The major core histone antigenic determinants in systemic lupus erythematosus are in the trypsin-sensitive regions

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Antibody responses against nucleosome core histones in systemic lupus erythematosus have been shown, by immunoblotting, to be directed largely against the trypsin-sensitive regions of the histones. These occur at the N-terminal regions of all 4 core histones and at the C-terminal ends of H2A and H3. Since these regions are often not the most antigenic when individual histones are used as immunogens, and appear to be exposed in the nucleosome, the active immunogens in systemic lupus erythematosus seem likely to be chromatin-bound, rather than free, histones.

Chromatin *Histone tail* *Anti-nuclear antibody* *Immunoblotting*

1. INTRODUCTION

A strong and frequent anti-histone response associated with systemic lupus erythematosus (SLE) is directed against histones H1 and H2B, and more particularly against determinants in the N-terminal 'half' (residues 1–59) of H2B and the C-terminal 'half' (residues 106–217) of H1 [1]. (The chicken H1 sequence has recently been deduced from the DNA sequence [2].) Antibodies to core histones other than H2B do occur, although less frequently and the response is often weak. We have now investigated the location of the antigens in all 4 core histones, H3, H4, H2A and H2B, by immunoblotting intact core histones and those from which the basic N-terminal regions, and in the case of H2A and H3 a basic C-terminal peptide, have been cleaved with trypsin. The role of these regions in chromatin is not clear [3] but in isolated core particles the N-terminal regions of some, or all, of the core histones appear to be relatively mobile [4,5]. We find that significant responses to H2B, H3 and H2A in the SLE sera examined are directed almost entirely against the trypsin-sensitive regions.

2. MATERIALS AND METHODS

2.1. *Human SLE sera*

These were fully described previously [1].

2.2. *Histones and trypsinized histones*

Histones were obtained from chicken erythrocyte nuclei [6,7], by extraction with 0.2 M H_2SO_4 [8]. To obtain histones from which the N-terminal 'tails' had been removed, chicken erythrocyte nuclei at A_{260} = 50 in 0.34 M sucrose, 3 mM $MgCl_2$, 50 mM triethanolamine·HCl (pH 8.0) were digested for 5 min at 37°C with trypsin (Sigma, TPCK-treated) at 25 μg/ml [1:100 (w/w) enzyme:histones]. Digestion was terminated by addition of phenylmethylsulphonyl fluoride (50 mM in propan-2-ol) to a final concentration of 1 mM and chilling on ice. The truncated core histones were extracted with 0.2 M H_2SO_4 as for intact histones [8]. They were dissolved in 5% (v/v) acetic acid, frozen rapidly (liquid N_2) and stored at −20°C.

For further characterization, the 3 components that could be resolved by sodium dodecyl sulphate

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(SDS)-polyacrylamide gel electrophoresis (bands A–C, see section 3) were electroeluted from an unstained gel using an ISCO sample concentrator. They were collected by precipitation with 25% (w/v) trichloroacetic acid, washed with acetone/0.1 M HCl and then with acetone. They migrated in SDS gels as single bands.

2.3. *SDS–polyacrylamide gels*

Gels were 12 cm long, 1 mm thick SDS–18% polyacrylamide gels [9].

2.4. *Amino acid analysis*

Histones and trypsinized histones were hydrolysed at 105°C for 24 h with 6 M HCl–0.1% (w/v) phenol [10], and amino acid analysis of the desiccated hydrolysate was carried out using an LKB 4400 automatic analyzer.

2.5. *Amino acid sequence determination*

Rapid sequencing of the first few amino acid residues in electroeluted trypsinized histones was carried out as in [11].

2.6. *Immunoblotting*

This was carried out as in [1], with the following modifications: (a) The 'blocking solution' for the nitrocellulose paper was 10% (v/v) newborn calf serum (Gibco) in phosphate-buffered saline (PBS)–0.25% (w/v) gelatin. (b) The PBS–Triton X-100 wash after exposure to serum was increased to 30 min, and the final PBS–Triton washes after exposure to ^{125}I-labelled staphylococcal protein A (^{125}I-SpA) were for 2 × 30 min. (c) The ^{125}I-SpA was from Amersham (code IM.144) and was used at 2×10^5 dpm per ml in PBS–0.25% (w/v) gelatin.

