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Systemic lupus erythematosus murine monoclonal DNA-binding antibodies recognize cytoplasmic and nuclear phosphorylated antigens that display cell cycle redistribution in HEp-2 cells

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SUMMARY

The immunological basis for the production of autoantibodies characteristic of systemic lupus erythematosus (SLE) against a wide range of antigens remains obscure. The specificity of (NZB × NZW)F_1 (BWF_1) or MRL/Mp-*lpr*/*lpr* (MRL/*lpr*) mouse monoclonal antibodies (mAb) was examined by immunofluorescence, immunoblotting and immunoprecipitation techniques. Using non-synchronized HEp-2 cells as substrate, the murine mAb were classified by indirect immunofluorescence into five groups on the basis of their staining patterns of subcellular components in interphase and mitotic stages of the cell cycle. The nature of the antigens recognized by the murine lupus was assessed by immunoblotting experiments in total, cytoplasmic and nuclear cell extracts from HEp-2 cells. The six antibodies used recognized in total cell extracts a range of polypeptides with apparent molecular weights from 25,000 to 210,000. Three polypeptides of 130,000, 110,000 and 45,000 MW were recognized by all six antibodies in both nuclear and cytoplasmic extracts. Immunoprecipitation of total cellular extracts labelled with [^{35}S]methionine showed almost the same pattern as obtained in the immunoblotting assay. The labelling *in vivo* of HEp-2 cells with [^{32}P], followed by the immunoprecipitation of the [^{32}P]cell lysate showed that these mAb recognized phosphorylated proteins. The progressive decrease in reactivity of these mAb following treatment with higher concentrations of alkaline phosphatase in both [^{32}P]cell lysate or nitrocellulose membranes indicates that these mAb recognize phosphorylated epitopes.

INTRODUCTION

Human systemic lupus erythematosus (SLE) and murine lupus are autoimmune diseases of unknown aetiology and cure. This disease is characterized by the production of autoantibodies against a wide range of antigens and the presence of circulating immune complexes. The antigens recognized include single-stranded (ss)DNA, double stranded (ds)DNA, RNA, phospholipids, histones, PCNA/cyclin, SS-A/Ro, SS-B/La, Sm, Ku, HSP90, HSP73, Ki/SL, ribosomal RNP, topoisomerase II, Alu RNA protein, nuclear lamins and U1-RNA.[1-3] Some of these antibodies are characteristic of SLE, like antibodies to dsDNA and Sm. Others, such as anti-ssDNA, anti-histones, anti-nuclear RNP, anti-SS-A/Ro, and anti-SS-B/La may be detected in other autoimmune diseases. The autoantibody frequencies vary over a wide range in SLE patients, up to 70% for antibodies to ssDNA and histones, 3% for antibody to PCNA, and some are very rare, such as those against topoisomerase II, Alu RNA protein, nuclear lamins and U1-RNA.[3]

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The DNA-binding antibodies in SLE have some characteristics and unusual properties. First, they recognize many different epitopic structures on the various physical forms of DNA itself: there are at least five major families of epitopes.[4] Second, the antibodies extensively share public idiotypes both between those of different specificity and between those from humans and mice.[5-7] Third, the antibodies are not all immunochemically specific for DNA by virtue of their reactions with cytoplasmic and membranous cell antigens and with some components of the extracellular matrix.[8,9]

The exact biological significance of the cross-reactions is not understood, but they do offer some interesting possibilities for the identification and analysis of molecules in cells that may have (epitopic) structures related to those of DNA itself.

In order to investigate the antibody specificity of several hybridoma antibodies from (NZB × NZW) F_1 and MRL/*lpr* mice obtained from different fusion experiments, immunofluorescence, immunoblotting and immunoprecipitation tech-

Table 1. Summary of staining patterns in immunofluorescence assay on HEp-2 cells

Group*	mAb	Interphase	Metaphase/Anaphase
A	228	Strong nucleolar faint cytoplasmic	Condensed chromosomes
B	88, 406, 152	Nucleolar, nuclear membrane and cytoplasmic dots	Material surrounding condensed chromosomes
C	33, 405, 410	Homogeneous nuclear, nuclear membrane and faint cytoplasmic	Condensed chromosomes
D	233, 212, 402, 112	Granular nuclear and area surrounding nucleolus	Condensed chromosomes
E	423, 58	Homogeneous nuclear excluding nucleolus and faintly cytoplastmic	Material surrounding condensed chromosomes

* Groups A, B, C, D and E correspond to Groups IV, V, I, II and III described previously.[4]

niques were applied. HEp-2 cells were used to define the ability of mAb to bind to subcellular components by indirect immunofluorescence. Total cell extracts, cytoplasmic and nuclear extracts from HEp-2 cells were used for immunoblotting and immunoprecipitation experiments.

