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Antibodies to histones in systemic lupus erythematosus: Localization of prominent autoantigens on histones H1 and H2B

(chromatin/antinuclear antibody)

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ABSTRACT **By the technique of immunoblotting we have assessed the ability of sera from 24 patients with systemic lupus erythematosus to bind nuclear proteins. Of the 11 patients who had antibodies to histones, 10 had antibodies to histone H1 and 9 of these also had antibodies to histone H2B. Antibodies to the other histones (H2A, H3, and H4) were less apparent. Five of the 11 patients (and two others in the remainder of the sample of 24) also had antibodies to a small number of nonhistone proteins that are probably components of ribonucleoprotein particles, but there was no obvious correlation between the presence of antihistone antibodies and the known antiribonucleoprotein activity of these sera. Separate determinants on H1 and H2B were demonstrated by immunoblotting with affinity-purified anti-H1 and anti-H2B antibodies derived from serum that showed both specificities. The localization of the determinants within the histone polypeptide chains was shown by immunoblotting with large fragments produced by specific proteolytic or chemical cleavage of the histones. The strongest determinant on H1 was located within the COOH-terminal half, with a weaker determinant being present within the NH_2-terminal half; the H2B determinant(s) was located entirely within the NH_2-terminal half of the molecule. The selectivity with which the antihistone antibodies in systemic lupus erythematosus are produced against the more exposed histones in the nucleosome (and perhaps against the most exposed regions of these histones) is consistent with the involvement of intact chromatin structures as immunogens in this disease.**

Antinuclear antibodies (ANAs) can be classified conveniently into groups directed against (*i*) ribonucleoproteins (RNP), (*ii*) deoxyribonucleoprotein, (*iii*) naked nucleic acids, and (*iv*) other constituents of the nucleus such as the nuclear matrix or enzymes. Recently, it has become clear that patients with systemic lupus erythematosus (SLE) produce at least nine different autoantibodies that recognize selected sets of RNP (1–3). Specificity within this system is based on the ability of the antibodies to identify proteins that bind with great selectivity to particular RNA molecules. These studies suggested that antibodies to deoxyribonucleoprotein might follow a similar pattern and represent a broad spectrum of specificities for different proteins that complex with DNA. In the present study, we have used the immunoblotting technique (4) to analyze the ability of antibodies in SLE sera to recognize nuclear protein antigens. The results indicate that the most prominent antigenic determinants of chromatin are located on histones, particularly H1 and H2B, within regions that are likely to be exposed in the structure of native chromatin.

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MATERIALS AND METHODS

Patients. We have studied 24 patients who fulfilled the American Rheumatism Association criteria for SLE (5). All had ANAs demonstrable by indirect immunofluorescence. The sera were stored at −70°C until assayed and transported from New Haven to Cambridge, England, in solid carbon dioxide.

Preparation of Nuclei. Nuclei were prepared from a fresh human term placenta essentially according to the procedure of Hewish and Burgoyne (6), from chicken erythrocytes as described by Bates *et al.* (7), and from the principal neurons of ox cerebral cortex as described by Pearson *et al.* (8).

Isolation and Specific Cleavage of H1 and H2B. H1 and H5 were extracted from chicken erythrocyte nuclei with 5% perchloric acid and separated by ion exchange chromatography on carboxymethylcellulose (CM-52, Whatman) in 10 mM sodium phosphate, pH 7/0.25 mM phenylmethylsulfonyl fluoride, using a gradient of 0.3 to 0.7 M NaCl; the two H1 species eluted first and appeared as a doublet in $NaDodSO_4$/polyacrylamide gels (see Fig. 2). H1 was cleaved with chymotrypsin at phenylalanine-106 (9); the NH_2- and COOH-terminal halves of the molecule (residues 1–106 and 107–220, respectively) were separated by chromatography on carboxymethylcellulose and characterized by comparison of their amino acid compositions obtained after acid hydrolysis with those expected from the amino acid sequences (10). The faster moving doublet and the slower moving singlet band on $NaDodSO_4$ gel electrophoresis (see Fig. 2*C*) were thus identified as arising, respectively, from the NH_2- and COOH-terminal halves of H1. (The two H1 species thus differ in their NH_2-terminal halves but probably share a common COOH-terminal sequence.)

