

## LAMIN B AUTOANTIBODIES IN SERA OF CERTAIN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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The nuclear envelope of eukaryotic cells separates the nuclear and cytoplasmic compartments, participates in the transport of molecules into and out of the nucleus, and may have a role in determining chromatin structure (1-4). Along with the double nuclear membrane and nuclear pore complexes, the nuclear lamina is a major component of the nuclear envelope (2, 5, 6). The nuclear lamina is a polymeric network of proteins interposed between the inner nuclear membrane and the chromosomes. In many types of cells, it consists of three proteins, lamins A, B, and C (4, 6). The nuclear lamina along with the nuclear pore complexes can be isolated as a supramolecular assembly (the pore complex-lamina [PCL]<sup>1</sup> fraction) by treatment of nuclei with nucleases, nonionic detergent, and high concentrations of salt (5). Although the lamina is highly stable in interphase cells, it is rapidly and reversibly depolymerized during mitosis, presumably through phosphorylation of the individual lamins (7-9).

The precise biological function of the nuclear lamina is not known, but molecular interactions with the nuclear pore complexes (10), chromatin (possibly mediated by lamins A and C [6, 11]), and the inner nuclear membrane (probably mediated by lamin B [1, 2, 12]) are likely. cDNA-derived protein sequences of lamins A and C indicate that they are nearly identical, differing only in their carboxy termini (13, 14). Although its amino acid sequence is not yet known, peptide mapping experiments indicate that lamin B is also related to lamins A and C, but less closely than lamins A and C are to one another (2). Monoclonal antibodies reactive with all three lamins provide further evidence that lamin B has some homology to lamins A and C (15-17).

In the present studies, we have characterized sera from patients with systemic lupus erythematosus (SLE) that display rim-pattern nuclear immunofluorescence. Although this pattern of reactivity has been associated with autoantibodies to DNA (18, 19), we demonstrate that, in some instances, rim-pattern immunofluorescence by sera from patients with SLE is related to the presence of autoantibodies to lamin B. Previously, autoantibodies specific for lamins A and C have

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<sup>1</sup> Abbreviation used in this paper: PCL, pore complex-lamina.

been described in a patient with linear scleroderma (20). Because most of the sera described here display only minimal reactivity with lamins A and C, the autoantibodies in these sera may be useful for examining the molecular structure and function of lamin B.

### Materials and Methods

**Sera.** Sera from ~200 patients treated at the Rockefeller University Hospital for collagen vascular diseases (including systemic lupus erythematosus, mixed connective tissue disease, scleroderma, polymyositis, and overlap syndromes) were screened by immunofluorescence for staining of the nuclear periphery. Sera that were strongly positive for homogeneous peripheral nuclear staining were selected for further study.

**Indirect Immunofluorescence.** Two types of immunofluorescence assays were used to detect serum autoantibodies reactive with the nuclear periphery. The first was the standard antinuclear antibody assay using human HEp-2 (laryngeal carcinoma) cells as substrate (21). Cells were grown on coverslips and fixed with methanol at  $-20^{\circ}\text{C}$  for 10 min. The cells were then overlaid sequentially with 1:50 diluted human serum for 30 min at  $22^{\circ}\text{C}$  followed by 1:40 diluted rhodamine or FITC-conjugated goat anti-human IgG antibodies (Tago, Inc., Burlingame, CA) for 30 min. at  $22^{\circ}\text{C}$ , and viewed with a Leitz Ortholux II epifluorescence microscope.

For the second immunofluorescence assay, HEp-2 cells were extracted in situ on coverslips using the method of Staufienbiel and Deppert (22) with minor modifications. This involves sequential treatment of the cells with 1% NP-40/KM (KM is 10 mM MES, pH 6.2, 10 mM NaCl, 1.5 mM  $\text{MgCl}_2$ , 1 mM EGTA, 5 mM dithiothreitol), deoxyribonuclease I (DNase, type IV; Sigma Chemical Co., St. Louis, MO; 50  $\mu\text{g}/\text{ml}$  in KM without EGTA or dithiothreitol), and DNase/2 M NaCl/KM (without EGTA or dithiothreitol). Cells were fixed and stained as above. Titers of autoantibodies to the nuclear lamina were determined by observing the immunofluorescent staining pattern of serially diluted sera using extracted nuclei as substrate.

