A monoclonal anti-double-stranded DNA autoantibody binds to a 94-kDa cell-surface protein on various cell types via nucleosomes or a DNA–histone complex

(autoimmune/disease)

LAURENT JACOB^{*†}, JEAN-PAUL VIARD^{*}, BÉNÉDICTE ALLENET^{*}, MARIE-FRANCE ANIN[‡],
FOUED B. H. SLAMA^{*}, JOËL VANDEKERCKHOVE[§], JACQUELINE PRIMO^{*}, JUDITH MARKOVITS[‡],
FRANÇOIS JACOB[¶], JEAN-FRANÇOIS BACH^{*}, JEAN-BERNARD LE PECQ[‡], AND DANIEL LOUVARD^{||}

^{*}Institut National de la Santé et de la Recherche Médicale Unité 25 and Centre National de la Recherche Scientifique Laboratoire Associé 122, Hôpital Necker, 75015 Paris, France; ^{||}Département de Biologie Moléculaire, Unité de Biologie des Membranes and [†]Unité de Génétique Cellulaire, Institut Pasteur, 75015 Paris, France; [‡]Centre National de la Recherche Scientifique Laboratoire Associé 147, Villejuif, France; [§]Riksuniversiteit Gent, Laboratorium Genetika, B-9000 Gent, Belgium; and [†]Département de Médecine Interne, Hôpital Cochin, 75014 Paris, France

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ABSTRACT A crude supernatant of hybridoma secreting a monoclonal anti-double-stranded (ds)DNA antibody (PME77 mAb), used to stain fibroblasts (CVI cells) in immunofluorescence, gives a punctuated staining of variable intensity. We had suggested that anti-DNA antibodies bind to cell-surface protein(s) of several cells. When the mAb of this crude supernatant was purified on a dsDNA-cellulose column and a histone-Trisacryl column, the mAb no longer bound to the cell surface. Only when dsDNA plus purified histones was added to the purified antibody did the immune complex strongly and uniformly stain again the cell surface of CVI cells. No significant staining was observed if either DNA or histones were omitted. A single 94-kDa protein from membrane fractions of CVI, Raji, and RINm cell lines was visualized in immunoblots when mAb–DNA–histone complexes were applied to the nitrocellulose strips. No polypeptide was seen if one component was omitted. This 94-kDa protein behaved like a plasma membrane protein since it required the use of detergent to be solubilized and was quantitatively recovered in the Triton X-114 detergent-rich phase. Moreover, a brief treatment of living cells with trypsin cleared off this protein. Purified nucleosomes could be substituted to DNA–histone complexes, giving rise to identical results. Finally, purified polyclonal anti-DNA antibodies from sera of systemic lupus erythematosus patients labeled a 94-kDa protein provided that DNA–histone complexes were added. Anti-DNA autoantibodies could be pathogenic when they are bound to nucleosomes.

Antinuclear antibodies are characteristic of murine and human systemic lupus erythematosus (SLE). Anti-double-stranded (ds)DNA antibodies are the major autoantibodies in SLE (1, 2) and are responsible for tissue lesions through the deposition of DNA–anti-DNA immune complexes (2).

Monoclonal anti-DNA antibodies (mAbs) against several antigens crossreact with a variety of unrelated molecules, such as phospholipids (3) or proteoglycans (4). It is possible that other target antigens rather than DNA may be involved in SLE. We have previously produced an anti-DNA mAb in autoimmune B/W mice, PME77, specific for dsDNA (5, 6). Using the cell-culture supernatant of PME77 mAb, we have observed in immunofluorescence a fine uniform punctate pattern at the surface of glomerular epithelial cells in culture, codistributed with coated pits stained with an anti-clathrin antibody (7). This pattern was unaffected by DNase I treatment and abolished by proteinase K treatment, suggesting

that the antigen recognized at the surface of cells is of a proteic nature. We have analyzed the antigen(s) recognized by PME77 mAb at the surface of cells in immunoblots. After mild treatment of living target cells with elastase, five polypeptides were visualized in the supernatant (8, 9). We assumed, as a consequence of the strategy used, that these polypeptides were distributed at the cell surface. We suggested that the binding of anti-DNA antibodies to the cell surface, either directly or indirectly via immune complexes, could be pathogenic and elicit tissue lesions in SLE (9). Finally, several groups have suggested that histones (10), DNA–histone complexes (11), or a putative DNA receptor (12) are present at the surface of leukocytes or monocytes. In this paper, we present evidence that the purified PME77 mAb binds to a 94-kDa cell-surface protein via nucleosomes or a DNA–histone complex.

MATERIALS AND METHODS

Cell Lines. Rat pancreas islet cell tumor line RINm (13), human Raji lymphoblastoid cell line, and monkey fibroblasts (CVI cells) were obtained and maintained as described (14).

