Human systemic lupus erythematosus sera contain antibodies against cell-surface protein(s) that share(s) epitope(s) with DNA
(autoimmune disease)

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ABSTRACT In previous work, a murine monoclonal anti-DNA antibody (PME77) with specificity for double-stranded DNA has been found to bind five polypeptides (34, 33, 17, 16, and 14 kDa) that are expressed at the surface of several human cell types involved in lupus pathogenesis. To determine more precisely the nature of the antigens recognized by the PME77 monoclonal antibody, and to release cell-surface-accessible fragments, we used a mild, controlled elastase treatment. We isolated several of these polypeptides by immunofinity chromatography. A polyclonal antibody was prepared by immunizing a rabbit with a mixture of these polypeptides (17, 16, and 14 kDa) adsorbed on nitrocellulose. This antibody was shown to react with 17-, 16-, and 14-kDa polypeptides. This antibody does not bind to double-stranded DNA, suggesting that most of the immunogenic determinants of these polypeptides are not shared by double-stranded DNA. Of six human systemic lupus erythematosus sera tested, all contained antibodies that recognized this cell-surface protein(s) and cross-reacted with double-stranded DNA. We suggest that this protein(s) be called LAMP [lupus-associated membrane protein(s)].

Most lesions observed in murine and human systemic lupus erythematosus (SLE) are considered to be associated with the presence of anti-double-stranded DNA (dsDNA) antibodies that are spontaneously produced in large amounts. The antibodies are believed to be deposited in the form of DNA–anti-DNA immune complexes in many organs (1). It is difficult, however, to produce antibodies to dsDNA by deliberate immunization (2).

We have reported (3, 4) that a murine monoclonal anti-DNA antibody (mAb PME77) with specificity for dsDNA recognizes five polypeptides of 34, 33, 17, 16, and 14 kDa at the surface of several human cell types involved in lupus pathogenesis, including normal human glomeruli, T- and B-lymphoblastoid cell lines, normal human erythrocytes, normal platelets, and rat neuronal tissue.

We now report the isolation of several of these polypeptides by immunofinity chromatography and preparative gel electrophoresis, as well as their use for the production of a rabbit polyclonal antibody. Finally, the detection of antibodies against these polypeptides in human and murine lupus erythematosus sera is shown.

MATERIALS AND METHODS

Sera. Sera were obtained from six SLE patients. Sera were heated for 30 min at 56°C to inactive complement and were stored at −20°C. Four normal human sera were used as controls.

Cell Lines. The human lymphoblastoid cell line Raji (5) was provided by G. Klein (Karolinska Institutet). The mouse erythroleukemic cell line IW32 (6) was kindly provided by N. Casadevall and O. Müller. Cell lines were maintained as described (5).

Murine mAb. Culture supernatant was obtained from hybridoma PME77. Hybridoma PME77 resulted from fusion of a nonsecreting myeloma line (P3X63Ag8.653) and (NZB × NZW)F1 spleen cells. PME77 mAb is an IgG2b, κ-chain antibody that is specific for dsDNA (7, 8).

Elastase Treatment of Raji and IW32 Cells. Ten million viable Raji or IW32 cells were incubated in 10 ml of RPMI 1640 medium with 200 μCi of [35S]methionine (800 Ci/mmole; 1 Ci = 37 GBq) for 4 hr. After this period, cells were centrifuged at 200 × g at 4°C and resuspended in 1 ml with elastase (Merck; final concentration 10 μg/ml) in 0.15 M NaCl/50 mM Tris Cl, pH 8.0. After 7 min on ice, cells were separated from surface proteins released by elastase, by centrifugation at 200 × g for 7 min. The “elastase supernatant” was kept and analyzed.

Immunoreplica Analysis of the Elastase Supernatant. An aliquot of the supernatant described above was analyzed for its polypeptide composition after NaDodSO4/PAGE (9) in slab gels. After electrophoresis, the immunoreplica technique was carried out as described by Burnette (10) and modified by Coudrier et al. (11).

Immunoprecipitation Analysis of the Elastase Supernatant. The elastase supernatant was incubated with PME77 mAb for 18 hr at 4°C. Immune complexes were precipitated by incubation with rabbit anti-mouse IgG (20 μg) for 30 min at room temperature and protein A-Sepharose 4B (40 μl, Pharmacia) under the same conditions. The samples were washed seven times in 190 mM NaCl/50 mM Tris Cl, pH 7.4/6 mM EDTA/2.5% (vol/vol) Triton X-100 and subjected to NaDodSO4/PAGE. For fluorography, the gel was soaked in ENHANCE (New England Nuclear), dried, and exposed to Kodak XAR-5 film at −80°C for 24 hr.