**Isolation of PCL and Lamin A, B, and C-enriched Fractions.** A crude PCL fraction was isolated from human K562 (erythroleukemia) cells by essentially the same procedure as described above for the in situ extractions (22). Cells were resuspended at  $5 \times 10^7$  cells/ml in 1% NP-40/KM containing 5 mM iodoacetamide and 0.5 mM PMSF on ice for 10 min, and nuclei were collected by centrifugation at 750 g. The nuclei were resuspended and incubated with DNase (50  $\mu\text{g}/\text{ml}$  in KM/0.5 mM PMSF) for 15 min. at  $22^{\circ}\text{C}$ , followed by the addition of NaCl to a final concentration of 2 M for 15 min at  $4^{\circ}\text{C}$ . The pellet was resuspended in loading buffer containing 2.5% SDS, and aliquots were analyzed on 10% SDS-polyacrylamide gels.

Lamin A, B, and C-enriched fractions were isolated from rat liver according to Fisher et al. (14). Briefly, rat liver nuclei were isolated (23), and the PCL fraction was obtained (5). The PCL fraction was solubilized in 6 M urea/20 mM dithiothreitol/20 mM Tris, pH 8.0, and insoluble material was removed by centrifugation at 14,000 g for 30 min. The supernatant was adsorbed on DEAE-cellulose (DE52; Whatman, Inc., Clifton, NJ) equilibrated with 6 M urea/5 mM dithiothreitol/20 mM Tris, pH 8.0. The column was eluted with a linear 0–0.2 M NaCl gradient in the same buffer. Under these conditions, lamins A and C eluted at low salt and lamin B at higher salt as determined by SDS-PAGE of aliquots of the fractions. Individual lamins were further purified by electroelution from preparative SDS-polyacrylamide gels (24).

**Western Blotting.** Subcellular fractions were resolved on 10% SDS-polyacrylamide gels and the proteins were transferred to nitrocellulose (25). The blots were probed with human sera at a dilution of 1:500 to 1:1,000, followed by incubation with either alkaline phosphatase-conjugated goat anti-human IgG antibodies (from Tago, at a dilution of 1:1,500) and nitroblue tetrazolium-indoxyl phosphate substrate (26) or by  $^{125}\text{I}$ -protein A (New England Nuclear, Boston, MA;  $10^5$  dpm/ml in PBS).

**Immunoprecipitation.** K562 cells were resuspended at  $1.5 \times 10^6$  cells/ml in methionine-deficient culture medium containing 50  $\mu\text{Ci}/\text{ml}$  of [ $^{35}\text{S}$ ]methionine (New England Nuclear)

and labeled for 12 h (27). Colcemid (0.05  $\mu\text{g}/\text{ml}$ ) was then added to the culture medium for an additional 4 h, and the cells were collected by centrifugation. A clarified cell extract was immunoprecipitated with human serum (27, 28), and the radioactive proteins eluted from the immunoprecipitate were analyzed on a 15% SDS-polyacrylamide gel followed by detection by fluorography.

*Reactivity of Serum Autoantibodies with Cytoplasmic Filaments.* Sera were diluted 1:20 and incubated with methanol-fixed PTK-2 (kangaroo rat) cells on coverslips as above, followed by 1:40-diluted FITC-conjugated goat anti-human IgG antibodies (21). In some experiments, the PTK-2 cells were treated with colcemid (100  $\mu\text{g}/\text{ml}$  for 12 h) before fixation and staining.

## Results

*Indirect Immunofluorescence.* Sera from four patients with SLE displayed a homogeneous peripheral pattern of reactivity with human HEP-2 cell nuclei by indirect immunofluorescence (Fig. 1A). Serum from patient 1 displayed no detectable staining of the nuclear interior (Fig. 1A), while the other three sera displayed speckled staining of the nuclear interior in addition to peripheral staining (not shown). The peripheral nuclear pattern of immunofluorescence gave rise to diffuse and punctate cytoplasmic immunofluorescence in metaphase cells (Fig. 1B). A similar staining pattern has been observed by Burke et al., (15) using a monoclonal antibody that reacts with lamins A, B, and C. In other metaphase cells (Fig. 1C), the cytoplasmic immunofluorescence was more diffuse. Cytoplasmic staining persisted through anaphase and early telophase (not shown), and no staining of the condensed chromosomes was observed.