MATERIALS AND METHODS

Monoclonal antibodies

Monoclonal antibodies (mAb), selected for their reactivity with DNA, were prepared from spleen cells of unstimulated (NZB × NZW)F_1 (BWF_1) or MRL/Mp-*lpr*/*lpr* (MRL/*lpr*) mice. Preparations used here were either culture supernatants or ascites fluids from hybridomas grown in either CBA/*nu*/*nu* or (NZW × Balb/c)F_1 or (MRL/*Mp* × Balb/c)F_1 mice. The mAb used were 58, 33, 88, 112 from an adult BWF_1 mouse; 152, 212, 228, 233, 402, 405, 406, 410 from adult MRL/*lpr* mice, and 423 from a 15-day MRL/*lpr* foetus. The properties of the antibodies are summarized in Table 1, and their preparation and characterization have been described previously.[4,10,11]

Cell culture

HEp-2 cells (human carcinoma of larynx) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM gluthamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37° in a humidified atmosphere in a 7% CO_2 incubator.

Preparation of protein extracts

Total HEp-2 cell extracts were prepared by four cycles of rapid freezing and thawing in cold TN buffer[12], and clarified by centrifugation at 8200 *g* at 4° for 10 min. The supernatant was collected, and the proteins were solubilized in Laemmli sample buffer.[13] Nuclear protein extracts from HEp-2 cells was performed according to the method described by Wu.[14] Cytoplasmic protein extracts from HEp-2 cells were prepared as described by Adlakha *et al.*[15]

DNase treatment

DNase treatment of culture supernatants or ascites fluids containing mAb, and of total extracts from HEp-2 cells was performed according to the method described previously.[16]

Indirect immunofluorescence (IF)

Antigens were localized by indirect immunofluorescence on HEp-2 cells grown on glass coverslips for 2–3 days. Two different fixation protocols were performed: (1) 5 min in a mixture of 50% methanol and 50% acetone; (2) 90 seconds in PHEM buffer[17,18] pH 6.9 containing 0.5% Triton X-100, prior to fixation in a mixture of 50% methanol and 50% acetone, for 5 min at −20°. The cells were exposed to mAb and to the second antibody [anti-mouse Ig fluorescein-linked whole antibody from sheep (Amersham International, Amersham, U.K.)], diluted in phosphate-buffered saline (PBS) containing 10% FCS and 0·1% Triton X-100, for 1 hr at room temperature. Double labelling was done with Hoechst 33258, for 10 min. Coverslips were mounted in 85% glycerol with 2·5% *n*-propylgallate.[19]

Electrophoresis and immunoblotting

One-dimensional electrophoresis was carried out essentially according to Laemmli,[13] by using 7·5% sodium dodecyl sulphate polyacrylamide gels (SDS–PAGE). Total cell extracts, cytoplasmic and nuclear extracts were solubilized in Laemmli sample buffer.[13] For immunoblotting, the polypeptides in the gel were transferred electrophoretically to nitrocellulose paper (0·45 μm; Hybond-C, Amersham) as described by Towbin *et al.*[20] The following human anti-sera were used as controls: anti-Sm; anti-RNP; anti-SS-A; anti-SS-B (Kallestad Diagnostic, Austin, TX). Secondary anti-mouse or human Ig horseradish peroxidase were obtained from Amersham.

Alkaline phosphatase treatment

Nitrocellulose immunoblots or cell extracts were treated with alkaline phosphatase,[21] from bovine intestinal mucosa (Sigma, Poole, U.K.), before addition of primary antibody.

Immunoprecipitation with [^{32}P] and [^{35}S]

Immunoprecipitation assays were performed according to published methods.[22] HEp-2 cells were labelled with [^{32}P]orthophosphate (Amersham) in phosphate-free DMEM at a final concentration of 3 μCi/ml, for 10 hr. HEp-2 cells were labelled with [^{35}S]methionine (Amersham) in MEM, at a final concentration of 300 μCi, for 6 hr. After lysing the cells, normal rabbit serum was used for preclearing the lysate. Half of the preclearing lysate was treated with different concentrations (from 0·01 U/ml up to 10 U/ml) of alkaline phosphatase.[21] In parallel, an equal volume of the preclearing lysate (without being treated with the alkaline phosphatase) was used as control. For each precipitation, 5 μl of mAb and the lysate from 5×10^7 HEp-2 cells were used. Pansorbin cells 10% (Calbiochem, Behring Diagnostics, La Jolla, CA) were used to collect the immune complex. The

immunoprecipitated proteins were separated by electrophoresis on 7·5% SDS–PAGE, dried and exposed to X-ray film.

RESULTS

Immunofluorescence staining of HEp-2 cells with murine lupus mAb

Murine lupus mAb were classified by indirect immunofluorescence assay into five different groups on the basis of their staining patterns of HEp-2 cells. Non-synchronized cells were used for this study so that cell cycle-dependent changes in the distribution of the antigens could be followed. Also, since it has been shown that the accessibility of some antigens increases by treatment of the cells with detergent prior to fixation,[18] staining patterns with or without pretreatment were compared. Table 1 gives a summary of the staining patterns of HEp-2 cells at interphase or during metaphase–anaphase. The results indicate that the majority of the staining patterns found were similar to those described previously.[4] The five groups showed very different staining of the interphase cells. Groups A and B showed strong nucleolar staining as well as cytoplasmic components (Fig. 1A, B) while Group C showed homogeneous nuclear staining (Fig. 1C), and both Groups D and E showed nuclear staining excluding the nucleolus (Fig. 1D, E). During metaphase–anaphase, there were essentially two staining patterns. Three groups (A, C and D) stained heavily the chromosomes (Fig. 1D1) and any previous cytoplasmic staining disappeared. The other two groups (B and E) did not stain the chromosomes themselves but the material which surrounds the chromosomes forming a stain halo (Fig. 1E).