Histone H2B extracted from chicken erythrocytes (11) was cleaved at methionine residues 59 and 62 with cyanogen bromide in 70% formic acid (12) and the mixture was then diluted 1:10 with water and freeze-dried. The NH_2- and COOH-terminal fragments (residues 1–59 and 63–125, respectively) were separated by elution from carboxymethylcellulose and characterized by comparison of their amino acid compositions with the amino acid sequence (10). The faster and slower moving bands on $NaDodSO_4$ gel electrophoresis (see Fig. 2*D*) were assigned, respectively, to the COOH- and NH_2-terminal fragments.

Affinity Purification of Anti-HI and Anti-H2B. Sepharose 4B (Pharmacia) was activated with cyanogen bromide as described by Porath *et al.* (13). Histones H1 and H2B were cou-

Abbreviations: SLE, systemic lupus erythematosus; ANA, antinuclear antibody; RNP, ribonucleoprotein(s); snRNP, small nuclear RNP; P_i/NaCl, phosphate-buffered saline; kDa, kilodalton(s).

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pled to the activated Sepharose, and antibodies were affinity purified essentially as described by Mazen *et al.* (14). The final concentration step was omitted. Anti-H1 antibodies were eluted from H1-Sepharose with roughly four times the volume of the initial serum. Anti-H2B antibodies were extracted from the material that did not adsorb to the H1-affinity column by passing the flow-through over H2B-Sepharose and then eluting with the same volume of pH 2.8 buffer that was used to elute anti-H1.

Immunoblotting. Proteins were resolved in a $NaDodSO_4$/18% polyacrylamide gel (8 cm long × 1 mm thick) (15) run at 25 mA. The gel was equilibrated with "transfer buffer" (25 mM Tris/0.19 M glycine/20% methanol)/0.1% $NaDodSO_4$ for 15 min at 37°C. Proteins were transferred from the gel to a sheet of nitrocellulose (membrane filter BA85, Schleicher & Schüll, Dassel, F.R.G.) in a Transblot box (Bio-Rad), for 3 hr at 250 mA. The nitrocellulose paper was rinsed briefly in cold phosphate-buffered saline (P_i/NaCl; 20 mM potassium phosphate/0.13 M NaCl, pH 7.4). A strip bearing a representative set of samples was cut off and stained with amido black [0.1% in methanol/acetic acid/water, 45:10:45 (vol/vol)]. The remainder was exposed for 1 hr at room temperature to "blocking solution" [3% (wt/vol) bovine serum albumin (Sigma, radioimmunoassay grade)/1% (wt/vol) human hemoglobin (Sigma, type IV) that had previously been adsorbed with staphylococcal protein A-Sepharose (Pharmacia)]. It was then cut into appropriate strips, each bearing a representative set of samples, and the strips were individually exposed for 30 min to a 1:40 dilution of the appropriate patient or normal control serum in P_i/NaCl/0.25% gelatin and passed through a Millipore filter (type G5; 0.22 μm) or to a 1:10 dilution of affinity-purified antibodies in P_i/NaCl. (Final concentrations are given in the figure legends.) The strips were washed (5 min in cold P_i/NaCl, 5 min in cold P_i/NaCl/0.1% Triton X-100, two 5-min periods in cold P_i/NaCl) and then exposed for 30 min to ^{125}I-labeled staphylococcal protein A (3×10^5 cpm/ml in P_i/NaCl/0.25% gelatin) iodinated by the lactoperoxidase method (Sigma) (16) <3 weeks earlier. The wash protocol was repeated. The strips were air-dried and autoradiographed for 12 hr (Fuji RX film) at −70°C with an intensifying screen.