*In Situ Extraction of Nuclei.* The homogeneous peripheral pattern of immunofluorescence in interphase cells and the diffuse cytoplasmic staining of mitotic cells displayed by these sera is similar to that previously described using antisera reactive with the nuclear lamina (1, 6, 20). Because the lamina is characteristically resistant to extraction with nonionic detergent, deoxyribonuclease I (DNase), and 2 M NaCl, the reactivity of these sera with nuclei that received this treatment (22) in situ was examined (Fig. 2). Serum from patient 1 displayed homogeneous staining of the nuclear periphery of both untreated and extracted cells. In contrast, sera containing antibodies reactive with DNA (anti-DNA) or the protein components of U1, U2, U4, U5, and U6 small nuclear ribonucleoproteins (snRNPs) (anti-Sm/RNP) stained the nuclei of untreated cells, but not those of extracted cells, indicating that immunoreactive DNA and extractable nuclear antigens (29) are largely removed by treatment with nonionic detergent, DNase and high salt. Sera from patients 2–4 also displayed homogeneous peripheral nuclear staining that was resistant to extraction in situ (not shown). These experiments suggested that the four sera contained autoantibodies to components of the PCL fraction. This possibility was tested more directly by subcellular fractionation and western blotting techniques.

*Identification of Polypeptides Reactive with the Sera.* A crude PCL fraction was isolated from human K562 cells by essentially the same technique as that used for the in situ extraction experiments described above. The insoluble material was solubilized in SDS and subjected to SDS-PAGE. Western blots of the gels were probed with sera from patients 1–4 or with normal serum (Fig. 3, N). Each serum reacted strongly with a prominent band of  $\sim 68$  kD (P68), and less strongly with bands at  $\sim 70$  and 60 kD (P70 and P60, respectively). Each of the sera also

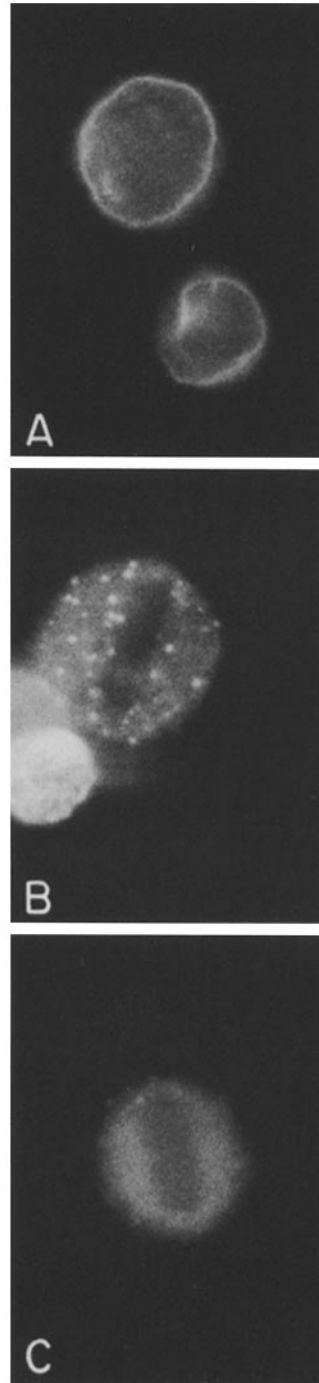


FIGURE 1. Indirect immunofluorescence staining of HEp-2 cells using patient 1 serum. (A) Staining of interphase cells showing peripheral (rim) pattern of immunofluorescence. (B and C) Metaphase cells demonstrating punctate (B) or diffuse (C) cytoplasmic staining. Patient 1 serum was used at a dilution of 1:50 for staining.

