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Immunology

Altered cell-surface protein(s), crossreactive with DNA, on spleen cells of autoimmune lupic mice

(systemic lupus erythematosus)

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ABSTRACT **A murine monoclonal anti-DNA antibody, PME77, with specificity for double-stranded DNA, has previously been shown to react with a protein(s) present at the surface of such cells involved in lupus pathogenesis as human glomeruli, T and B lymphocytes, erythrocytes, and platelets. Mild elastase treatment of lymphoid cells from non-autoimmune (CBA/ca or BALB/c) mice releases a series of crossreactive polypeptides (34, 33, 17, 16, and 14 kDa) recognized by PME77. These polypeptides are not formed after treatment of the same cells with papain or trypsin. When lymphoid cells from autoimmune [MRL-*lpr/lpr* or (NZB × NZW)F_1 B/W] mice are treated with elastase, trypsin, or papain, PME77 detects, in all supernatants, a single polypeptide of about 55 kDa. Antibodies present in the sera of autoimmune MRL-*lpr/lpr* and B/W mice and IgG eluted from kidneys of MRL-*lpr/lpr* mice react with the same polypeptides. Neither sera nor eluted IgG of normal BALB/c mice react with these polypeptides. These results suggest that an altered cell-surface protein(s), which we call LAMP [for lupus-associated membrane protein(s)], may be involved in lupus pathogenesis.**

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, especially the kidneys (1). It is difficult, however, to produce antibodies to dsDNA by deliberate immunization (2). To study these antibodies, we analyzed the properties of PME77, a monoclonal antibody (mAb) with anti-dsDNA specificity, prepared from spleen cells of an autoimmune (NZB × NZW)F_1 B/W mouse. mAb PME77 has been shown to react with a protein(s) present at the surface of a variety of normal cell types known to play a role in lupus pathogenesis (3, 4). Furthermore, it has been shown (13) that the ectodomain of this membrane bound protein(s) can be released as a series of polypeptides by mild treatment with elastase. However, this protein(s) appears to be destroyed by mild treatment of the same cells with trypsin or papain.

In this paper we show that the protein(s) present at the surface of cells from autoimmune mice (MRL-*lpr/lpr* and B/W) displays different properties with respect to protease treatment. Furthermore, IgG with the same specificities as PME77 can be detected in the sera of MRL-*lpr/lpr* and B/W lupus mice as well as in the kidneys of MRL-*lpr/lpr* lupus mice.

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MATERIALS AND METHODS

Mice. MRL-*lpr/lpr* mice (16 weeks old) were kindly provided by J. L. Guenet (Institut Pasteur, Paris). B/W mice were the offspring of NZB females and NZW males and were 6 months old at the onset of experiments. NZB and BALB/c mice were obtained from the CSEAL Centre National de la Recherche Scientifique (Orléans, la Source, France). CBA/ca mice were bred in the laboratory. NZW mice were kindly provided by P. Verroust (Hôpital Tenon, Paris).

Serum. MRL-*lpr/lpr*, B/W, and BALB/c sera were heated for 30 min at 56°C to inactivate complement and then stored at −20°C.

Murine mAb. The hybridoma secreting anti-DNA antibody PME77 was obtained after fusion between a nonsecreting myeloma line (P3X63Ag8.653) and B/W spleen cells. PME77 mAb was shown to be an IgG2b, κ-chain antibody specific for dsDNA (5, 6).