The immunofluorescence patterns did not change when the cells were permeabilized by brief exposure to detergent prior to fixation. However, the amount of mAb required to give a comparable intensity of staining was much lower.

Murine lupus DNA-binding mAb recognized multiple polypeptides

The mAb used in the immunofluorescence study were originally isolated by their DNA affinity; however, their staining patterns on HEp-2 cells showed that they recognized antigens some of which were not associated with the nucleus. Therefore, immunoblotting assays were performed to assess the nature of antigens in total, cytoplasmic and nuclear HEp-2 cell extracts.

The antibodies used in these experiments recognized in total cell extracts a range of polypeptides with apparent molecular masses from 25,000 to 210,000 (Fig. 2A). A summary of all the immunoblotting experiments is presented in Table 2.

There were three polypeptide bands of 130,000, 110,000 and 45,000 MW recognized by all mAb, and present in both nuclear and cytoplasmic extracts. Monoclonal antibody 228 from Group A was the only one that recognized antigens of exactly the same MW in nuclear and cytoplasmic extracts. Group B mAb 88, 406 and 152 recognize antigens in the cytoplasmic extracts that had the same MW. However, in the nuclear extracts mAb 406 and 152 reacted with one additional polypeptide band, compared with the only one recognized by mAb 88 which was used as a culture supernatant. The polypeptides recognized by Group C mAb 405 and 410 had apparent molecular masses of 100,000 and 70,000 in cytoplasmic extracts, and 80,000 and 60,000 in nuclear extracts. Group D mAb 233 and 212 recognized an antigen of 90,000 MW in the cytoplasmic extracts in addition to those of 80,000, 70,000 and 50,000 MW recognized in nuclear extracts. Finally, Group E mAb 58 and 423, recognized two antigens of 90,000 and 80,000 MW in cytoplasmic extracts.

When the antigen loading was increased to 80–100 μg protein/lane there were also polypeptide bands corresponding to 210,000, 180,000 and 170,000 MW that appeared in cytoplasmic extracts, and a polypeptide band of 150,000 MW that was detected in nuclear extracts (data not shown).

Human serum anti-Sm, anti-RNP, anti-SS-A and anti-SS-B were used as controls, and the polypeptides they recognized showed, respectively, 28,000, 62,000, 52,000 and 47,000 MW (Fig. 2B).