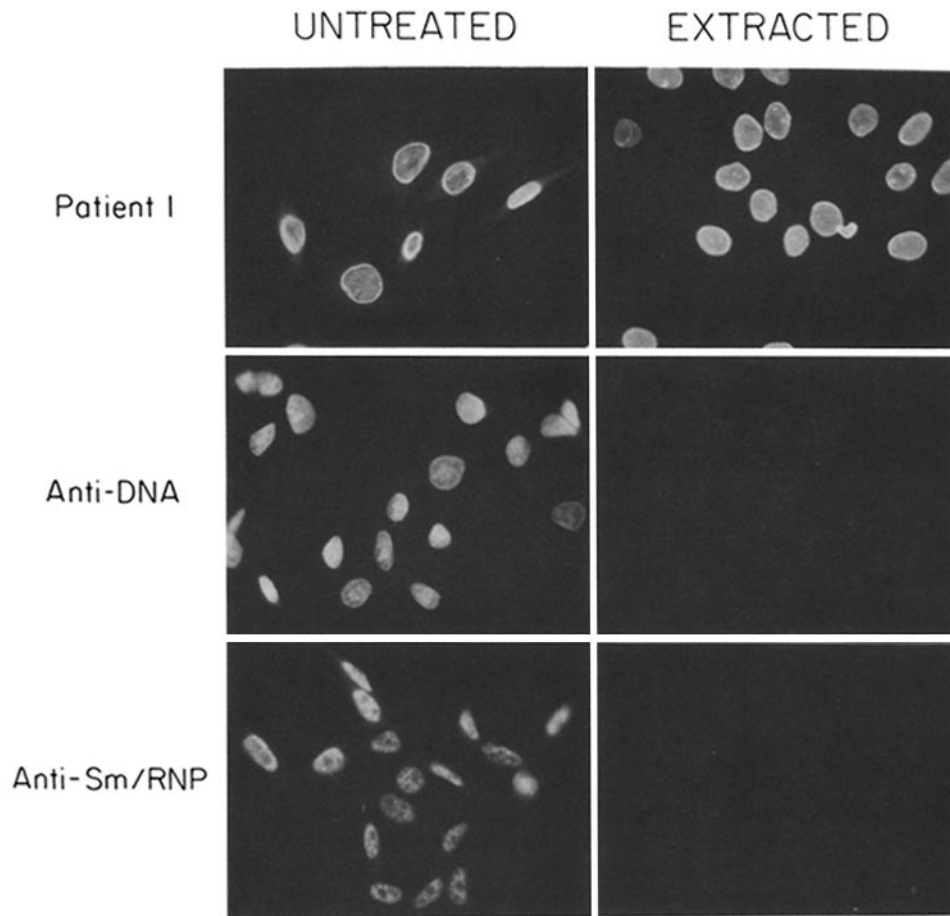


FIGURE 2. Indirect immunofluorescence staining of HEP-2 cells extracted in situ with non-ionic detergent, nuclease, and high salt. Immunofluorescence patterns displayed by various human autoimmune sera diluted 1:50 were determined using as substrate either untreated, methanol-fixed cells (*left*) or cells that were extracted with nonionic detergent, nuclease, and 2 M NaCl by the method of Staufenbiel and Deppert (22) (*right*). (*Top*) Serum from patient 1, showing reactivity with the nuclear periphery of both untreated and extracted cells. (*Middle*) Serum containing a high titer of antibodies to single and double-stranded DNA (*anti-DNA*). (*Bottom*) Serum containing a high titer of antibodies to protein components of U1, U2, U4, U5, and U6 snRNPs (*Anti-Sm/RNP*). Anti-DNA and anti-Sm/RNP sera reacted with untreated but not with extracted cell nuclei.

reacted with a band of ~45 kD that may be a proteolytic degradation product of the lamins (15), and individual sera displayed reactivity with other proteins as well.

A more highly purified PCL fraction was isolated from rat liver nuclei (5). This material was solubilized in 6 M urea, and fractions enriched in lamins A, B, and C, respectively, were isolated by ion-exchange chromatography and electroelution from SDS-polyacrylamide gels (Fig. 4, *right*). As previously described (5), lamins A, B, and C were found to be prominent constituents of the PCL fraction by Coomassie blue staining of SDS-polyacrylamide gels (Fig. 4, *right*;