Elution of Immunoglobulins from Kidneys of MRL-*lpr/lpr* Autoimmune Mice and BALB/c Mice. Eight MRL-*lpr/lpr* mice and eight BALB/c mice were perfused with 20 ml of 0.9% NaCl and their kidneys were frozen in nitrogen liquid. Sixteen kidneys of MRL-*lpr/lpr* mice and 16 kidneys of BALB/c mice were pooled, cut into small pieces while still frozen, suspended in phosphate-buffered saline (PBS:0.15 M NaCl/150 mM phosphate, pH 7.2), and then homogenized in a chilled Waring Blendor at 0°C. The homogenate was washed at 4°C with 500 ml of PBS by centrifugation at 4000 × *g*. This washing procedure was repeated three times, and the pellet was resuspended and incubated for 2 hr at 37°C in 20 volumes of 0.02 M citrate buffer at pH 3.2 (7). After elution, the suspension was centrifuged (4000 × *g* for 30 min), and the eluates were neutralized (pH 7.0) with 1 M Na_2HPO_4, dialyzed against PBS overnight, concentrated, adjusted to 2 ml, and stored at −20°C. The optical density at 280 nm was approximately 3.7 for MRL-*lpr/lpr* and 1.1 for BALB/c.

ELISA. The assay for the detection of eluted IgG from kidneys was performed as described (8). In brief, microtest plates (Luxlon, CEB, Nemours, France) were coated with goat anti-mouse IgG antiserum (Cappel Laboratories, Cochranville, PA) diluted to a concentration of 2 μg/ml in borate buffer (pH 8.2) containing 0.2 M NaCl. After an overnight incubation at 4°C, the plates were washed in PBS containing 1% bovine serum albumin at room temperature for 1 hr. Eluted IgG was then added. After the plates were incubated for 1 hr at 37°C and then washed three times with PBS containing 0.1% (vol/vol) Tween 20, 100 μl of β-galactosidase-labeled goat anti-mouse IgG antiserum (Biosis, Compiègne, France) diluted 1:500 was added and left to incubate 1 hr at 37°C. Plates were then washed three times in PBS/Tween 20 before the

Abbreviations: dsDNA, double-stranded DNA; mAb, monoclonal antibody; SLE, systemic lupus erythematosus.

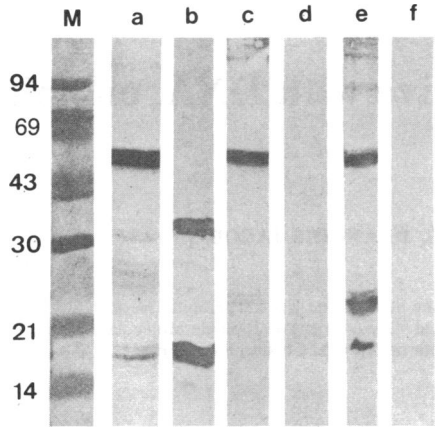

FIG. 1. Immunoreplica analysis, using mAb PME77, of the elastase supernatant (lanes a and b), the trypsin supernatant (lanes c and d), and the papain supernatant (lanes e and f) from spleen cells of MRL-*lpr/lpr* (lanes a, c, and e) and CBA/ca (lanes b, d, and f) mice. Lane M: standard proteins used as molecular mass markers (values in kDa at left).

addition of 100 μl of *o*-nitrophenyl β-D-galactopyranoside (Sigma) as substrate for 30 min at 37°C.

Cell Lines. Mouse erythroleukemic cell line IW32 was kindly provided by N. Casadevall and O. Muller (Hôpital Cochin, Paris) (9). The lymphoblastoid B-cell line Raji was obtained as described (3). Cell lines were maintained in suspension culture in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (5%), penicillin (100 units/ml), and streptomycin (100 μg/ml), all from GIBCO.

Gel Electrophoresis and Immunoreplica Analysis of IW32 Cell Extract. The extract of IW32 cells was prepared as described (3). $NaDodSO_4$/polyacrylamide gel electrophoresis (10) was done in a slab gel. After electrophoresis, the immunoreplica technique was carried out as described by Burnette (11) and modified by Coudrier *et al.* (12). After electrophoretic transfer of polypeptides, the nitrocellulose sheet was incubated with the supernatant of PME77 mAb-secreting hybridoma or with eluted IgG of MRL-*lpr/lpr* mice for 90 min at room temperature. Antigen–antibody complexes were detected by a peroxidase technique (3).