Murine mAb. The hybridoma PME77 secreting anti-DNA was isolated after fusion between a nonsecreting myeloma line (P3X63Ag8.653) and (NZB × NZW)F₁ B/W spleen cells. The selection of hybrid, cloning and subcloning of the line, purification of the antibody, characterization of its class and subclass, and its specificity have all been described in detail (5, 6). PME77 mAb has been shown to be an IgG2b κ -chain antibody and specific for dsDNA (6).

Preparation of the Purified Membrane Fraction. The membrane fraction was prepared as described (15). Ten million viable cells were washed three times in phosphate-buffered saline (PBS). Cells were suspended in 7 ml of buffer A containing 10 mM Tris-HCl, 10 mM CaCl₂, and 0.25 M sucrose adjusted to pH 7.4. Homogenization was performed in a Dounce homogenizer in the presence of protease inhibitors (phenylmethylsulfonyl fluoride and inhibitor cocktail). The homogenate was centrifuged at 1000 × *g* for 20 min. Then, the supernatant was centrifuged at 20,000 × *g* for 60 min. The pellet was resuspended in PBS (pH 7.4). This fraction corresponds to an enriched preparation in plasma membrane proteins.

Extraction of the Purified Membrane Fraction by Triton X-114 (TX-114). The fraction prepared as described above was subjected to solubilization with 1% TX-114 (Fluka) (16)

in PBS at 4°C, centrifuged on a 20% cushion sucrose for 1 hr at $100,000 \times g$ at 4°C, and then transferred to a fresh tube at 37°C and incubated for 3 min. After centrifugation for 3 min at 3000 rpm at room temperature, the pellet and the supernatant were kept and analyzed. Hydrophobic membrane proteins were recovered in the detergent phase and the soluble proteins were recovered in the aqueous phase.

Trypsin Treatment. Living RINm cells were treated with trypsin at 50 $\mu\text{g}/\text{ml}$ for 5, 10, and 20 min at 0°C. Cell viability was estimated by trypan blue exclusion assay, which was 98% in all preparations and unchanged at the end of the treatment with trypsin. Cellular extracts were subjected to extraction by TX-114 as described above. We analyzed in immunoblots the detergent phase obtained after trypsin treatment.

DNA-Cellulose Column. Briefly, the supernatant of the PME77 mAb was passed through a DNA-cellulose column (Sigma). After washing in PBS, the PME77 mAb was eluted in 2 M NaCl (pH 8.0).

Histones Covalently Linked to *p*-Nitrophenyl Chloroformate-Activated Trisacryl Beads. The eluted PME77 mAb from the DNA-cellulose column was passed through histones covalently linked to an activated Trisacryl (IBF) column in a PBS buffer. The effluent corresponded to the purified PME77 mAb used in the experiments.

DNA and Histone Preparation. DNA (Boehringer Mannheim) was purified by proteinase K digestion (Boehringer Mannheim) (100 $\mu\text{g}/\text{ml}$ for 1 hr at 4°C) followed by extraction with phenol. Histones were provided by J. Hardin (Yale University) and treated with DNase I (Worthington) dissolved in Hanks' medium containing 10 μM MgCl_2 (200 μg) for 1 hr at 4°C.

Immunoblot Analysis. This was performed as described (8).

Immunofluorescence Assay. CVI cells were subjected to an indirect immunofluorescence procedure. Unpermeabilized cells were fixed with freshly prepared paraformaldehyde solution, incubated with purified PME77 mAb, and stained by a mouse anti-IgG labeled with fluorescein (Biosys, Compiègne, France).

Chromatin and Nucleosome Preparations. Chromatin and nucleosomes were prepared from nuclei of L1210 leukemic cells grown in cell culture as described (17). To analyze the purity of the preparation, 0.8% agarose gel electrophoresis and 3.6% NaDodSO_4 /polyacrylamide gel electrophoresis in ethidium bromide were performed. Only one band was visualized with mononucleosomes. Their purity, confirmed by a Joyce-Loebl Scann, was at least 95%.

ELISA for Anti-DNA and Anti-Histone Antibodies. This was performed as described (18).

RESULTS

To analyze the mechanism by which an anti-DNA antibody could recognize a proteic antigen at the cell surface, we determined the partial sequence of the polypeptides released by elastase and recognized by the mAb in immunoblots. These polypeptides had sequences identical to intact histones (data not shown) and the cell culture supernatant of PME77 mAb also reacted with purified histones in immunoblots (data not shown). This suggested that the binding of the cell surface by PME77 mAb could involve the formation of DNA-histone complexes.