Purification of Cell-Surface Protein(s) from IW32 Cells. Immunoabsorbant preparation. Culture supernatant of the anti-DNA-secreting hybridoma PME77 was passed through a protein A-Sepharose 4B column. Then the mAb was covalently linked to the protein A by using dimethyl suberimidate as crosslinking agent, as described by Coudrier et al. (12).

Purification procedure. The elastase supernatant was passed through the immunoabsorbant column. The column was washed with 0.5 M NaCl/150 mM phosphate, pH 7.4/0.1% Triton X-100. Bound material was eluted in a buffer containing 0.2 M HCl adjusted to pH 2.2 with glycine. The eluate was neutralized by addition of 1 M Tris.

Gel electrophoresis and immunoprecipitation analysis of the eluted polypeptides. An aliquot of the eluted [35S]methio-

Abbreviations: SLE, systemic lupus erythematosus; mAb, monoclonal antibody; dsDNA, double-stranded DNA.
nine-labeled polypeptides was subjected to NaDodSO₄/PAGE in slab gels and fluorography. In another experiment, an aliquot of these polypeptides was also immunoprecipitated with the PME77 mAb.

Preparation of Rabbit Polyclonal Antibody Against 1W32 Cell-Surface Protein(s). After NaDodSO₄/PAGE and transfer of the separated proteins to nitrocellulose, three polypeptides (17, 16, and 14 kDa) were visualized with Ponceau S according to Coudrier et al. (11). The bands were then excised, soaked in liquid nitrogen, and crushed with a pestle in a mortar cooled in liquid nitrogen. The pulverized nitrocellulose was resuspended in 500 μl of 0.9% NaCl and emulsified with 500 μl of complete Freund’s adjuvant (Difco). This emulsion was used to immunize a rabbit. For the first injection, material was injected into the popliteal lymph node (about 5 μg of protein) and in the back intradermally along the spine (about 5 μg) in several spots. Three weeks later, antigen adsorbed on pulverized nitrocellulose emulsified in incomplete Freund’s adjuvant (Difco) was administered by subcutaneous (5 μg) and intraperitoneal (5 μg) injections. On day 30, nitrocellulose-adsorbed antigen suspended in phosphate-buffered saline (pH 7.4) was injected subcutaneously (10 μg). Blood was collected 1 week later and subsequently every week for 1 month.

Binding of Antibody to Intact Cells. Cell-binding experiments were done with PME77 mAb, rabbit polyclonal antibody, and nonimmune rabbit serum as described previously (7).

Immunoreplica Analysis of Rabbit Polyclonal Antibody and Human SLE Sera. A total extract (3) of 1W32 cells and an aliquot of the elastase supernatant were prepared. After NaDodSO₄/PAGE and electrophoretic transfer of polypeptides, the nitrocellulose sheet was incubated with the polyclonal antibody (diluted 1:100) or with one of the six human SLE sera (diluted 1:50) for 90 min at room temperature. Antigen-antibody complexes were detected by the peroxidase technique (11).

Immunofinity Purification of the Polyclonal Antibody Bound to 17-kDa Band. A microscale affinity purification of antibodies from blots was performed. Several samples of a total extract of 1W32 cells were subjected to immunoreplica analysis as described (3). The 17-kDa bands, specifically recognized by the polyclonal antibody, were located precisely on the filter. Each of these bands was cut out and processed as described (11).

DNA-Binding Capacity of Polyclonal Antibody. This was measured by using a solid-phase radioimmunoassay (8).

RESULTS

Antigens Recognized by PME77 mAb Are Released from Raji Cells by Elastase. We have previously shown the cell-surface accessibility of antigens recognized by PME77 mAb, using proteinase K digestion of intact living cells (3). To determine more precisely the nature of the antigens recognized by the PME77 mAb, and to release cell-surface accessible epitopes, we used several protease treatments. These antigens were found to be very sensitive to papain and trypsin. After digestion with enzyme at 10 μg/ml for 5 min at 4°C, no detectable fragments were recovered in the supernatant. These antigens were removed from the cell surface, since they could not be detected in the cell pellet recovered and analyzed after proteolysis (data not shown). However, when a mild elastase treatment was used, cell-surface-accessible fragments were released and recovered in the supernatant. The largest fragments were obtained and recovered efficiently when elastase was used at pH 8.0 at a concentration of 10 μg/ml for 7 min. A family of polypeptides, most often migrating at 34, 33, 17, 16, and 14 kDa, was usually detected in the elastase supernatant with the PME77 mAb by immunoblotting analysis (Fig. 1). However, a few other bands were sometimes seen in addition to the major ones, depending on the experimental conditions. So far, we have not been able to design an experimental protocol allowing us to avoid the occasional generation of only one large proteolytic fragment or the presence of minor bands of variable intensity (for example, in Fig. 4B, a crossreacting polypeptide at 23 kDa is visible; this species is usually not observed).