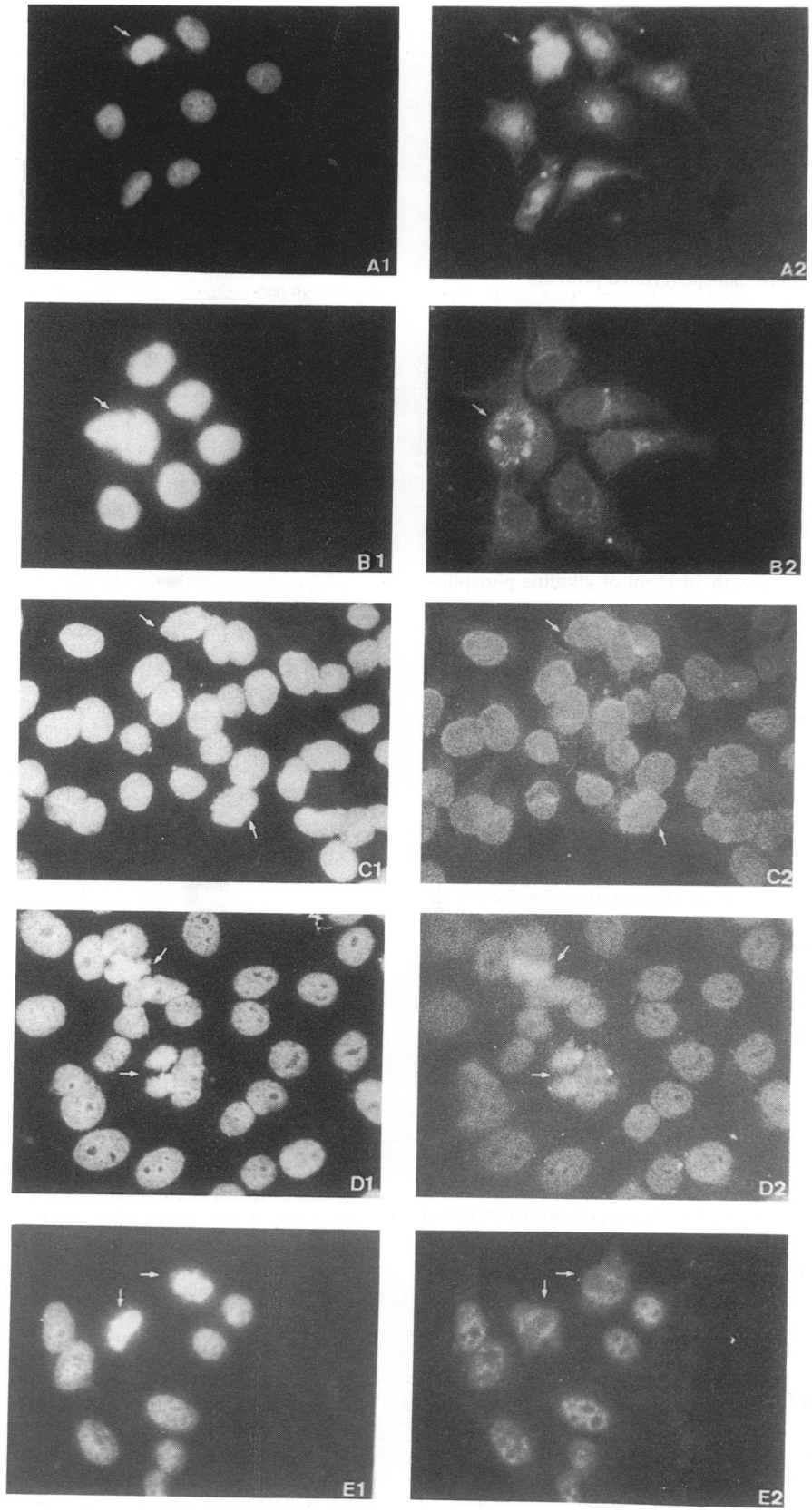
Effect of the DNase treatment

In order to investigate whether any of the observed reactions of the mAb with blotted proteins were due to non-specific binding of antibodies through DNA fragments trapped in their binding sites, the mAb were treated with DNase I. As a consequence, the antigens of 80,000, 50,000 and 35,000 MW in the nuclear extracts, and those of 70,000 and 35,000 MW in the cytoplasmic extracts were no longer detected with mAb 233 and 88 tested as culture supernatants (Fig. 2C). The antigen with 35,000 MW was preferentially recognized when these mAb were used as supernatant samples. This indicates that many cross-reactions of DNA-binding antibodies with intracellular structures depend upon the involvement of DNA: it follows that the molecules identified here to which this applies are almost certainly DNA-binding proteins themselves. The pattern of immunoblotting did not change after DNase treatment of ascites mAb or pretreatment of cell extracts (data not shown).

Immunoprecipitation of [^{35}S]Met-labelled cellular extracts with mAb

Immunoprecipitation of total cellular extracts labelled for 6 h with [^{35}S]Met was used to identify the antigens recognized by these mAb which were not detected by immunoblotting. This

Figure 1. (*opposite*) Staining pattern of interphase and mitotic (arrows) HEp-2 cells stained with Hoechst 33258 (A1, B1, C1, D1, E1) and immunofluorescence staining of interphase and mitotic (arrows) HEp-2 cells (A2, B2, C2, D2, E2) fixed in a mixture of 50% methanol and 50% acetone, 5 min at −20°. (A) (mAb 228)—shows strong staining of the nucleolus and faint cytoplasmic staining in interphase cells, and chromosomal staining in metaphase cells. (B) (mAb 406)—shows faint nucleolar and nuclear membrane staining with cytoplasmic dots in interphase cells, and metaphase cells show staining of material surrounding condensed chromosomes. (C) (mAb 410)—shows homogeneous nuclear staining with peripheral accentuation and faint cytoplasmic staining, and metaphase cells show chromosomal staining. (D) (mAb 233)—shows nuclear granule staining with peripheral accentuation surrounding nucleolus without staining them, and metaphase cells show chromosomal staining. (E) (mAb 423)—shows homogeneous nuclear staining (excluding nucleolus) and faint cytoplasmic staining in interphase cells, and metaphase cells show staining of material surrounding condensed chromosomes.


A1
A2
B1
B2
C1
C2
D1
D2
E1
E2

experiment was done with mAb 228, 152, 410, 212 and 58, belonging respectively to groups A, B, C, D and E (Fig. 3). The results suggest that representative mAb from all five groups identify almost the same polypeptide bands as those recognized by immunoblotting (Table 3). Some of the antigens detected by immunoblotting when high antigen concentration was used, were also detected by immunoprecipitation, like the antigen of 210,000 MW. Only three polypeptide bands of 85,000, 55,000 and 43,000 MW were immunoprecipitated but not detected by immunoblotting.