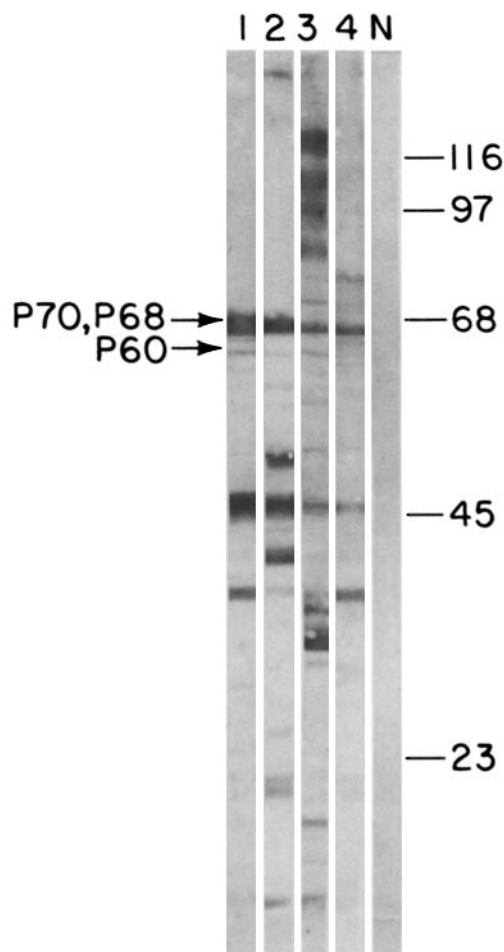


FIGURE 3. Western blots of crude PCL fraction. K562 cells were extracted sequentially with nonionic detergent, nuclease, and 2 M NaCl (22), and the crude PCL fraction was analyzed on a 10% preparative SDS-polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose, and strips of the blot were probed with sera from patients 1–4 or with normal human serum (*N*) at a dilution of 1:750. Sera from patients 1–4 reacted strongly with a band at ~68 kD (P68) and less strongly with bands at ~70 and 60 kD (P70 and P60, respectively). Sera from patients 1–4 also reacted with a prominent ~45 kD band, and individual sera reacted with other bands, as well. Molecular mass markers (in kD) are indicated on the right.

*PCL*). Numerous minor bands were also present in the *PCL* fraction, as well as contaminating histones (indicated by dots). Reactivity of the sera with the *PCL* fraction and the individual lamins was then determined by western blotting. Three of the four sera displayed the pattern illustrated by patient 1 (Fig. 4, *left*). This serum reacted strongly with a 68 kD protein in the *PCL* fraction and with lamin B, but displayed only minimal reactivity with the lamins A and C. Serum from patient 2 (*center*) also reacted strongly with P68 in the *PCL* and with purified lamin B. However, this serum also displayed substantial reactivity with lamins A and C.

Table I summarizes the specificities of the four sera determined by western

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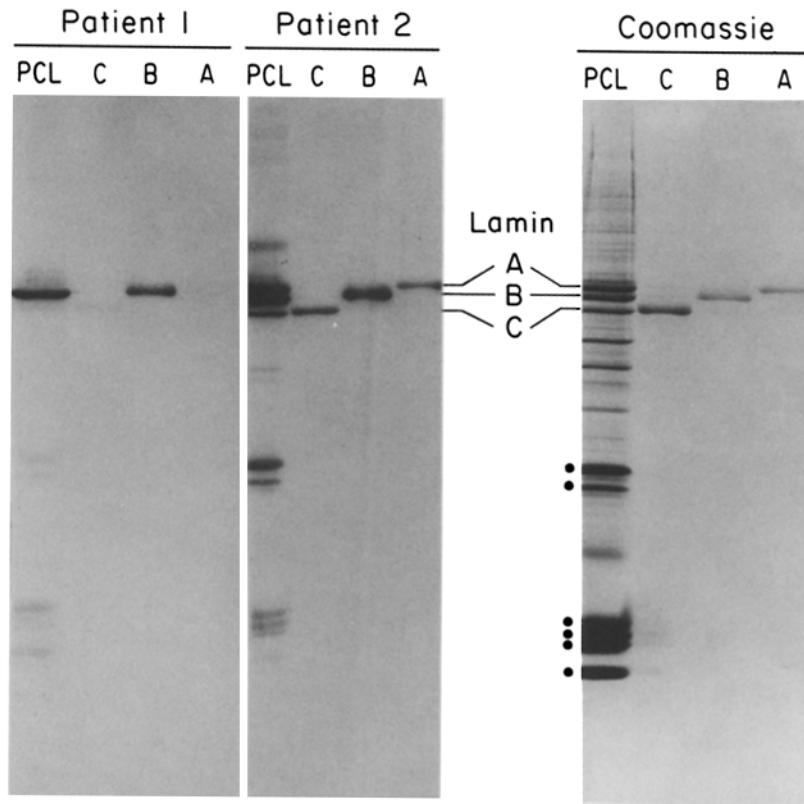