Immunoprecipitation Analysis of the Elastase Supernatant. The PME77 mAb specifically immunoprecipitated three polypeptides, of 17, 16, and 14 kDa, from the elastase supernatant (Fig. 2). The absence of polypeptides at 34 and 33 kDa could be explained by proteolysis of this material.
Immunology: Jacob et al.

Fig. 3. Analysis and immunoprecipitation of the immunoaffinity-purified polypeptides from IW32 cells. Polypeptides were visualized by fluorography. Lane a: polypeptides eluted from the PME77 mAb column. Lane b: immunoprecipitation analysis of the eluted polypeptides with PME77 mAb. Lane c: immunoprecipitation analysis of the eluted polypeptides with anti-Golgi mAb. Lane M: standards.

during the long incubation periods and washings imposed by this technique, the epitopes recognized on the 34- and 33-kDa polypeptides may not be accessible on the native form of the antigen but only recognized after NaDodSO₄ denaturation. Conversely, these polypeptides were not immunoprecipitated by the anti-Golgi mAb used as control (13).

Isolation of Cell-Surface Protein(s). At least four major polypeptides, of 43, 17, 16, and 14 kDa, released from the surface of IW32 cells could be recovered by immunoaffinity chromatography with the PME77 mAb crosslinked to protein A-Sepharose 4B. The presence of these polypeptides was demonstrated when an aliquot of the eluted polypeptides was subjected to electrophoresis (Fig. 3, lane a). Conversely, in the same experiment, when the anti-Golgi mAb crosslinked to protein A-Sepharose 4B was used, no polypeptides were nonspecifically trapped on the immunoabsorbent. In another experiment, the eluted polypeptides were immunoprecipitated and analyzed using the PME77 mAb (Fig. 3, lane b). Polypeptides at 17, 16, and 14 kDa were recognized but not that at 43 kDa. This observation is consistent with the notion that the 43-kDa protein recovered in this experiment may be converted into smaller fragments during the immunoprecipitation procedure.

Properties of a Polycional Antibody to Cell-Surface Protein(s) Crossreacting with dsDNA. An antiserum was produced by immunizing rabbits with the three immunologically related polypeptides at 17, 16, and 14 kDa recovered by immunoaffinity chromatography and preparative electrophoresis.

We have previously reported the binding of PME77 mAb to Raji cells (3). Similar cell-binding experiments, with the polyclonal antibody and ¹²⁵I-labeled protein A as a tracer, indicated specific binding to cells (Table 1).

Table 1. Binding of rabbit polyclonal antibody to Raji cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Binding, cpm</th>
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<tbody>
<tr>
<td>None (phosphate-buffered saline)</td>
<td>228 ± 24</td>
</tr>
<tr>
<td>PME77 mAb (100 μg/ml)</td>
<td>8520 ± 529</td>
</tr>
<tr>
<td>Rabbit polyclonal antibody</td>
<td></td>
</tr>
<tr>
<td>Dilution 1:100</td>
<td>5302 ± 412</td>
</tr>
<tr>
<td>Dilution 1:500</td>
<td>2478 ± 223</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td></td>
</tr>
<tr>
<td>Dilution 1:100</td>
<td>1417 ± 221</td>
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<tr>
<td>Dilution 1:500</td>
<td>567 ± 89</td>
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Binding was assayed by use of ¹²⁵I-labeled protein A, as described in ref. 5.

At least four major bands (43, 17, 16, and 14 kDa) were detected by the polyclonal antibody in immunoreplica analysis of a total extract of IW32 cells (Fig. 4A, lane d). The

Fig. 4. (A) Analysis of specificity of rabbit polyclonal antibody. Each lane was loaded with 20 μg (protein) of IW32 cell extract. Lanes a and c: extract of untreated IW32 cells, total protein pattern. Lane b: immunoreplica analysis of extract of untreated IW32 cells, using PME77 mAb. Lane d: immunoreplica analysis of extract of untreated IW32 cells, using rabbit polyclonal antibody diluted 1:100. Lane e: extract of elastase-treated IW32 cells, total protein pattern. Lane f: immunoreplica analysis of extract of elastase-treated IW32 cells, using rabbit polyclonal antibody diluted 1:100. Lane g: immunoreplica analysis of untreated-cell extract, using nonimmune serum diluted 1:100. The doublet seen in lane h, whose position corresponds to >43 kDa, presumably represents a nonspecific interaction with an antigen contained in the cell extract and an antibody present in this normal rabbit serum. Lane M: standards. (B) Affinity purification of the polyclonal antibody from the blotted specific 17-kDa band. Lane a: IW32 cell extract, total protein pattern. Lane b: immunoreplica analysis of IW32 cell extract with the affinity-purified rabbit polyclonal antibody, diluted 1:100. Lane M: standards.
bands at 43 and 17 kDa showed strong reaction, and the two others, weaker reaction. When a nonimmune rabbit serum was used at the same dilution, no specific band was detected (Fig. 4A, lane h). To determine the cell-surface accessibility of the antigen recognized by the polyclonal antibody and to confirm its proteinc nature, live cells were treated with elastase (100 μg/ml). The four polypeptides were removed almost completely (lane f). To test the reactivity of the polyclonal antibody with DNA, the antibody was preincubated with dsDNA (1 mg/ml). The intensities of the four protein bands were not affected (Fig. 4A, lane g). Moreover, the antibody did not bind to dsDNA, as assessed by radioimmunoassay (Table 2). Thus, it appears that the rabbit antisera contains primarily antibodies that recognize epitopes unrelated to dsDNA.