Murine lupus mAb recognize phosphorylated proteins

In order to investigate whether the polypeptide bands recognized by these DNA-binding mAb included phosphorylated proteins, immunoprecipitations of total cellular extracts were performed, after labelling *in vivo* HEp-2 cells, in the presence of inorganic [^{32}P]. The results of experiments in which mAb from Groups A, B and C were used are shown in Fig. 4. It can be seen that many of the polypeptides recognized by murine lupus are indeed phosphorylated including antigens of 210,000, 170,000, 130,000, 110,000, 90,000, 80,000, 60,000, 50,000, 45,000 and 32,000 MW. None of these bands appeared after treatment of the [^{32}P]total cellular lysate with 10 U/ml of alkaline phosphatase.

Murine lupus mAb recognize phosphorylated epitopes

Since many of the bands recognized by these mAb correspond to phosphorylated proteins it was tested whether the ability of the mAb to recognize multiple bands in immunoblotting could be altered by pretreatment of total cellular extracts with increasing quantities of alkaline phosphatase. The results of such an experiment in which mAb 228 from Group A and 405 from Group C were used are presented in Fig. 5. The use of increasing amounts (0·01 U/ml to 10 U/ml) of alkaline phosphatase caused a progressive decrease in reactivity and changed the apparent molecular masses of some of the antigens. Incubation with 0·01 U/ml of alkaline phosphatase eliminated the mAb 405 reactivity to antigens of 150,000, 100,000 and 70,000 MW while it continued to recognize all the other antigens. Using 0·1 U/ml of alkaline phosphatase the antigens of 110,000 and 45,000 MW were still recognized, but changed their apparent molecular masses to 105,000 and 42,000 MW. Incubation with 1 U/ml changed the 105,000 MW to 100,000 MW while the other was not detected. Treatment of extracts with 10 U/ml of phosphatase removed all reactivity of this mAb in Western blotting clearly indicating that these mAb do recognize phosphorylated epitopes. Similar results were obtained with all other mAb previously described in this study (data not shown).

DISCUSSION

In this study the antibody specificity of several hybridoma antibodies reactive with DNA from BWF$_1$ and MRL/*lpr* mice has been investigated by immunofluorescence and immunoblotting techniques, with a view to define the nature of some of the proteins with which they cross-react.

These mAb were classified into five groups on the basis of their reactivity with different subcellular structures and with purified polynucleotides, mononucleotides and nucleosides.[4]

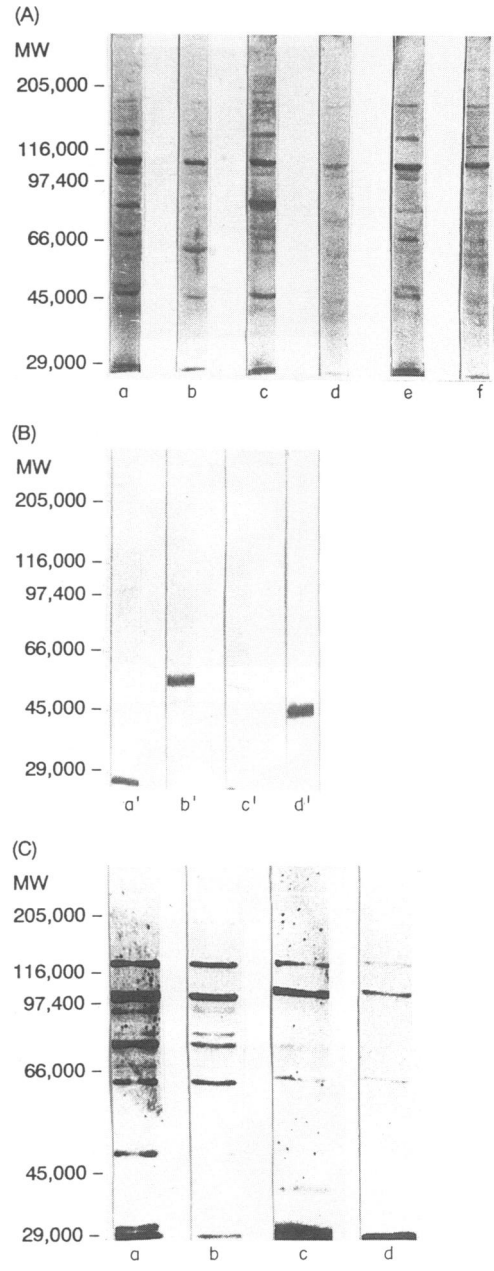


Figure 2. (A) Immunoblotting pattern showing the reactivity of seven mAb with whole HEp-2 total cell extract (each lane was loaded with 50 μg protein). Monoclonal antibodies used (diluted 1/100): a, 228; b, 410; c, 58; d, 152; e, 233; f, 405. These antibodies recognized a family of polypeptides with apparent molecular masses ranging from 25,000 to 210,000 MW. There are three polypeptide bands of 130,000, 110,000 and 45,000 MW, that react with all the antibodies. (B) Immunoblotting pattern of controls: anti-Sm serum, diluted 1/32 (a′); anti-RNP serum diluted 1/64 (b′); anti-SS-A serum, diluted 1/200 (c′); and anti-SS-B serum, diluted 1/64 (d′). (C) Immunoblotting patterns showing the reactivity of mAb 233 (supernatant) in cytoplasmic and nuclear extracts of HEp-2 cells, before (lanes a and c) and after (lanes b and d) DNase treatment. The cytoplasmic antigens of 70,000 and 35,000 MW (lane b) disappear, and the nuclear antigens of 80,000, 50,000 and 35,000 MW (lane d) are also absent. The molecular weight markers (Sigma) are myosin (205,000 MW), β-galactosidase (116,000 MW) phosphorylase b (97,400 MW), BSA (66,000 MW), ovalbumin (45,000 MW) and carbonic anhydrase (29,000 MW).

