0.876	Active transverse myelitis, +STS, MRI abnormal signal in midthoracic spinal cord T1–T8, CSF protein 486 mg%, ischemic demyelination of cord likely
5. WF 19. DPGN documented on renal biopsy 12/1992, intermittent signs of clinical disease activity	10-31-92	0.484	Arthritis, rash, mucosal lesions, 4+ proteinuria, RBC casts, ANA 1:5120, Crithidia 1:160, C3 40, C4 10
	03-26-93	0.792	Prednisone 60 mg/day, 8 g/24 h, proteinuria, RBC casts in urine, creatinine 1.4, considered to have active glomerulonephritis
	03-11-94	0.004	Status post 3 cyclophosphamide pulse treatments, still has 3+ proteinuria, no casts, rash and joint symptoms absent, C3 70, C4 20, considered in relative remission
6. BF 28. MGN WHO Class V	06-24-94	0.294	Already had MGN documented by renal biopsy, ANA 1:1280, Crithidia 1:160, had 1 cyclophosphamide treatment
	07-15-94	0.679	Still has active SLE nephritis, 3 g/24 h proteinuria, RBC in urine, ANA 1:1280, Crithidia 1:80
	09-09-94	0.099	Received 2 IV cyclophosphamide treatments, disease activity considered less, ANA 1:320, Crithidia 1:80; C3 80; C4 30
	02-07-95	0.239	Still has active nephritis, develops episodes of digital vasculitis, C3 40, C4 9, ANA 1:1280, Crithidia 1:160
	02-25-95	0.487	Active nephritis, C3 30, C4 8, continued proteinuria and RBC casts
	02-15-96	1.538	Active nephritis, C3 60, C4 10, serum creatinine 7.6, ANA 1:1280, Crithidia 1:160, on dialysis
	04-17-96	2.761	Still on dialysis with heavy proteinuria, 6 g/24 h, ANA 1:2560, anti-Crithidia 1:80, C3 40, C4 14

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Table 3. Continued

Patient	Date of Sample	ELISA Optical Density of Assay	Disease Activity
7. Hispanic F 38. Longstanding proteinuria and nephrotic syndrome ending in renal failure	03-13-90	0.011	Admitted for kidney biopsy, membranous GN, no joint symptoms; C3 40, C4 14, 3 g proteinuria, microscopic exam of urine no RBC or casts, ANA 1:160, Crithidia (–)
	08-01-95	0.347	Increasing proteinuria 5 g/24 h, ANA 1:2560, Crithidia 1:80, creatinine 2.0
	01-29-98	0.839	Marked interval increase in proteinuria, cyclophosphamide pulse treatment every 6–8 weeks, serum creatinine 3.5; 8 g proteinuria/24 h
8. WF 50. Chronic chorea and athetoid movement disorder, no renal disease	02-26-92	0.016	CNS symptoms quiescent; ANA 1:320, anti-DNA Crithidia (–), C3 65, C4 30, urine negative
	10-15-92	1.057	Flagrant, almost continuous chorea; MRI shows multiple small infarctions, (+) antiphospholipid profile, ANA 1:2560, Crithidia 1:80, C3 30, C4 15
	06-04-93	0.056	Occasional chorea, no joint symptoms, considered in remission, Crithidia 0, ANA 1:160
9. WF 21. Longstanding progressive renal disease culminating in renal failure	02-19-96	0.047	Blood sample taken immediately after dialysis; patient admitted to hospital day before with renal failure
	02-20-96	0.074	Blood sample obtained 1 h after dialysis, patient having no CNS activity; oliguria, serum creatinine 8 mg%, hematuria, RBC casts
	02-21-96	1.481	Patient not dialyzed on day sample obtained, still oliguric, microscopic hematuria, no seizures
	02-23-96	1.678	Dialysis occurred 24 h before sample obtained; still has clinical evidence of active renal disease
10. WM 21 (East Indian). Admitted 05-16-97 to hospital with renal failure, serum creatinine 8 mg%, 4+ proteinuria, microscopic hematuria, ANA (+) 1:5120, Crithidia + > 1:160, C4 8, C3 20	05-17-97	0.421	Sample obtained 2 h after dialysis, patient oliguric, receiving 1 g solumedrol per day × 3, considered to have active SLE nephritis
	05-22-97	0.517	Sample obtained 3 h after dialysis, patient felt to have active SLE nephritis, on prednisone 120 mg/day, C3 28, C4 109
	06-13-97	1.509	SLE nephritis still active, 4+ proteinuria, C4 12, C3 30, ANA +1:1280, Crithidia +1:160, 2 days before sample obtained developed clinical signs of transverse myelitis
	06-23-97	1.418	Spinal cord MRI shows diffuse lesions T3–T8, lupus nephritis still active, sample obtained before dialysis
	07-21-97	1.632	Urinalysis 4+ protein, 24 h urine 6 g protein, signs of transverse myelitis persist, C4 18, C3 40