Since the PME77 mAb could have a very high affinity for DNA-histone complexes, free antibodies could not be obtained after conventional purification procedures. We therefore selected the following methods for purification of PME77 mAb anti-DNA antibody. Antibodies were first bound to a DNA-cellulose column. The eluted mAb contained heavy and light chains in NaDodSO_4 /polyacrylamide gel electrophoresis and no histone (data not shown). The eluted mAb was later passed through histones covalently linked to an

Fig. 1. Reaction of PmAb with dsDNA, histones, and dsDNA + histones in ELISA. Δ , DNA (1 $\mu\text{g}/\text{ml}$) + histone (2.5 $\mu\text{g}/\text{ml}$); $\blacksquare$, DNA (1 $\mu\text{g}/\text{ml}$); $\circ$, histone (2.5 $\mu\text{g}/\text{ml}$).

activated Trisacryl column to remove any contaminating DNA.

The effluent obtained is referred to as "purified PME77 mAb" (PmAb). The PmAb reacted with dsDNA (Fig. 1) but did not react with purified histones in immunoblots (see Fig. 4A, lane C) and ELISA (Fig. 1). PmAb plus DNA reacted with purified histones in immunoblots (see Fig. 4A, lane d) and ELISA in contrast to PmAb in the absence of DNA (see Fig. 4A, lane c). Thus, the PmAb was able to form a complex with DNA and histones. The PmAb reacted better with dsDNA plus purified histones than with DNA alone in ELISA (Fig. 1). Since the PmAb reacted with the DNA-histone complex, we tested the possibility that the PmAb also reacts with chromatin and nucleosomes. As shown in Fig. 2, the PmAb bound to chromatin, polynucleosomes, oligonucleosomes, oligonu-

Fig. 2. Reaction of PmAb (15 $\mu\text{g}/\text{ml}$) with dsDNA (Δ), chromatin ($\blacksquare$), polynucleosomes ($\blacktriangle$), oligonucleosomes ($\circ$), and mononucleosomes ($\blacklozenge$) in ELISA.

FIG. 3. Binding of PmAb to cell surface in immunofluorescence using CVI cells. (a) PmAb (20 μ g) + DNA (1 μ g) + histone (5 μ g). (b) PmAb (20 μ g) + mononucleosomes (8 μ g). (c) PmAb (20 μ g).

cleosomes, and mononucleosomes in ELISA. These results (Figs. 1 and 2) suggest that the PmAb has a better affinity for DNA–histone complexes and particularly for mononucleosomes and oligonucleosomes than for DNA alone.

Binding of the PmAb–DNA–Histone Complexes to the Cell Surface. The PmAb did not bind to the cell surface in immunofluorescence assays (Fig. 3c). If, however, dsDNA plus purified histones were added to the PmAb, the immune complexes strongly and uniformly stained the cell surface (Fig. 3a). No significant staining was observed if DNA or histone was omitted. These data demonstrate that the PmAb binds to the cell surface via a DNA–histone complex.

Binding of PmAb–DNA–Histone Complexes to a 94-kDa Protein. Binding of PmAb in the presence or absence of DNA–histone complexes was also probed on the TX-114 detergent-rich phase from purified membrane fractions of RINm cells. A single 94-kDa protein was detected by immunoblotting procedures when PmAb–DNA–histone complexes were applied to the nitrocellulose strips (Fig. 4B, lane c). No polypeptide was seen if DNA was omitted (Fig. 4B, lane a).

A weak reaction was observed in the presence of PmAb–DNA complexes (Fig. 4B, lane b).

The 94-kDa Protein Had the Properties of a Cell-Surface Protein. This 94-kDa protein behaved like a plasma membrane protein since it required the use of detergent to be solubilized and was quantitatively recovered in the TX-114 detergent-rich phase (Fig. 5A, lane b). Moreover, brief treatment of living cells with trypsin, which did not modify cell viability, cleared off this protein showing that it is exposed on the external face of the plasma membrane (Fig. 5B). Immunoblot analysis of the trypsin supernatant in the course of trypsin treatment failed to reveal a tryptic fragment, suggesting that the binding site of nucleosomes on the 94-kDa protein is very sensitive to trypsin. The 94-kDa protein was found on the surface of various cell types: human B-lymphoblastoid Raji cell lines, monkey CVI cells, and rat pancreas islet tumoral cell line RINm.

Binding of the PM77 mAb–Nucleosome Complex to the 94-kDa Protein. When chromatin, polynucleosomes, oligonucleosomes, and purified mononucleosomes were substi-

FIG. 4. (A) Reaction of PmAb with an extract of Raji cells and purified histone, in immunoblots, in the presence or absence of DNA. Lanes: a, reaction of PmAb (20 μ g) with an extract of Raji cells; b, reaction of PmAb (20 μ g) + DNA (1 μ g) with an extract of Raji cells; c, reaction of PmAb (20 μ g) with purified histone (1 μ g); d, reaction of PmAb (20 μ g) + DNA (1 μ g) with purified histone (1 μ g). (B) Binding of PmAb to a 94-kDa protein, in the presence or absence of DNA–histone complexes, in immunoblots. An extraction of the purified membrane fraction from RINm cells by TX-114 was performed. The TX-114 detergent-rich phase was used as antigen in the experiment. Lanes: a, PmAb (20 μ g); b, PmAb (20 μ g) + DNA (1 μ g); c, PmAb (20 μ g) + DNA (1 μ g) + histones (1 μ g). LMW, low molecular weight markers.