RESULTS

Initially, we screened the 24 sera in immunoblots using total nuclear proteins as substrates. Nuclei from two different sources were used, those of human origin, from placenta (P, in Fig. 1), to ensure that any species-specific effects would not be overlooked and those from (ox) neurons (N, in Fig. 1) to increase the likelihood of detecting antibodies to nuclear components other than histones, because these active nuclei are especially rich in nonhistone proteins (Fig. 1, stained nitrocellulose paper). Representative results with four sera are shown in Fig. 1. There is a low "background" level of radioactivity over every histone band, even with normal (nonimmune) serum, probably due to nonspecific interaction between histones and serum IgG. In addition, certain patient sera give more intense radioactivity over one or more of the histone bands, indicating the presence of specific antibodies to particular histones. Thus, serum B.M. reacts strongly with histones H1 and H2B and weakly with H4, serum J.P. reacts moderately with H1 and H2A and weakly with H3, H2B, and H4, and serum I.L. reacts moderately with H1 and H3 and weakly with H4. In contrast, serum P.S. probably contains little or no antibody to histones. Although antibody titers were not determined, the most intense blots were reproducibly obtained with sera that recognized H1 and H2B, suggesting that antibodies to these particular histones occur in especially high concentrations.

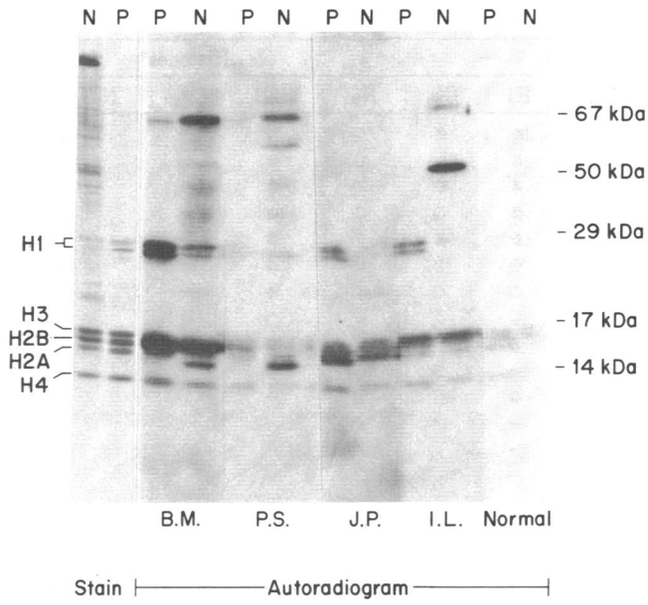

FIG. 1. Patient sera identify histone and nonhistone proteins. Replicate samples of nuclei from ox cerebral cortex neurons (N) and human placenta (P) were fractionated by $NaDodSO_4$/18% polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose paper, and the paper was then cut into strips. One strip was stained with amido black and the remaining strips were blotted with patient (B.M., P.S., J.P., and I.L.) or normal control sera. Equal quantities of total protein were applied to each lane of the gel. Note that the neuronal nuclei contain more nonhistone proteins than the placental nuclei. Because the electrophoretic mobilities of histones H3 and H2B are similar, variable periods of exposure to x-ray film were sometimes necessary to distinguish between these histones on the autoradiogram. The proteins used as relative molecular mass markers in the gel (positions indicated) were bovine serum albumin (67 kDa), glutamic dehydrogenase (53 kDa), carbonic anhydrase (29 kDa), hemoglobin (17 kDa), and cytochrome *c* (14 kDa).

Seven sera recognized nonhistone proteins in the immunoblots. For example (Fig. 1), sera B.M. and P.S. identify a protein migrating near the 67-kilodalton (kDa) marker and another just ahead of H2A; serum I.L. identifies a protein migrating near the 53-kDa marker. These proteins are likely to be components of small nuclear ribonucleoprotein (snRNP) particles, because sera B.M. and P.S. contain anti-RNP and anti-Sm antibodies (Table 1), which bind, respectively, to the 68- and 16-kDa polypeptides found within these particles (17–19), and serum I.L. contains anti-La antibodies, which are specific for a 53-kDa ribonucleoprotein (20–22).