FIGURE 4. Western blots of highly purified lamin fractions. The PCL fraction was isolated from rat liver nuclei, and lamin A, B, and C-enriched fractions were obtained (14). The far right panel shows a Coomassie blue-stained gel of the PCL fraction, and lamin C, B, and A-enriched fractions. Similar gels were blotted onto nitrocellulose and probed with patient 1 or patient 2 sera, each at a dilution of 1:500. Patient 1 serum reacted strongly with lamin B, but only weakly with lamins A and C. Patient 2 serum also reacted strongly with lamin B, but displayed substantial reactivity with lamins A and C, as well.

TABLE I  
*Reactivity of Human Autoantibodies with Components of  
the Nuclear Lamina*

| Patient | Diagnosis | Anti-nuclear<br>lamina titer* | Specificity<br>(western blots) |                   |
|---------|-----------|-------------------------------|--------------------------------|-------------------|
|         |           |                               | Lamin B                        | Lamins A<br>and C |
| 1       | SLE       | 1:6,250                       | ++                             | +/-               |
| 2       | SLE       | 1:36,250                      | ++                             | +                 |
| 3       | SLE       | 1:1,250                       | ++                             | +/-               |
| 4       | SLE       | 1:1,250                       | ++                             | +/-               |

\* Titer of antibodies to nuclear lamina by immunofluorescence using HEP-2 cells.

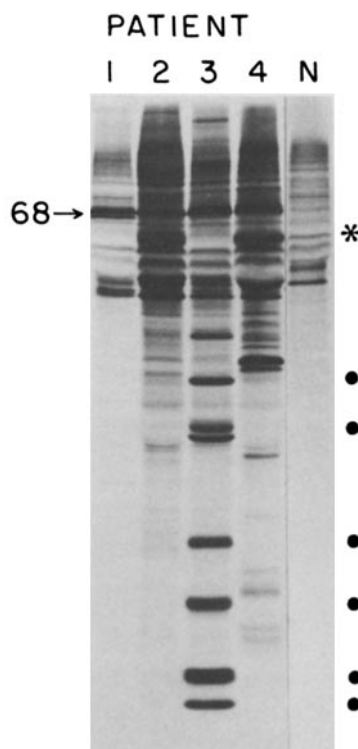


FIGURE 5. Immunoprecipitation of lamins. K562 cells were metabolically labeled with [ $^{35}$ S]-methionine and treated with colcemid to enrich for mitotic cells. Cleared cell lysate was immunoprecipitated with 10  $\mu$ l of sera from patients 1–4, or with normal human serum (N). Sera from patients 1–4 each immunoprecipitated heavily labeled proteins at ~68 kD (arrow). Serum from patients 2 and 4 immunoprecipitated a prominent protein at ~60 kD (\*), which is the Ro antigen. Serum from patient 3 immunoprecipitated protein components of U1, U2, U4, U5, and U6 snRNPs (dots) with molecular masses of ~33, 25 (doublet), 19, 16, 12 (doublet), and 9 kD, respectively.

blot analysis. All of the sera reacted with lamins A, B, and C, but reactivity with lamins A and C was detectable in only trace amounts in three of the four sera. The titers of antibodies to the nuclear lamina in the sera were determined by indirect immunofluorescence using extracted HEP-2 cell nuclei, and are also shown in Table I. These ranged from 1:1,250 to 1:36,250, indicating that the sera contain large amounts of anti-nuclear lamina activity.