Table 2. Apparent MW of the antigens recognized by mAb in cytoplasmic and nuclear extracts

Group	mAb	Ig class	Antigens detected in total cellular extracts	Antigens detected exclusively in	
				Cytoplasmic extracts	Nuclear extracts
A	228	(Ig G2a)	180,000, 170,000, 150,000, 130,000, 110,000, 90,000, 80,000, 70,000, 60,000, 50,000, 45,000, 30,000–25,000		
B	88	(Ig G1)	130,000, 110,000 [80,000, 70,000, 60,000, 50,000, 45,000, 35,000]*	70,000	60,000
	406	(Ig G2a)	210,000, 180,000, 150,000, 130,000, 110,000, 90,000, 80,000, 70,000, 60,000, 50,000, 45,000, 30,000–25,000	70,000	90,000, 60,000
	152	(Ig G2a)	180,000, 170,000, 130,000, 110,000, 90,000, 80,000, 70,000, 60,000, 50,000, 45,000, 30,000–25,000	70,000	90,000, 60,000
C	405	(Ig G2a)	210,000, 180,000, 170,000, 150,000, 130,000, 110,000, 100,000, 80,000, 70,000, 60,000, 50,000, 45,000, 30,000–25,000	100,000, 70,000	80,000, 60,000
	410	(Ig G2a)	180,000, 170,000, 150,000, 130,000, 120,000, 110,000, 100,000, 80,000, 70,000, 60,000, 50,000, 45,000, 30,000–25,000	100,000, 70,000	80,000, 60,000
D	233	(Ig G1)	180,000, 150,000, 130,000, 110,000, 90,000, [80,000, 70,000, 50,000, 45,000, 35,000], 30,000–25,000	90,000	
	212	(Ig G2a)	180,000, 140,000, 130,000, 110,000, 90,000, 80,000, 70,000, 50,000, 45,000, 30,000–25,000	90,000	
E	58	(Ig G2a)	210,000, 170,000, 150,000, 130,000, 120,000, 110,000, 100,000, 90,000, 80,000, 60,000, 45,000, 35,000 30,000–25,000	90,000, 80,000	
	423	(Ig G3)	210,000, 150,000, 130,000, 110,000, 100,000, 90,000, 70,000, 45,000, 30,000–25,000	90,000, 80,000	

* Apparent MW of antigens shown in square brackets indicate that they could be eliminated by pretreatment of mAb (used as culture supernatant) with DNase.

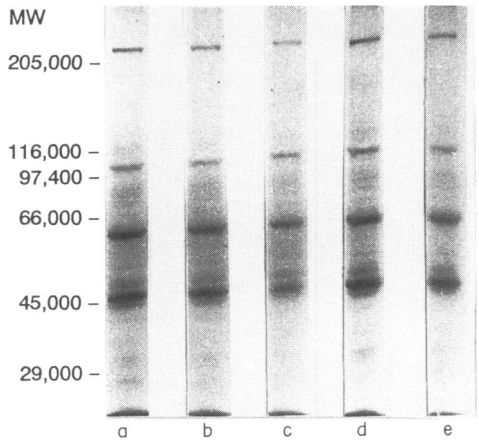


Figure 3. Immunoprecipitation of total cellular extracts of HEp-2 cells labelled with [^{35}S]Met. mAb used: 152 (lane a), 410 (lane b) 58 (lane c), 112 (lane d) and 228 (lane e). The apparent MW of the main immunoprecipitated proteins is described in Table 2. Three of these antigens (85,000, 55,000 and 43,000 MW) were not detected in the immunoblotting assay (see Fig. 2). The MW markers are as in Fig. 2.

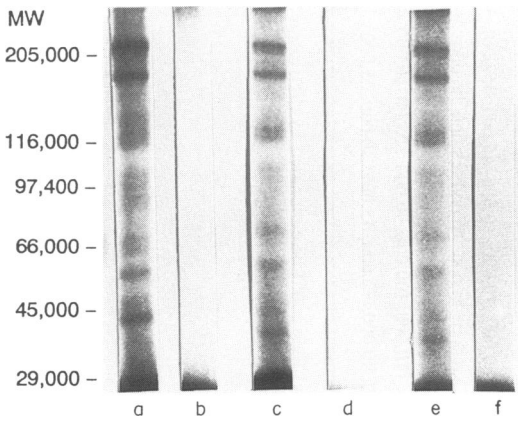


Figure 4. Autoradiography of 7·5% SDS–PAGE of total cellular extracts from HEp-2 cells (each lane was loaded with 50 μg of protein) labelled with [^{32}P]orthophosphate, and the proteins immunoprecipitated with different mAb, before and after alkaline phosphatase treatment (10 U/ml). The reactivity of immunoprecipitated proteins of 210,000, 170,000, 130,000, 110,000, 90,000, 80,000, 50,000, 45,000 and 32,000 MW (lanes a, c, and e) is completely removed after alkaline phosphatase (10 U/ml) treatment (lanes b, d, and f). Lanes a and b were immunoprecipitated with mAb 228, lanes c and d were immunoprecipitated with mAb 152, and lanes e and f were immunoprecipitated with mAb 410. The MW markers are as in Fig. 2.