The occurrence of antibodies to histones, RNP, and native DNA in this group of patients is summarized in Table 1. Of the 24 sera, 11 contained antibodies that identified one or more histones in immunoblots, 12 immunoprecipitated selected sets of RNP particles, and 5 contained antibodies to native DNA. Among the histones, H1 and H2B were identified most prominently. All of the sera that contained anti-DNA antibodies also contained antihistone antibodies (although the converse was not true) but there was no obvious correlation between antihistone and anti-RNP antibodies.

Because the most prominent responses against histones appeared to be directed against H1, H2B, or both, we sought to identify the antigenic site(s) on these particular molecules more precisely by immunoblotting of fragments obtained by specific cleavage of purified H1 and H2B. In these experiments, all seven sera that contained both anti-H1 and anti-H2B antibodies were tested. The histones (Fig. 2*A*) were from chicken erythrocytes, because these gave results identical with those for human pla-

Table 1. Antibodies to nuclear components in SLE

	Histones*									RNA proteins†					
Patient	H1	C_{H1}	N_{H1}	H2B	C_{H2B}	N_{H2B}	H3	H2A	H4	RNP	Sm	Ro	La	rRNA	DNA‡
S.B.	●	●	○	●	−	●	○	−	○	−	−	+	−	−	+
I.O.	●	◒	○	●	−	◒	−	○	○	−	−	−	−	+	−
B.M.	●	●	○	●	−	●	−	−	○	+	+	+	−	−	+
R.M.	●	○	○	◒	−	−	−	−	−	−	−	−	−	−	+
T.D.	●	●	○	●	−	○	○	−	◒	−	−	−	−	−	−
S.D.	○	○	−	●	−	◒	−	−	−	−	−	+	−	−	+
N.T.	○	○	○	●	−	◒	−	−	−	−	−	+	−	−	−
J.P.	◒			○			○	◒	○	+	−	−	−	−	−
Y.K.	●			●	−	○	−	◒	○	+	+	−	−	−	−
C.H.	−			−			◒	○	◒	+	+	−	+	−	+
I.L.	◒			−			◒	−	○	−	−	−	+	−	−
M.C.	−			−			−	−	−	+	−	−	−	−	−
P.S.	−			−			−	−	−	+	+	+	−	−	−
S.M.	−			−			−	−	−	−	+	−	−	−	−
L.M.	−			−			−	−	−	+	−	−	−	−	−
T.M.	−			−			−	−	−	−	−	−	+	−	−
E.F.	−			−			−	−	−	−	−	+	−	−	−
L.F.	−			−			−	−	−	−	−	−	+	−	−
N.D.	−			−			−	−	−	+	−	−	−	−	−
A.C.	−			−			−	−	−	−	−	−	−	−	−
M.D.	−			−			−	−	−	−	−	−	−	−	−
D.J.	−			−			−	−	−	−	−	−	−	−	−
D.D.	−			−			−	−	−	−	−	−	−	−	−
M.F.	−			−			−	−	−	−	−	−	−	−	−

C_{H1} and N_{H1}, COOH- and NH_2-terminal halves of histone H1; C_{H2B} and N_{H2B}, COOH- and NH_2-terminal halves of histone H2B. ●, Strong response; ◒, intermediate response; ○, weak response; +, positive; −, negative.
*Determined by immunoblotting (this paper).
†Determined by immunoprecipitation (3).
‡Determined by radioimmunoassay (unpublished data).

cental histones (results not shown) in immunoblots such as those in Fig. 1; this crossreactivity reflects the high evolutionary sequence conservation in the histones (even H1, which is the most variable) (10). An immunoblot of purified H1 and H2B with serum B.M. is shown in Fig. 2*B*. Only a single band coinciding with the intact histone is visible on the autoradiogram in each case, showing that the histones are free of other immunoreactive material, such as histone fragments.