*Race, sex and age. B: black, W: white, DPGN: diffuse proliferative glomerulonephritis, MGN: membranoproliferative glomerulonephritis, RBC: red blood cells, CNS: central nervous system.

8, with intermittent severe chorea, showed normal serum nucleosome levels while her movement disorder was quiescent, but during an extended period of severe chorea 8 months later showed high serum nucleosome levels. Eight months later, with resolution of chorea, serum nucleosomal levels were almost normal. Patient 9 is perhaps one of the most instructive of all cases studied. She had experienced symptoms and signs of severe lupus nephritis and CNS involvement since age 8, with a long history of increasing proteinuria, finally oliguria, and then emergency admission to hospital in renal failure. The first 2 blood samples were obtained within 30 min after 2 successive days of hemodialysis and showed low nucleosomal serum levels. The last 2 samples were obtained on subsequent days close together when no dialysis had occurred, and were markedly elevated for serum nucleosome levels. Patient 10 also showed marked elevations of serum nucleosomal levels over a 2 month interval in conjunction with initial emergency dialysis (first 2 samples) and later with onset of extensive transverse myelitis. In this patient, first with renal involvement and then very active transverse

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myelitis, serum nucleosome levels remained elevated throughout most of his hospital course.

When individual SLE serum levels of anti-4H7 reactive nucleosomal antigen were directly compared with the SLEDAI, a positive, highly significant correlation was observed ($R = 0.404$, $p < 0.0001$) (Figure 6). The statistical test for correlation was Pearson's correlation coefficient between X and Y. The p value resulted from a standard significance test for correlation showing that the correlation was significantly greater than zero. This finding, as well as the serial studies of individual patients, appeared to indicate that 4H7 reactive nucleosomal antigen elevation often coincided with major SLE clinical flares.

Renal biopsy. We studied 12 renal biopsy tissue eluates from SLE patients with nephritis, and in parallel 12 control subjects with other forms of kidney disease including 2 with Wegener's granulomatosis, 3 renal transplant rejections, one with IgA nephropathy, 2 diabetic glomerulosclerosis, one hemolytic uremic syndrome, and 3 with chronic membranous glomerulonephritis. No SLE or control renal biopsy eluate showed significant ELISA reactivity with Mab 4H7. However, many of these renal biopsy eluates contained detectable IgG with antinuclear or anti-DNA reactivity as measured by sensitive ELISA, but no positive reaction was noted with Mab 4H7, indicating that, at least under the experimental conditions employed, there was no evidence for glomerular deposition of nucleosomal antigens reacting with Mab 4H7. It is possible that the very low protein content within these renal biopsy eluates made it impossible to detect Mab 4H7 reactivity.

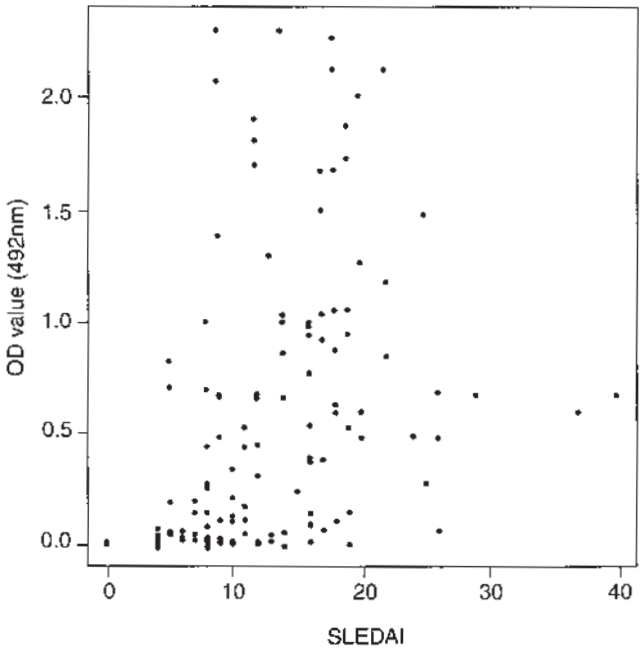

Figure 6. Correlation between OD 492 nm values measured using 1:10 dilution of single SLE sera and 4H7 (5 µg/ml) versus SLEDAI ($p < 0.0001$).