Our immunofluorescence results confirm and extend those results. Furthermore, a clear cell cycle redistribution was shown of some of the antigens recognized by murine lupus mAb.

During interphase, antibodies from different groups showed different patterns of reactivity (described in Table 1). Groups A and B stained the nucleolus very strongly and the cytoplasm faintly. Group C showed a homogeneous staining of the nucleus along with a strong peripheral accentuation, and Group D showed numerous speckles in the nuclear staining. Finally, Group E showed a faint cytoplasmic reaction, and a homogeneous nuclear staining that spares the nucleolus. The same

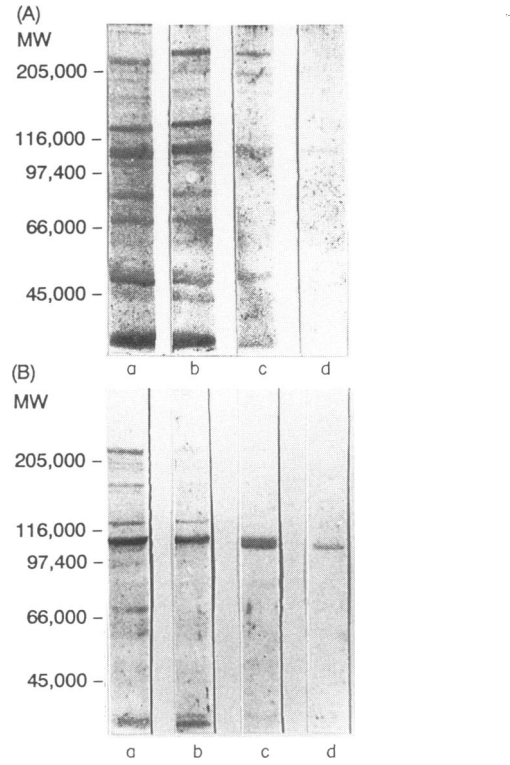


Figure 5. (A) Immunoblotting pattern of total cell extract from HEp-2 cells not treated (lane a) and treated with 0·01 U/ml (lane b), 0·1 U/ml (lane c) and 1 U/ml (lane d) of alkaline phosphatase using mAb 228 (diluted 1/100). (B) Immunoblotting pattern of total cellular extracts from HEp-2 cells not treated (lane a) and treated with 0·01 U/ml (lane b), 0·1 Uml (lane c) and 1 U/ml^{-1} (lane d) of alkaline phosphatase using mAb 405 (diluted 1/100). Each lane was loaded with 50 μg of total cellular extracts. The MW markers are myosin (205,000 MW), β-galactosidase (116,000 MW) phosphorylase b (97,400 MW), BSA (66,000 MW), and ovalbumin (45,000 MW).

staining patterns were obtained after pretreatment of the cells by a brief exposure to detergent prior to fixation. Under these conditions, and working with lower concentrations of antibody, we believe that non-specific binding reactions were avoided,[18] and more selective staining was obtained.

During metaphase, the staining patterns of mAb from Groups A, C and D in dividing cells were restricted to the condensed chromosomes. The other mAb from Groups B and E stained particulate material which formed a halo around the metaphase plate and appeared to line the chromosomes. Recent data have shown that nucleolar ribonucleoproteins might be associated with this staining pattern.[23] None of the murine lupus mAb tested show specific localization to known structures associated with the mitotic apparatus as described for the other autoantibodies (CREST).[2]

DNase treatment of culture supernatants shows that some cross-reactions of DNA-binding antibodies with intracellular structures depend upon the involvement of DNA in their binding site.

Although these mAb were originally selected for their DNA-binding ability, it was found that they also recognize various other nuclear and cytoplasmic components. Immunoblotting revealed reactivity with a wide range of polypeptides (25,000–210,000 MW). There were three polypeptide bands recognized

Table 3. Apparent MW of the antigens in total cell extracts recognized by mAb in immunoblotting and immunoprecipitation assays