A representative study using serum B.M. in immunoblots with chymotrypsin-cleaved H1 and cyanogen bromide-cleaved

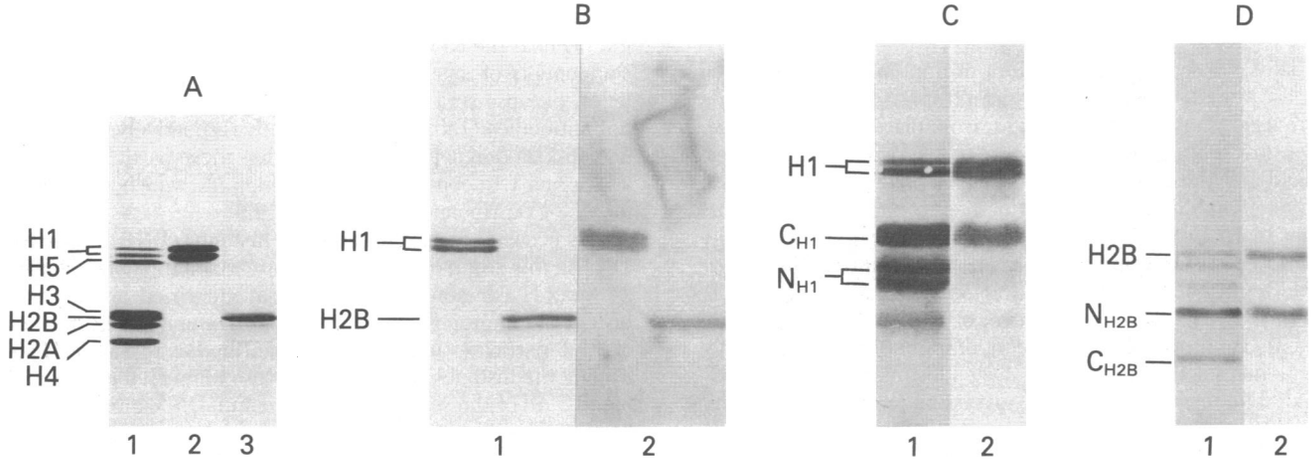

FIG. 2. Patient sera containing antibodies to histones H1 and H2B recognize the COOH-terminal half of H1 and the NH_2-terminal half of H2B. (*A*) $NaDodSO_4$/18% polyacrylamide gel electrophoresis of chicken erythrocyte histones. Lanes: 1, total acid extracted histones; 2, pure H1; 3, partially pure H2B, with traces of the other three core histones. The gel was stained with Coomassie brilliant blue. (*B*) An immunoblot of H1 and H2B. Lanes: 1, proteins were transferred to nitrocellulose paper and stained with amido black; 2, autoradiogram of an identical nitrocellulose strip after exposure to serum B.M. and ^{125}I-labeled protein A. (*C*) An immunoblot of chymotrypsin-digested H1. Lanes: 1, amido black stain; 2, the corresponding autoradiogram. N_{H1} and C_{H1}, NH_2- and COOH-terminal halves of the molecule. The unmarked fast-migrating band in lane 1 arises from further digestion of the NH_2-terminal fragment. (*D*) An immunoblot of cyanogen bromide-cleaved H2B. Lanes: 1, amido black stain; 2, the autoradiogram. N_{H2B} and C_{H2B} the NH_2- and COOH-terminal "halves" of the molecule. The unmarked bands in lane 1 arise from H2A (upper) and residues 1–90 of H3 (lower). In each case (*B–D*), the serum used for immunoblotting was B.M. Similar results were obtained with six of the other seven sera tested (Table 1).