DISCUSSION

We outline direct evidence for increased concentrations of nucleosomal related antigen in serum samples from patients with active SLE. Whether the presence of residual antigens related to nucleosomes in serum relates to the parallel status of the disease state itself among individual patients with SLE remains to be investigated in detail. A number of investigators had provided evidence for the presence of DNA in SLE serum or plasma before the definitive studies of Fournier[7] and Rumore and Steinman[8]. However, the latter authors reported that DNA in SLE plasma or serum was present as nucleosome subunits. Our study supports this concept in yet another way. Nucleosome particles consist of a histone octamer (two H2A-H2B dimers and one histone [H3-H4] tetramer), 146 base pairs of DNA wrapped 1.75 times around the octamer (forming the so-called core particle), and linker DNA (between 20 and 60 bp of DNA) joining adjacent core particles. Histones H1 (and/or H5) bind to core particles and to the linker DNA. Many of the antigenic materials recognized by autoantibodies encompassed in nucleosomes can be reconstituted or successfully expressed by admixtures of DNA, histones, and other nuclear components, as supported by several studies[12,14,27]. Our observations may indicate that portions of nucleosomes, like the surface exposed H2B and H3 domains we describe, may provide a stronger antigenic stimulus in the naturally occurring disease than some of the whole histone or DNA determinants within nucleosomal particles themselves[28,29]. Whether these same surface exposed histone regions may also be important as T cell reactive regions would also be of interest. Remarkably, peptide 10–33 of H2B was recently found to be recognized by CD4 T cells from patients with lupus[11].

A recent report by Amoura and coworkers[16] also provided strong evidence for the occurrence of detectable levels of plasma nucleosomes in patients with SLE using a capture ELISA and a Mab called 13D10, which was said to react specifically with nucleosomes but not to the individual nucleosomal components (dsDNA and histones). In that study, levels of plasma nucleosomes were higher in SLE patients than in normal subjects. However, the plasma nucleosome levels appeared to be higher in patients with inactive rather than active disease[16]. By contrast, our data seem to indicate that the serum levels of the nucleosome antigens studied here with 4H7 were most often highest in SLE patients with active CNS disease or renal involvement. Moreover, serial studies of serum samples obtained from individual SLE patients sometimes over periods of several years or months clearly indicated elevated levels of nucleosomal antigen when SLE disease activity increased, as shown in Table 3. Data presented here indicate that the specificity of Mab 4H7 appears to be directed, at least in part, against histone H2B and H3 peptide sequences, which are surface accessible in the native nucleosome structure[18]. The Mab 4H7 also reacts with whole nucleosome preparations. When whole nucleosomes were added to normal human serum, their presence could still be detected

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using the standard ELISA with Mab 4H7, and virtually identical results were obtained when increasing quantities of nucleosomes were added to normal human serum. Thus, our failure to identify Mab 4H7 reactive material within SLE glomerular eluates was disappointing. It may be due either to the low concentration of circulating nucleosomes in renal eluates, or to damage resulting from the low pH treatment used to elute nucleosomal antigens, or to the fact that most nucleosomal structures are tightly bound to the glomerular basement membrane[30,31] or complexed to antibodies, preventing their detection with 4H7. Finally, it is also possible that although nucleosomal antigen exposure may in some way trigger the immune response in SLE, the nucleosomal antigens recognized by 4H7 do not persist in SLE within diseased glomerular deposits.

We consistently found elevated levels of serum or plasma nucleosome antigens with increased SLE disease activity, whereas previous investigators did not[16]. This may relate to the fine specificity of anti-nucleosome antibody used to detect circulating nucleosomes and to basic differences in the assay employed. Amoura, *et al*[16] used a sandwich ELISA technique in which their nucleosome reactive mouse Mab 13D10 was coated on the ELISA plate, test plasma was then placed on the plate, and the final reaction developed with a different mouse Mab with anti-DNA specificity. It seems possible that the DNA antigenic determinants presumably on nucleosomes, which were expected to be reacting with the capture anti-DNA Mab, were not visible or accessible in all nucleosome antigens present in plasma, and thus results different from ours were found. No serial studies extending over a period of several years were provided by Amoura, *et al*. Our serial studies clearly indicate disease activity correlated with elevations in serum or plasma nucleosomal antigen. We found no significant difference between serum and plasma nucleosome quantitations in the same patients, but all our serum samples were collected in serum tubes with a serum-cell gel separator preventing contact between supernatant serum and cell pellet, even during centrifugation.