3. RESULTS

3.1. *Characterization of trypsinized histones*

Histones and their cleavage products isolated from native and trypsin-treated chicken erythrocyte nuclei are shown in fig.1a. H1 and H5 were rapidly and completely degraded by trypsin [12,13] and the 4 core histones were replaced by limit peptides. These have been shown to arise by excision of ~20–30 amino acid residues from the basic N-terminal regions of H3, H4 and H2B, as well as of the 6 C-terminal residues of H3, and of 11 residues from the N-terminus and 10 residues from the C-terminus of H2A [12,14–17]. The amino acid composition of the unfractionated mixture of limit peptides (table 1) was in excellent agreement with that expected from cleavage at the positions indicated (see footnote to table 1).

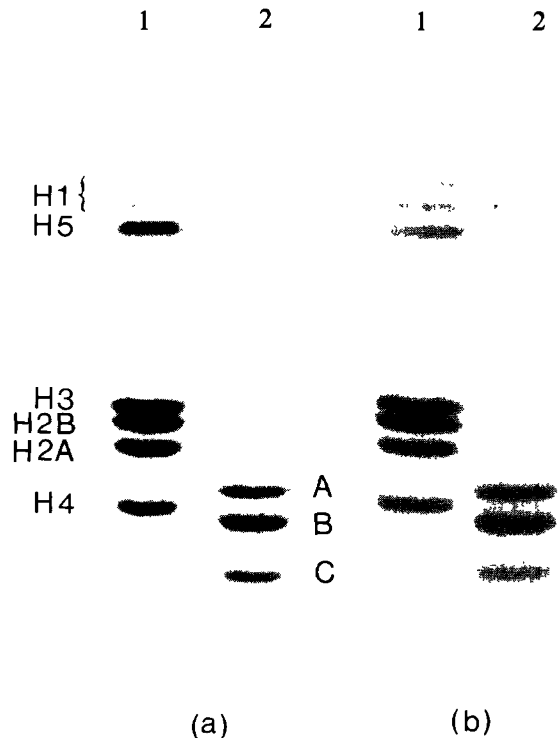

Fig.1. Tryptic digestion of chicken erythrocyte histones and the efficient transfer of limit peptides of the core histones to nitrocellulose. (a) SDS–18% polyacrylamide gel stained with Coomassie blue. (b) Nitrocellulose paper after electrophoretic transfer, stained with Amido black. In each case lane 1 contains intact histones; lane 2 contains limit peptides of core histones from nuclei treated with trypsin.

SDS-gel electrophoresis (fig.1a) showed 3 limit peptide bands, A–C, staining roughly in the ratio 1:2:1; from their relative electrophoretic mobilities these seemed likely to contain, respectively, the limit peptides of H3, (H2A + H2B) and H4. This assignment was supported by the amino acid compositions of the components electroeluted from a gel, and by limited amino acid sequence determination. The material in band A had the N-terminal sequence X-Ser-Ala-Pro-Ala- and the amino acid composition shown in table 1, and was thus the limit peptide of H3, arising from cleavage

Table 1

Amino acid composition of trypsinized core histones

Amino acid	Total limit peptides		H3 limit peptide	Band A	H2A:H2B limit peptides (1:1)	Band B
	Calc. (%)	Obs. (%)	Calc. (%)	Obs. (%)	Calc. (%)	Obs. (%)
Asp	5.9	6.2	4.9	5.6	6.6	7.6
Thr	5.7	5.4	5.8	5.7	4.7	4.7
Ser	5.2	5.8	4.9	6.1	7.1	7.4
Glu	9.4	9.7	11.7	11.6	9.0	9.5
Pro	3.2	3.7	4.9	4.2	3.3	3.4
Gly	7.2	7.6	3.9	6.6	7.1	7.8
Ala	10.4	10.5	10.7	11.0	11.3	11.1
Cys	0.2	–	1.0	–	0	–
Val	7.5	7.1	5.8	5.3	7.5	5.7
Met	1.2	1.2	1.9	0.8	0.9	0.9
Ile	6.2	5.7	5.8	5.4	5.7	5.2
Leu	9.9	9.9	10.7	10.0	10.4	10.6
Tyr	3.7	3.5	2.9	3.1	3.8	3.8
Phe	2.2	2.2	3.9	3.3	1.4	1.8
His	2.0	2.7	1.9	2.4	2.4	1.8
Lys	9.1	8.5	7.8	7.3	10.4	10.4
Arg	10.6	10.1	11.7	11.3	8.5	8.3