Group	mAb	Imunoblotting	Immunoprecipitation
A	228	180,000, 170,000, 150,000, 130,000, 110,000, 90,000, 80,000, 70,000, 60,000, 50,000, 45,000, 30,000–25,000	210,000*, 180,000, 170,000*, 150,000, 130,000*, 120,000, 110,000*, 110,000*, 90,000*, 85,000, 80,000*, 70,000, 60,000*, 55,000, 50,000*, 45,000*, 43,000, 30,000–25,000
B	152	180,000, 170,000, 130,000, 110,000, 100,000 90,000, 80,000, 70,000, 60,000, 50,000, 45,000, 30,000–25,00	210,000*, 180,000, 170,000*, 150,000, 130,000*, 120,000, 110,000*, 90,000*, 85,000, 80,000*, 70,000, 60,000*, 55,000, 50,000*, 45,000*, 43,000, 30,000–25,000
C	410	180,000, 170,000, 150,000, 130,000, 110,000, 90,000, 80,000, 70,000, 60,000, 45,000, 30,000–25,000	210,000*, 180,000, 170,000*, 150,000, 130,000* 120,000, 110,000*, 90,000*, 85,000, 80,000*, 70,000, 60,000, 55,000, 50,000*, 45,000*, 43,000, 30,000–25,000
D	212	180,000, 150,000, 130,000, 110,000, 90,000, 80,000, 70,000, 50,000, 45,000, 30,000–25,000	210,000, 180,000, 150,000, 130,000, 120,000, 110,000, 90,000, 85,000, 80,000, 70,000, 60,000, 55,000, 50,000, 45,000, 43,000, 30,000–25,000
E	58	180,000, 150,000, 130,000, 110,000, 90,000, 80,000, 70,000, 60,000, 50,000, 45,000, 30,000–25,000	210,000, 180,000, 150,000, 130,000, 120,000, 110,000, 90,000, 85,000, 80,000, 70,000, 60,000, 55,000, 50,000, 45,000, 43,000, 30,000–25,000

* Phosphoproteins (see next section).

by all the antibodies (130,000, 110,000 and 45,000 MW). The reaction of mAb with polypeptides in both cytoplasmic and nuclear extracts could relate to the fact that non-synchronized cells were used. The major polypeptide bands recognized by all mAb relate directly to the immunofluorescence patterns, and not to the immunoglobulin class or fusion experiment that generated each mAb.

In SLE, the identity of many autoantigens has been defined: Sm;[2,3] nuclear RNP of U1 snRNP,[24] SS-A/Ro,[2,3] and SS-B/La, a phosphoprotein;[2,3] ribosomal RNP phosphoproteins;[2,3] Ku has DNA-binding proteins;[2,3] Ki/Sl;[2,3] PCNA/cyclin;[2,3] heat-shock protein 90 (hsp90) and hsp73;[2,3] Alu-RNA protein;[2,3] topoisomerase II;[2,3] nuclear lamins A, B, and C.[2,3] It remains to be defined whether any of these known antigens correspond to those observed in this study. Some mAb raised against mitotic cells, like MPM-1 and MPM-2, also recognize multiple antigens which show cell cycle-dependent rearrangement.[21,25,26] Recent data have also indicated that these antigens undergo cell cycle-dependent phosphorylation and dephosphorylation.[27] The identity of only one of these antigens is known, and probably corresponds to the scaffold-associated protein topoisomerase II.[28]

HEp-2 cells were labelled with [^{32}P]orthophosphate in order to detect whether any of the antigens recognized by these DNA-binding mAb were phosphorylated. Immunoprecipitation of [^{32}P]cell lysate showed that most of the antigens recognized by immunoblotting were phosphorylated (see Table 3). These polypeptides do not include all those detected by immunoprecipitation after [^{35}S]Met labelling, suggesting that either not all antigens are phosphorylated or that the phosphorylated form of the missing ones is not sufficiently represented in the cell lysates.

Further indication that most of the antigens were indeed phosphoproteins was obtained after treatment of either nitrocellulose blots or cellular lysates before SDS–PAGE with increasing amounts of alkaline phosphatase. A decrease in the reactivity of these mAb was observed, as well as changes in the apparent MW of the antigens recognized by them. The results obtained lead to the conclusion that these mAb recognize a set of antigens that have the presence of phosphorylated epitopes in common.

It has been suggested that the multiple cross-reactions of individual lupus antibodies imply that DNA is not necessarily the immunogenic stimulus for their production.[29]. Indeed some forms of DNA are poorly immunogenic in experimental model systems.[30] Analysis of V-gene sequences of DNA-binding mAb derived from individual mice by Shlomchik *et al.*[31] has shown that each animal uses a restricted set of germ line genes to encode the antibodies it makes and the appearance of mutations affecting affinity strongly argues that the DNA antibody response is driven to the DNA itself.

The molecular basis of DNA-binding specificity in these antibodies is incompletely understood. Some are rich in arginine residues which establish hydrogen bonds to bases in DNA.[8] Recently, it was shown that rheumatic disease-associated autoantigens display a very high occurrence of highly charged segments, either in the form of very long runs of charged residues or in the form of charged clusters.[24,32,33] However, other mAb have no charged residues (specially arginine) in their binding site but may have tyrosines in abundance. The mAb 88 used in this study is one such example (N. A. Staines, unpublished data). The comparison of cross-reactive antigen structures may help to elucidate the structural basis of DNA antibody specificity.

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