Clearly, antibodies to DNA may react with nucleosomes, since the latter contain dsDNA. Our Mab 4H7 recognizes separate antigens related to nucleosomes, but does not react directly with DNA, whole histones themselves, or other nuclear proteins such as Sm or RNP. However, the exposed areas of H2B residues and H3 residues must contribute to an important conformational epitope recognized by our Mab 4H7. The epitope reacting with Mab 4H7 therefore represents another antigenic component common to nucleosomal elements, since highly purified chicken erythrocyte nucleosomes and calf thymus nucleosomes, as well as nucleosomes from human peripheral blood mononuclear cells, all provide strong expression of the reactive nucleosomal antigens recognized by Mab 4H7.

The curious observation that our Mab 4H7 reacts with externally accessible H2B and H3 peptides but not with the whole H2B or H3 proteins may relate to the possibility that these 2 short histone peptide segments are actually presented in a 3 dimensional shape, on the outside or shell of the nucleosome, that these same segments do not assume in the unfolded isolated H2B and H3 proteins. Thus these particular antigenic determinants may not be accessible when the individual H2B or H3 proteins are isolated and thereby are possibly slightly altered from their active native shape when fixed within the nucleosome structure they were destined for. There are many examples in the literature of reactive peptide segments of proteins in the face of no reactivity with the whole cognate protein. Many of these observations have involved so-called autoantigens. In addition, previous observations in some systems have indicated that in patients' sera, antibodies reacting with peptides of a self-protein, but not with the cognate protein itself, co-existed with antibodies actually reacting with the whole parent protein. In many instances the presence of the former antibody population has been ignored because, in general, investigators select sera reacting with a particular protein, and only subsequently examine the reactivity of positive sera with peptides to delineate the epitopes recognized within the parent protein. However, when sera are tested systematically with peptides and the native cognate protein, such antibody subsets, e.g., those reacting with a peptide from the cognate protein but not with the whole protein itself, have been revealed. This same phenomenon has been described with respect to histones[29,32] as well as for Sm BB′N[33,34], for SmDl protein[35-37], and also for U1A protein[38]. Similar observations have been published for Ro52 protein[39] and for poly(ADP ribose polymerase)[40,41]. It could be argued that these observations only reflect the possibility that certain cross-reactions are better recognized when peptides bearing a major epitope, rather than whole purified or recombinant proteins, are assayed in different test conditions. An alternative explanation for the presence of high levels of specific peptide antibodies in the serum of some patients with autoimmune disorders is that antibodies in such conditions show stronger reactivity with denatured, rather than native proteins. Thus it is possible that in such diseases "non-native" proteins may have pathogenic significance in either the initiation or propagation of an autoimmune response. Thus, the literature already supports the presence of antibodies in various situations where the antibody reacts with a peptide derived from the cognate protein, but not with the whole protein itself.

Our Mab 4H7 reacts with the 2 N-terminal tails of histones H2B and H3, which are mobile and therefore difficult to localize within the crystal structure. The 4 amino-terminal tails of H2B and H3 exit through narrow channels formed by the aligned major grooves of adjacent turns of the DNA superhelix[42]. An adaptation of some of these relationships from a recent review by Luger and Richmond[43] is shown in Figure 7; this may help the reader visualize at least part of the epitopes reacting with our Mab 4H7. The other parts of the epitope