Comparison of Immunoreplica Analysis Before and After Elastase Treatment of Living Cells. Two total cellular extracts of IW32 cells were prepared as described above. One was from cells that had been treated with elastase (100 μg/ml) for 1 hr at 0°C; the second extract was from cells not subjected to elastase digestion. In both cases, cell viability was at least 95%.

Elastase, Trypsin, and Papain Treatment of Cells. One hundred million viable spleen cells obtained from CBA/ca, BALB/c, MRL-*lpr/lpr*, or B/W mice (after red-cell lysis) were treated in 1 ml of 0.15 M NaCl/50 mM Tris Cl, pH 8.0, for 7 min with elastase (final concentration, 10 μg/ml; Merck) on ice. One hundred million viable spleen cells from CBA/ca, MRL-*lpr/lpr*, or B/W mice were treated in 1 ml of PBS (pH 7.5) for 7 min with trypsin (final concentration, 10 μg/ml; Boehringer Mannheim) on ice. One hundred million viable spleen cells from CBA/ca or MRL-*lpr/lpr* mice were treated in 1 ml of PBS/5 mM EDTA/2.5 mM cysteine, pH 7.5, for 7 min with papain (final concentration, 10 μg/ml; Boehringer Mannheim) on ice.

Cell surface proteins released by these mild protease treatments were separated from the cells by low-speed centrifugation (200 × *g*, for 7 min). Aliquots of the "elastase supernatant," the "trypsin supernatant," and the "papain supernatant" were subjected to $NaDodSO_4$/polyacrylamide slab gel electrophoresis followed by immunoreplica analysis.

DNA-Binding Capacity. The DNA-binding capacity of sera and hybridoma supernatants was measured by a solid-phase radioimmunoassay (5).

RESULTS

Polypeptides Released by Protease Treatment from the Surface of Cells of Non-Autoimmune or Autoimmune Mice. We first compared the polypeptides recognized by mAb PME77 after treatment of cells from non-autoimmune (CBA/ca and BALB/c) or autoimmune (MRL-*lpr/lpr* and B/W) mice with proteases. Cells were treated as described in *Materials and Methods* with either elastase, trypsin, or papain. After centrifugation, the supernatants were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis followed by transfer to nitrocellulose and immunodetection with mAb PME77 (Fig. 1). As previously shown (13), in the supernatant derived from "normal" cells, PME77 detected a series of five polypeptides of 34, 33, 17, 16, and 14 kDa. However, PME77 detected no polypeptide either in the supernatant or in the pellet of the same cells treated with trypsin or papain, a result indicating a high sensitivity of the surface protein(s) to protease digestion.

In contrast, when spleen cells from autoimmune MRL-*lpr/lpr* and B/W mice were treated in a similar way, a major polypeptide of 55 kDa was detected by PME77 in the supernatants obtained with any of the three proteases. This result indicates that the protein(s) expressed at the surface of the cells of autoimmune mice displays different sensitivity to proteases than those observed in non-autoimmune mice.

Sera from Autoimmune Mice Recognize the Same Polypeptides as mAb PME77. If the polypeptides released by protease treatment were involved in lupus pathogenesis, one would expect to find in the sera of autoimmune, but not of normal mice, antibodies with specificity similar to that of PME77. We investigated this question by immunoreplica analysis of sera from autoimmune and normal mice. As shown in Fig. 2,