Residues are expressed as mol%. The limit peptides are taken to be residues 27–129 of H3 [16], 20–102 of H4 [16], 12–118 of H2A [15] and 21–125 of H2B [17]. The calculated amino acid compositions are based on the known amino acid sequences of the histones. Complete sequences have been determined for chicken erythrocyte H3 [21], H2A [29] and H2B [30]. In the case of H4 the sequence of the calf thymus histone [21] is assumed

at Arg(26)–Lys(27) and Arg(129)–Ile(130) [16]. The amino acid composition of band B (table 1) was in good agreement with that expected for a roughly equimolar mixture of H2B cleaved at Lys(20)–Ala(21) [17] and H2A cleaved at both Arg(11)–Ala(12) and Lys(118)–Lys(119) [15]; consistent with this, the N-terminal sequence analysis showed Ala-Lys-Ala-Lys-Ser- and Ala-. Band C material gave X-Val-Leu- as a prominent N-terminal sequence, and was probably largely the limit peptide from H4 cleaved at Arg(19)–Lys(20) [16] with other peptides [12]. [The absence of any recognizable spot on thin-layer chromatography for the N-terminal residue in A and C, which in both cases turned out to be lysine (see above), is almost certainly due to the known heterogeneous (mono-, di- and perhaps tri-) methylation at these positions in H3 and H4.]

3.2. *Immunoblotting of intact and trypsinized histones*

Fig.1b shows similar efficiency of electrophoretic transfer of intact and trypsinized core histones from the SDS-polyacrylamide gel to nitrocellulose. Although H1 also transferred well, transfer of H5 was rather less than optimal, but since we are concerned here only with the core histones, this is not a problem.

Immunoblots of intact and trypsinized histones with 10 different SLE sera and one normal serum are shown in fig.2. The right-hand track in each case contains the intact histones. All sera were used at the same dilution (1:40) so the strength of the response clearly varies from one serum to another. Four sera (T.D., R.M., Y.K. and S.B.) had strong anti-H1 and anti-H2B activity, a combination found previously to be a common anti-histone