*Immunoprecipitation of Lamins.* The nuclear lamina is reversibly depolymerized during mitosis (7). Because the depolymerized lamins are soluble in physiologic buffers, we enriched for mitotic cells by treating with colcemid, and then immunoprecipitated [ $^{35}$ S]methionine-labeled extracts of these cells with the four sera (Fig. 5). Each serum immunoprecipitated prominent bands at ~68 kD (Fig. 5, arrow). Serum from patient 2 also immunoprecipitated less strongly labeled bands at ~70 and 60 kD; the 60 kD band was partially obscured by a strongly labeled band migrating slightly ahead of it. The more rapidly migrating 60 kD protein (Fig. 5, asterisk) is the Ro antigen (30), which was also immunoprecipitated by serum from patient 4. In addition to the ~68 kD bands, serum from patient



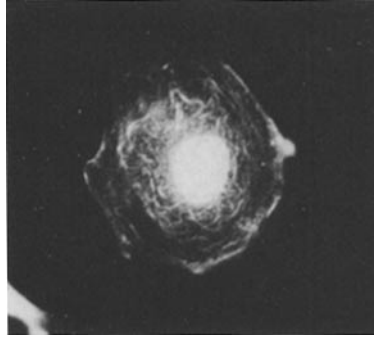


FIGURE 6. Indirect immunofluorescence staining of PTK-2 cells using patient 1 serum. Patient 1 serum at a dilution of 1:20 was used to stain methanol-fixed PTK-2 cells. In addition to nuclear rim staining, the serum displayed prominent staining of cytoplasmic filaments.

3 also immunoprecipitated protein components of U1, U2, U4, U5, and U6 snRNPs (29), ranging from ~9–33 kD (Fig. 5, *dots*). The additional reactivities present in sera from patients 2–4 were confirmed by immunoprecipitation of  $^{32}\text{P}$ -labeled small RNAs as described by Lerner and Steitz (31) (not shown). In contrast to the results obtained with sera from patients 2–4, the immunoprecipitate using patient 1 serum contained the prominent ~68 kD bands, but was otherwise nearly indistinguishable from the immunoprecipitate using normal human serum (Fig. 5, *N*), indicating that serum from patient 1 was the most nearly monospecific for lamin B. It is also notable that each of the four sera consistently immunoprecipitated two closely migrating proteins at ~68 kD from K562 cell extracts (Fig. 5). The significance of this observation is unclear at present. These may represent two different lamin B proteins (32), a precursor of lamin B and mature lamin B, or partially degraded lamin B. Alternatively, the slightly different mobilities may reflect the different degrees of phosphorylation of lamin B in mitotic versus interphase cells (7), or else the presence of coexisting autoantibodies reactive with an additional (nonlamin) 68 kD protein.

*Reactivity of the Sera with Filaments.* Lamins A and C are homologous to intermediate filament proteins (13, 14), and protein sequencing experiments suggest that lamin B is also related to intermediate filaments (N. Chaudhary, unpublished observation). We therefore tested the sera for autoantibodies reactive with cytoplasmic filaments of PTK-2 cells. Two of the sera (from patients 1 and 3) reacted with cytoplasmic filaments by indirect immunofluorescence at a dilution of 1:20 but not at 1:50 (Fig. 6). The staining pattern was unaffected by pretreatment of the cells with colcemid for 12 h (21), suggesting that the antibodies may be specific for prekeratins rather than vimentin (33).

### Discussion

Sera from patients with SLE contain autoantibodies reactive with a variety of nuclear structures (29). Some of these sera react with the nuclear periphery, producing what has been termed rim-pattern immunofluorescence (18, 19). Previously, this pattern of immunofluorescence was attributed to anti-DNA reactivity. In the present studies, we have examined the specificities of four sera

that displayed typical rim-pattern immunofluorescence. Although each of the sera contained high levels of anti-DNA antibodies (our unpublished observation), indirect immunofluorescence combined with a cell fractionation approach suggested that the rim-pattern immunofluorescence displayed by these sera was most probably related to the presence of autoantibodies reactive with the nuclear envelope (Figs. 1 and 2). Each serum reacted with nuclei in a homogeneous, peripheral pattern that is distinct from the punctate peripheral pattern obtained with antibodies that react with the nuclear pore complexes (26). Serum from patient 1 displayed only a homogeneous peripheral pattern, while the other sera contained autoantibodies to components of small ribonucleoproteins (either U1, U2, U4, U5, and U6 snRNPs, or Ro small cytoplasmic ribonucleoproteins, see Fig. 5) in addition to antibodies to structures at the nuclear periphery. The distribution of the antigen that was recognized by these patients' autoantibodies in interphase and mitotic cells (Fig. 1), as well as its resistance to nonionic detergent, DNase, and 2 M NaCl (Fig. 2) strongly suggested that the autoantibodies reacting at the nuclear periphery might be specific for the nuclear lamina rather than DNA. This possibility was further supported by western blotting (Fig. 3) and immunoprecipitation (Fig. 5).