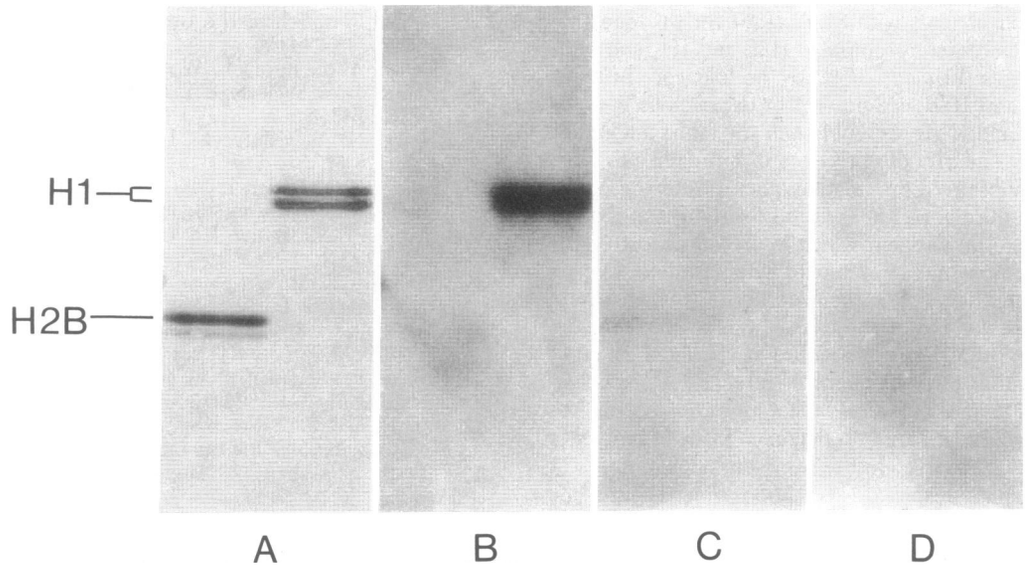

FIG. 3. Immunoblots with affinity-purified anti-H1 and anti-H2B antibodies. (*A*) Amido black stain of H1 and H2B (from chicken erythrocytes) after transfer to nitrocellulose paper. (*B–D*) Autoradiograms of identical nitrocellulose strips after exposure to anti-H1 (14 μg/ml) (*B*), anti-H2B (4 μg/ml) (*C*), and normal serum (*D*).

H2B is shown in Fig. 2. Of the two fragments of roughly equal length produced by chymotrypsin cleavage of chicken erythrocyte H1 at phenylalanine-106, the NH_2-terminal fragment (residues 1–106) is blotted very weakly while the COOH-terminal fragment (residues 107–220) is recognized strongly (Fig. 2*C*, lane 2). This highly basic COOH-terminal region of H1 is likely to play a major role in chromatin condensation; the NH_2-terminal half of the molecule has a basic NH_2-terminal domain that probably has a role in DNA binding and a central globular region that stabilizes the formation of two complete turns of DNA around the histone octamer in the nucleosome (23). Of the two fragments produced by cyanogen bromide cleavage of H2B at methionine residues 59 and 62, the NH_2-terminal fragment (residues 1–59) alone is recognized (Fig. 2*D*, lane 2). This region includes the basic NH_2-terminal 20- to 30-residue "tail," which is readily removed by tryptic digestion and whose structural role is as yet unclear (24).

The pattern of binding shown in Fig. 2 for serum B.M. was typical of all eight sera tested in this way. The results are summarized in Table 1. Thus, with H1, the most pronounced reaction was always with the COOH-terminal half, although weaker determinants were present in the NH_2-terminal half, whereas recognition of H2B was solely dependent on an NH_2-terminal site.

Because individual sera tended to identify H1 and H2B simultaneously, it seemed possible that these proteins might share a common determinant. To examine this possibility, serum B.M. was passed over a column of H1-Sepharose and then the flow-through was passed over a column of H2B-Sepharose. Antibodies eluted from these columns were tested in immunoblots using purified chicken erythrocyte H1 and H2B as substrates (Fig. 3*A*). The antibodies recovered from the H1 affinity column recognized only H1 (Fig. 3*B*), while those from the H2B column recognized only H2B (Fig. 3*C*). Two separate antigenic determinants therefore give rise to the dual antibody specificity of the serum.