Relationship Between the 43-kDa and the 17-kDa Polypeptides Recognized by the Polyclonal Antibody. A microscale affinity purification of the polyclonal antibody from the blotted specific 17-kDa band was carried out as described in Materials and Methods. A total extract of IW32 cells was then subjected to immunoreplica analysis using the purified antibody. A family of polypeptides, at 43, 34, 17, 16, and 14 kDa, were specifically detected (Fig. 4B), demonstrating that these polypeptides are antigenically related. A 23-kDa band was observed in this particular experiment, presumably due to variation in the experimental conditions.

Immunoreplica Analysis of Human SLE Sera. Immunoblotting of the elastase supernatant, using a human SLE serum (diluted 1:50) as probe detected bands at 34, 17, and 16 kDa (Fig. 5, lane d). When the human SLE serum (diluted 1:30) was preincubated with dsDNA (1 mg/ml), the intensities of the bands at 34 and 17 kDa were strongly reduced, while that of the 16-kDa band was less reduced (lane e). Similar results were obtained with five other human SLE sera, clearly demonstrating the presence of antibodies against this cell-surface protein(s) in human SLE sera. No band was observed when four normal human sera were used (lane g).

**DISCUSSION**

We previously reported that PME77 mAb antibody to dsDNA recognizes five polypeptides, of 34, 33, 17, 16, and 14 kDa, that are exposed at the surface of various human cell types involved in SLE (3, 4). These observations and those of others (14, 15) emphasize the involvement of cell-surface components in murine and human SLE.

In the present work, we found that mild elastase treatment of human lymphoblastoid (Raji) cells releases at least five major polypeptide fragments, of 34, 33, 17, 16, and 14 kDa, that can be recognized by the PME77 mAb in immunoreplica analysis. We also obtained four major polypeptides, of 43, 17, 16, and 14 kDa, expressed at the surface of murine erythroleukemia (IW32) cells, by immunoaffinity chromatography. Of these, all but the 43-kDa polypeptide can be reimmunoprecipitated by the PME77 anti-DNA mAb. We used a mixture of the 17-, 16-, and 14-kDa polypeptides, released from the surface of IW32 cells by mild elastase treatment, to elicit a rabbit polyclonal antiserum. We used a mixture of the three polypeptides because we assumed that they are derived from a common precursor. The observation that antibody purified by binding to the isolated 17-kDa polypeptide reacts with the 34- and 43-kDa polypeptides is consistent with this assumption. However, the structural relationship of the polypeptides remains to be established. The polyclonal antibody does not react with dsDNA, showing that the major epitopes of the polypeptides are not shared by dsDNA. We suggest that this lupus-associated membrane protein(s) be termed LAMP and that it may act as a potent immunogen instead of DNA itself when the physiopathological conditions associated with the development of SLE are encountered. Antibodies against cell-surface protein(s) and crossreacting with dsDNA were detected in six out of six human SLE sera. These data have recently been confirmed in murine lupus. Moreover, immunoglobulins eluted from kidneys of autoimmune MRL/lpr/lpr mice react specifically with the lupus-associated membrane protein(s) described above.

Taken together, these results indicate that the lupus-associated membrane protein(s) may trigger an autoimmune response similar to those reported for the acetycholine receptor in myasthenia gravis (16, 17). We suggest that this protein(s) may play a pathogenic role in the onset of SLE.

**Note Added in Proof.** We have analyzed the elastase supernatant obtained after mild proteolysis of lymphocytes from lupus mice (MRL/lpr/lpr). A single polypeptide (55 kDa) was found by immunoblotting analysis. The same peptide was also released from the cell surface of these lupus mice by using papain or trypsin. This is in contrast with our observations with normal lymphocytes. Indeed, a different fragment can be removed by protease cleavage in lupus mice if the primary structure of the lupus-associated membrane protein is changed slightly or via its association with other cell-surface proteins. Such a modified protein could trigger the immune response and the onset of SLE.

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