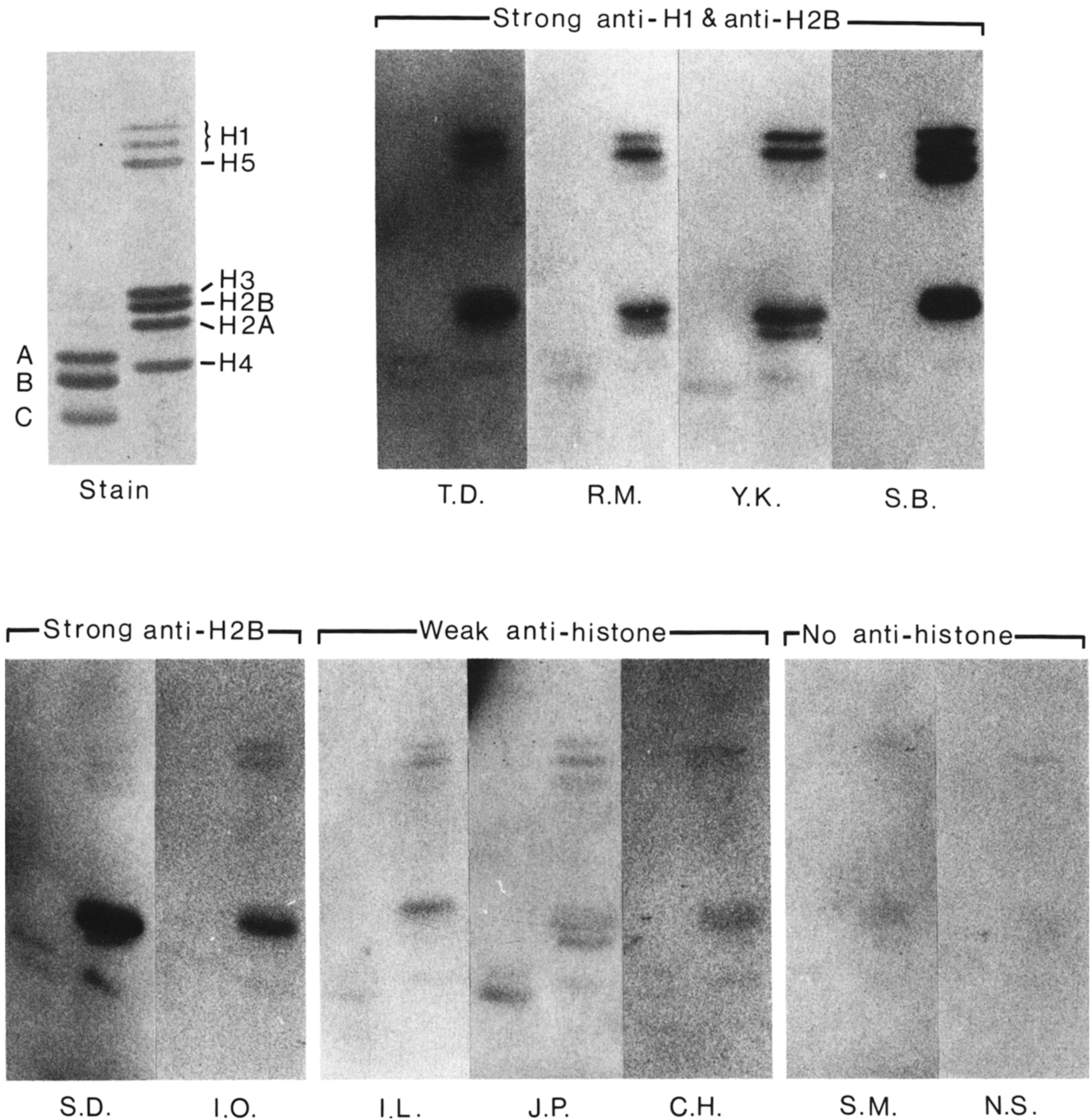

Fig.2. Immunoblotting of intact and trypsinized histones with SLE sera and normal sera. Nitrocellulose strips generated in parallel with that shown in the top left panel were exposed to 10 SLE sera and one normal serum (N.S.), and the bound IgG located by exposure to ^{125}I-SpA followed by autoradiography. All sera were used at the same dilution (1:40). The right-hand and left-hand lanes in each case contain, respectively, the intact and trypsinized histones.

response in SLE [1]. These 4 sera also reacted less strongly with other histones: T.D. with H3 and slightly with H4, R.M. with H2A, Y.K. with H2A and slightly with H4, and S.B. with H3 and with H5.(S.B. probably contains antibodies to H1°. Antibodies to H5 are known to cross-react with H1° [18].) Two sera (S.D. and I.O.) reacted strongly and moderately strongly, respectively, with H2B; S.D. also reacted weakly with H4 and H2A, and I.O. with H1, H3 and perhaps H4. Three sera

had weak anti-histone activity: I.L. against H3 and H1 (and to a lesser extent H4); J.P. against H1 and H2A (and to a lesser extent H2B, H3 and H4); and C.H. against H1, H2B, H3, and H4. S.M. had no antibodies to histones, since the blot showed no distinct bands and was very similar to that obtained with normal serum (N.S.) from a healthy donor. Patient S.M. does, however, have anti-Sm antibodies [1] but since acid-extracted histones were used here for blotting, rather than whole nuclear proteins, the Sm and other protein constituents of ribonucleoprotein particles were not visualized.

Wherever a strong reaction was observed with intact histones there was extensive loss of immunoreactivity after treatment with trypsin, which removes N-terminal tails and, in the case of H2A and H3, a C-terminal fragment as well (cf. the left track of each pair in fig.2 with the track on the right). In some cases (S.B. and I.O.) this loss was complete; in others (T.D., R.M., Y.K. and S.D.) up to about 10% of the reactivity persisted. Similarly in two of the 3 weak sera (C.H. and I.L.) the trypsinized core histones were essentially non-immunoreactive. In contrast, however, when blotted with the third of the weak sera (J.P.) the trypsinized product appeared to be as immunoreactive as the intact H2A, the most strongly reacting of the core histones.