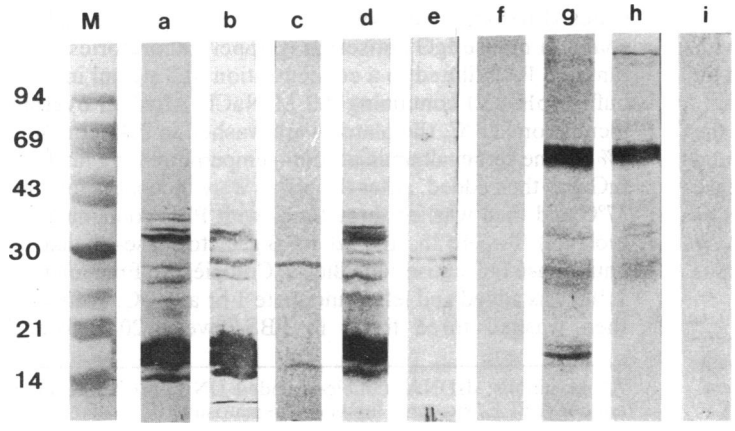

FIG. 2. Immunoreplica analysis, using sera from autoimmune (MRL-*lpr/lpr* and B/W) and control (CBA/ca) mice, of the elastase supernatant from IW32 cells and spleen cells of MRL-*lpr/lpr* mice. Lanes a–f: elastase supernatant from IW32 cells, analyzed with mAb PME77 (lane a), a pool of MRL-*lpr/lpr* sera (IgG concentration, 400 μg/ml; lane b), a pool of MRL-*lpr/lpr* sera preincubated with dsDNA (lane c), an individual B/W serum (IgG concentration, 400 μg/ml; lane d), an individual B/W serum preincubated with dsDNA (lane e), and an individual CBA/ca serum (IgG concentration, 400 μg/ml; lane f). Lanes g–i: elastase supernatant from spleen cells of MRL-*lpr/lpr* mice, analyzed with mAb PME77 (lane g), pooled MRL-*lpr/lpr* sera (lane h), and an individual CBA/ca serum (lane i). Lane M: molecular mass markers.

Table 1. DNA-binding capacity measured by solid-phase radioimmunoassay

Sample*	Binding, cpm†
MRL-*lpr/lpr* serum	3018 ± 325
B/W serum	3408 ± 270
Normal (BALB/c) serum	527 ± 74
PME77 hybridoma supernatant	5378 ± 285
Anti-Golgi hybridoma supernatant‡	214 ± 73

*Serum samples were diluted 1:100.
†Values are mean ± SEM.
‡Anti-Golgi mAb in ref. 15.

the same series of five polypeptides was detected in the elastase supernatant from cells of normal mice by mAb PME77 (lane a) and by pooled sera of MRL-*lpr/lpr* mice (lane b), three individual MRL-*lpr/lpr* sera (data not shown), and four B/W sera (one shown in lane d). No reaction was observed with sera from two CBA/ca mice, used as controls (one shown in lane f). The reaction was strongly reduced when the MRL-*lpr/lpr* and B/W sera were preincubated with 1 mg of dsDNA per ml (lanes c and e). The same major polypeptide of 55 kDa was recognized in the elastase supernatant of spleen cells from MRL-*lpr/lpr* mice by the mAb and by sera from MRL-*lpr/lpr* mice (lanes g and h), but no reaction was observed with CBA/ca sera as control (lane i).

IgG Eluted from Kidneys of Autoimmune MRL-*lpr/lpr* Mice, but Not of Normal BALB/c Mice, Recognizes the Same Series of Polypeptides at the Surface of Normal Cells as mAb PME77. SLE pathogenesis is often considered as being linked to the presence of DNA–anti-DNA immune complexes deposited in kidney glomeruli. However, while the presence of IgG in the kidney of patients has been demonstrated, that of extracellular DNA has not been clearly established (14). Therefore, we analyzed the properties of the IgG eluted from kidneys of autoimmune MRL-*lpr/lpr* mice.