DISCUSSION

We have shown that the nuclear proteins recognized most prominently in immunoblots with SLE sera are histones and a few additional proteins that are likely to be components of RNP. As shown in Table 1, about half of the 24 SLE patients studied have antihistone antibodies detectable by immunoblotting. The most prominent responses were against the COOH-terminal half of H1 and the NH_2-terminal half of H2B. A similarly high incidence of selectively produced antihistone antibodies in SLE has been observed previously. In studies using an enzyme-linked immunosorbent assay, particularly strong responses were found against H2B and H2A (H1 not tested) (25) and to the H2B–H2A complex (26). Initially, our results raised the possibility that H1 and H2B possess crossreacting antigenic determinants. Indeed, monoclonal antihistone antibodies show extensive crossreaction with different histone types (27). However, the results in Figs. 2 and 3 show that the antihistone antibodies in human serum are specific for selected segments of individual histones. This focused response strongly suggests the operation of a specific immune stimulus.

However the possible limitations of the immunoblotting method must be borne in mind when considering the range of ANAs detected in this study. As with most other immunoassays, sensitivity is determined by substrate (antigen) concentration and stability and by antibody titer and affinity. Although the predominance of histones in total nuclear proteins used as substrates for immunoblotting might favor detection of antihistone antibodies, the assay is, however, sufficiently sensitive to detect antibodies to proteins not visible on stained gels (e.g., the nonhistone protein blotted with sera B.M., P.S., and I.L.; see Fig. 1). Moreover, when we used large quantities of the high-mobility group proteins HMG-14 and -17 from chicken erythrocytes as substrates, we detected no antibodies to these potential nuclear autoantigens (data not shown). However, it remains possible that other important antinuclear antibodies were not detected for one or more reasons: because of insufficient antigen on the nitrocellulose strips, because the antigen is a tertiary structural determinant, or because the antibodies are of an immunoglobulin class that does not interact with ^{125}I-labeled staphylococcal protein A. Finally, it is not clear to what extent, if any, the denatured proteins renature on nitrocellulose before and during exposure to serum.

The relative abundance of histones in the nucleus might explain why antihistone antibodies constitute a major autoim-

mune response in patients with SLE. However this does not account for a preferential response to H2B and H1 [and H2A (25)] among the five histones, because the four core histones (H3, H4, H2A, and H2B) are present in the nucleus in equimolar quantities and H1 is present in half the molar amount of the others (28). Similarly, the presence of antibodies to RNP bears little relationship to the relative quantities of these particles: about one-third of patients with SLE produce antibodies to the cytoplasmic Ro antigen, which is present in extremely low concentrations, while an abundant cytoplasmic RNA particle, the ribosome, is rarely recognized by ANA-positive sera (3).

The relatively strong antigenic nature of H1 and H2B compared with the other histones might be rationalized if nucleosomal structures themselves (rather than, for example, free histones) were the antigenic elements that elicit many of the ANAs found in patients with SLE. In the complete nucleosome, H1 occupies a particularly exposed external position; its antigenic COOH-terminal tail is probably bound to the linker DNA between nucleosomes. Within the nucleosome core, H2B (with H2A) is also in a relatively exposed position, with one copy being accessible on each face (29), and more readily accessible to H2B antiserum than are H3 and H4 to their antisera (30). Antibodies raised in rabbits against the NH_2-terminal half of H2B bind to chromatin, indicating accessibility of the antigenic determinants in this region whereas antigenic determinants in the COOH-terminal half are inaccessible (31). The presence of antigenic determinant(s) on the NH_2-terminal 20–30 trypsin-sensitive residues of one or more of the core histones in nucleosomes is also suggested by an earlier study of antibodies in a SLE serum (32). Some support for the possibility that chromatin bound rather than free histones are immunogens in SLE comes from a study in which nucleosome core particles from chicken erythrocytes used as immunogens in rabbits produced an immune response similar to that observed here, with antibodies to H5, H2B, and H2A being prominent (33). When individual histones (complexed to tRNA) are separately used as immunogens, antibodies to H1 and each of the respective core histones are produced (30, 34).

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