Using partially purified lamin fractions, the autoantibodies in the sera displayed strong reactivity with lamin B and only minimal reactivity with lamins A and C in three of four cases (Fig. 4). This contrasts with the results of a previous study, in which serum from a patient with linear scleroderma was found to react specifically with lamins A and C, but not with lamin B (20). In view of the fact that lamins A and C are nearly identical proteins (13, 14), it is not surprising that reactivity with both lamins A and C was found in that study.

Although its amino acid sequence is not yet known, peptide maps of lamin B suggest that portions of the molecule may be structurally similar to lamins A and C (2), and several monoclonal antibodies reactive with a conserved region of all three lamins have been obtained (4, 15, 17). There are, however, significant differences in the peptide maps of lamin B on one hand, and lamins A and C on the other. The present study suggests that autoantibodies reactive with the nuclear lamina in three of the four patients with SLE bind almost exclusively to portions of lamin B that are not homologous to lamins A and C. Thus, the major autoantigenic epitopes recognized by these sera from patients with systemic lupus erythematosus lie outside the conserved domains that are shared by each of the different lamins. A minor component of the autoantibodies may, however, be reactive with the conserved domains. The presence in two of the four sera of low levels (1:20 titer) of autoantibodies that react with cytoplasmic filaments (probably prekeratins) may reflect weak reactivity with the homologous  $\alpha$ -helical domains present in both intermediate filament proteins and the lamins (13, 14). Crossreacting antibodies of this type might also provide an explanation for the low levels of anti-lamin A and C activity detectable in these sera (Figs. 3 and 4). We cannot be certain, however, whether the reactivities with intermediate filaments and lamins A and C are due to a single autoantibody species that crossreacts with the  $\alpha$ -helical domains of lamin B, lamins A and C, and intermediate filaments, or whether there are different populations of antibodies in the sera that react with each antigen. Experiments to resolve this issue are in progress.

The reactivity of these sera with various epitopes of the lamins will most easily be examined after molecular cloning of lamin B. Since sera from three of these patients react predominantly with lamin B, and one is nearly monospecific, they may be useful for screening cDNA expression libraries. A similar approach has been used successfully to clone cDNA for lamins A and C (13). In addition, the reactivity of the autoimmune sera with regions that are unique to lamin B may allow these to be used for studies of the biological functions of lamin B. Finally, an understanding of why the autoimmune response to the nuclear lamina is directed mainly against nonconserved regions of lamin B in patients with SLE, but is specific for lamins A and C in linear scleroderma may provide clues to the causes of autoimmune diseases.

### Summary

Sera from four patients with systemic lupus erythematosus containing antibodies that yield nuclear rim staining of HEP-2 cells by indirect immunofluorescence were identified and characterized. Each serum contained autoantibodies reacting strongly with lamin B on western blots. One of the four sera displayed weaker reactivity with lamins A and C, while the other three displayed only minimal reactivity with lamins A and C. Titers of antilamin antibodies ranged from 1:1,250 to 1:36,250. Two of the sera also reacted at a dilution of 1:20 with cytoplasmic filaments of PTK-2 cells, suggesting that a small fraction of the autoantibodies in these sera may bind to  $\alpha$ -helical domains of the lamins that are homologous to those of intermediate filaments. The majority of the antilamin antibodies in these patients' sera are specific for portions of the lamin B molecule that are not homologous to lamins A and C, however. The findings suggest that autoantibodies to the nuclear lamina may, in some instances, be responsible for a rim pattern in the fluorescent antinuclear antibody assay. In addition, autoantibodies to the nuclear lamina in sera of certain patients with systemic lupus erythematosus may be useful for defining the molecular structure and biological functions of lamin B, as well as for studying mechanisms of autoimmunity.

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