The amount of IgG eluted was measured by ELISA. Whereas 185 μg was recovered from autoimmune MRL-*lpr/lpr* mice, only 1.8 μg was recovered from BALB/c mice. The antibodies eluted from MRL-*lpr/lpr* mice, but not those from BALB/c mice, were found to bind to dsDNA, by solid-phase radioimmunoassay (Table 1). As shown in Fig. 3, five major bands, at 34, 33, 17, 16, and 14 kDa, were detected with mAb PME77 (lane b) and with eluted IgG of MRL-*lpr/lpr* mice (lane d), in a total cellular extract of IW32 cells. To determine the cell-surface accessibility of the antigen recognized by eluted IgG of MRL-*lpr/lpr* mice and to confirm its protein nature, live IW32 cells were treated with elastase (100 μg/ml). This treatment almost completely removed the immunoreactive polypeptides (lane g). When eluted MRL-*lpr/lpr* IgG preincubated with dsDNA (1 mg/ml) and eluted IgG of BALB/c mice were used as controls, the reaction was strongly reduced (lanes e and h).

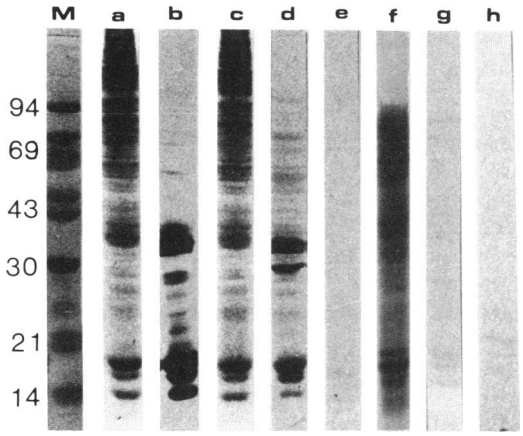

FIG. 3. Immunoreplica analysis, using IgG eluted from kidneys of MRL-*lpr/lpr* mice, of IW32 cell extract (20 μg of protein per lane). Lanes a and c: untreated IW32 cell extract, total protein pattern (Ponceau S staining). Lane b: untreated IW32 cell extract, immunoreplica analysis with mAb PME77. Lane d: untreated IW32 cell extract, immunoreplica analysis with eluted IgG of MRL-*lpr/lpr* mice. Lane e: untreated IW32 cell extract, immunoreplica analysis with eluted IgG of MRL-*lpr/lpr* mice, preincubated with dsDNA. Lane f: elastase-treated IW32 cell extract, total protein pattern. Lane g: elastase-treated IW32 cell extract immunoreplica analysis with eluted IgG of MRL-*lpr/lpr* mice. Lane h: untreated IW32 cell extract, immunoreplica analysis with eluted IgG of BALB/c mice.

DISCUSSION

The first observation on the double specificity—anti-DNA and anti-protein—of IgG, involved in the SLE pathogenesis, was made with a mAb prepared using spleen cells of an autoimmune B/W mouse. Here we show that circulating antibodies of autoimmune B/W and MRL-*lpr/lpr* mice, as well as IgG present in kidneys of MRL-*lpr/lpr* mice, exhibit similar properties. [Such antibodies are also present in sera of 25 SLE patients (even two without anti-DNA antibodies) (data not shown).] These antibodies are not detected, however, either in the sera or in the kidneys of normal mice. Since the presence of extracellular DNA in the kidney of autoimmune animals has not been demonstrated (14), it is at least reasonable to consider, as an alternative, the role of a protein rather than DNA in SLE pathogenesis.

This point is strengthened by the finding that the protein(s) expressed at the surface of cells from autoimmune B/W and MRL-*lpr/lpr* mice displayed altered sensitivity to proteases. This could result from a change either in the structure of the protein(s) itself or in its environment in the membrane [such as, for example, circulating autoantibodies against the lupus-associated membrane protein(s) (LAMP)]. It is clear, however, that, whatever their origin, such modifications might result in the appearance of a non-self antigen and therefore elicit an autoimmune response. We suggest that a similar situation may apply to other autoimmune diseases (for instance, to the acetylcholine receptor in myasthenia gravis).

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