In summary, the strong response to H2B in SLE (sera T.D., R.M., Y.K., S.B., S.D. and I.O.) is largely to a determinant in the N-terminal 20 residues; this also appears to be the case for the very weak response to H4 (sera C.H., J.P., T.D. and Y.K.). The response to H3 (sera T.D., S.B. and C.H.) is largely to determinant(s) in the N-terminal 26 residues and/or C-terminal 6 residues. Similarly, the strong response to H2A in Y.K. is to the N-terminal 11 residues and/or C-terminal 10 residues; the situation for R.M. is unclear since the immunoreactive limit peptides may contain contributions from one or both of H2A and H2B. Only one serum (J.P.) of the 9 with anti-histone reactivity revealed a major determinant clearly not removed by trypsin. This determinant is therefore in the trypsin-resistant core region (residues 12–118) of H2A.

4. DISCUSSION

Immunoblotting of intact histones and their limit digestion products from trypsin-treated nuclei shows that antibodies to nucleosome core histones arising in SLE are mainly directed against the trypsin-sensitive N-terminal regions of H2B and H4, and the N-terminal and/or C-terminal regions of H2A and H3. The strongest response was against the N-terminus of H2B. An earlier study using a solid-phase radioimmunoassay had shown that trypsin treatment abolishes the reaction of mononucleosomes with anti-nuclear antibodies, but in that case individual histones were not distinguished [19]. The antibodies detected by immunoblotting are, of course, likely to be directed against primary structural determinants, as noted earlier [1]; tertiary structural antigens per se will not be detected (unless there is considerable renaturation on nitrocellulose, which seems unlikely), nor will combined DNA/protein determinants. The antigen(s) being recognised are clearly not species-specific (the blots are of chicken histones with human sera), although some, if not all, reside within the trypsin-sensitive N-terminal regions which contain most of the species variation in histones, especially H2A and H2B [20].

The trypsin-sensitive regions of the core histones are precisely those regions which, in the core particle at least, appear to be exposed and relatively loosely bound. NMR spectroscopy suggests that the N-terminal tails of some, or all, of the core histones are relatively mobile [4,5]. The trypsin-sensitive site, Lys(118)–Lys(119), in the H2A C-terminal region is adjacent to the ubiquitin attachment point, Lys(118) [21], and hence this is likely to be in a relatively exposed position to permit access to enzymes that add and remove ubiquitin. An exposed location for the C-terminal hexapeptide of H3 is shown by binding to the nucleosome of antibodies to this peptide [22].

A focused response against those regions of the histones that are known to be exposed in chromatin may mean that chromatin structures per se, rather than free histones, are the immunogens in SLE. When purified histones (complexed with RNA) are used as immunogens, the antibodies directed against the major determinants often do not bind well to chromatin, suggesting that the determinants are largely masked [23]. For exam-

ple, 90% of the antibodies in an anti-H2B serum were directed against the C-terminal 'half' of H2B (residues 63–125) and only 10% against the N-terminal 'half' (residues 1–58) [24], but the determinants in the C-terminal 'half' proved to be relatively inaccessible in chromatin, whereas those in the N-terminal half were exposed and bound by antibody [25]. However, although some or all of the region containing residues 30–56 of H2B is accessible in nucleosomes [26], this region is evidently not strongly immunogenic in SLE, in which the antigen(s) is confined to residues 1–20. Two of the 3 antigenic determinants identified on free H3 [27] (within residues 40–60 and 80–100) do not appear to be antigenic in SLE, on the basis of the sera examined here; a third, in the C-terminal 15 residues (residues 121–135) may, however, be recognized in SLE, in which the H3 antigen may be within the last 6 residues (and/or the first 26 residues), and these 6 residues are known to be exposed in chromatin [22]. It would appear, therefore, that in SLE the histones are presented to the immune system in a form which is not free; the antigens are restricted to regions of the histones known to be relatively exposed in nucleosomes, and one plausible explanation, although by no means the only one, is that the immunogen in SLE is chromatin itself [1]. The more strongly antigenic nature of the N-terminus of H2B than of the other core histones could perhaps arise from a particularly exposed position in chromatin.

Whatever the nature of the immunogen in SLE, antibodies directed against the N-terminal and/or C-terminal regions of the core histones, which may easily be affinity-purified, should prove to be useful probes for the location (exposed or otherwise) of these regions within chromatin higher-order structures. They may prove no less useful for this purpose than monoclonal antibodies, of which one (of the IgM class) directed against a determinant in the N-terminal region of H2B has recently been reported [28].

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