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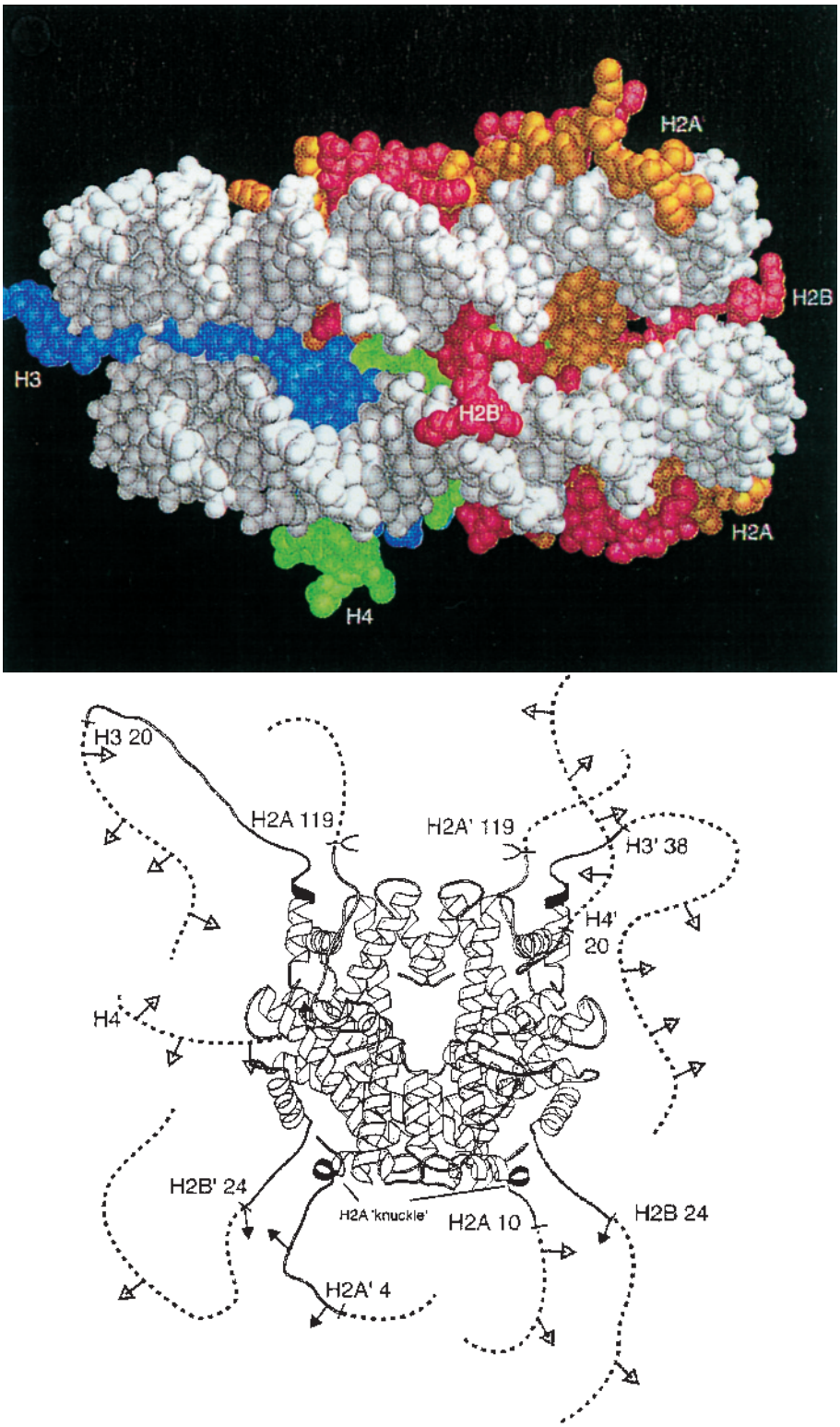

Figure 7. The histone tails of the nucleosome. Luger, *et al*[42] solved the structure of the nucleosome core to a resolution of 2.8 A. Top panel shows histone tails between DNA gyres. The H2B and H3 N-terminal tails pass through channels in the DNA superhelix formed by aligned minor grooves (reproduced with permission from Nature 1997;389:251-60). Bottom panel shows the orientation of each histone tail observed in the crystal structure[43]. The regions of the tails and histone-fold extension that are in contact with the DNA are shown (solid lines). Amino acids that could not be interpreted in the model are shown in arbitrary positions (broken lines). Arrows show the acetylation sites (reproduced with permission from Current Opinion in Genetics and Development 1998;8:140-6).

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reacting with the Mab must be other 3 dimensional shapes within the nucleosome particle.

A number of lines of evidence point to the central role of chromatin and/or nucleosomal elements as presumptive antigenic stimuli for the initiation of abnormal immune responses in SLE[9-13]. Our finding of detectable circulating nucleosome related antigens in serum of patients with active SLE lends credence to the important influence of nucleosomal elements in the disease state. Exactly which antigenic stimuli occur first, and the subsequent sequence of other stimulating antigens in the ongoing SLE disease state, await further investigation.

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