FIG. 5. Presence of the 94-kDa protein in the plasma membrane fraction. (A) Detection of the 94-kDa protein in immunoblots after extraction of the purified membrane fraction from RINm cells by TX-114. Binding of PmAb (20 μ g) + DNA (1 μ g) + histone (1 μ g), before trypsin treatment, in immunoblots. Lanes: a, TX-114 supernatant before the separation phase; b, TX-114 detergent-rich phase; c, TX-114 aqueous phase. LMW, low molecular weight markers. (B) Binding of PmAb (20 μ g) + DNA (1 μ g) + histone (1 μ g) after trypsin treatment at a concentration of 50 μ g/ml for 5, 10, and 20 min at 0°C in immunoblots. Lanes: a, TX-114 detergent-rich phase without trypsin; b, TX-114 detergent-rich phase after trypsin treatment (50 μ g/ml for 5 min); c, TX-114 detergent-rich phase after trypsin treatment (50 μ g/ml for 10 min); d, TX-114 detergent-rich phase after trypsin treatment (50 μ g/ml for 20 min).

tuted to DNA-histone complexes, identical results were observed in immunofluorescence assays (Fig. 3b) and in immunoblots. However, a weaker reaction was observed in immunoblots with PmAb mononucleosomes. This demonstrates that PmAb-nucleosome complexes react with the cell

surface in immunofluorescence assays and with the 94-kDa cell-surface protein in immunoblots.

Binding of Anti-DNA Antibodies from Active SLE Patient Sera in Active Phase to a 94-kDa Protein via DNA-Histone Complexes. Human SLE sera were passed on a DNA-cellulose column. The antibodies were eluted in 2 M NaCl. The purified antibodies labeled a 94-kDa protein provided that DNA-histone complexes were added (Fig. 6, lane c). Interestingly, the effluents, which probably contained antibodies complexed to DNA and histones, from three patients' sera in acute phase reacted with the 94-kDa protein, but those from two patients' sera in inactive phase did not (data not shown).

FIG. 6. Binding of a purified polyclonal anti-DNA antibody from an active human SLE serum to the 94-kDa cell-surface protein, in the presence and absence of DNA-histone complexes, in immunoblots. TX-114 detergent-rich phase from a purified membrane fraction of RINm cells was used as antigen. The human SLE serum was first bound to a DNA-cellulose column. The eluted polyclonal antibody was later passed through histones covalently linked to an activated Trisacryl column. The effluent obtained is referred to as purified polyclonal anti-DNA antibody. Lanes: a, PmAb (20 μ g) + DNA (1 μ g) + histone (1 μ g); b, PmAb (20 μ g); c, purified polyclonal anti-DNA antibody (20 μ g) + DNA (1 μ g) + histone (1 μ g); d, purified polyclonal anti-DNA antibody (20 μ g).

DISCUSSION

In this paper, we have shown that PmAb binds to the cell surface in immunofluorescence assays and to a 94-kDa protein in immunoblots, but only as a complex with DNA and histones or, more likely, with nucleosomes. This 94-kDa protein behaves like a plasma membrane protein since it requires the use of detergent to be solubilized and is quantitatively recovered in the TX-114 detergent-rich phase. Moreover, a brief treatment of living cells with trypsin cleared off this protein, showing that it is exposed on the external face of the plasma membrane. This 94-kDa membrane protein was found at the surface of human B-lymphoblastoid Raji cell lines, monkey CVI cells, and rat islet pancreas cell line RINm. Nucleosomes, rather than free histones and DNA, are likely to represent the major immunogen in SLE, as suggested by Hardin and Thomas (19). This is also consistent with our results showing that PmAb has a higher affinity for nucleosomes than for DNA alone. The distribution of PmAb-DNA-histone immune complex at the cell surface has been shown in double labeling experiments to be superimposable to that of clathrin (7). Clathrin-coated pits are known to play a key role in receptor-mediated endocy-

tosis (20). The working model that we can sketch out of these results is then as follows. The 94-kDa cell-surface protein acts as a receptor for nucleosomes. After fixation of PME77 mAb, the antibody–nucleosome–94-kDa receptor complex becomes internalized. Inside the cell, nucleosomes are enzymatically digested. After reappearance of fragments at the cell surface, any nucleosome components, or nucleosome-associated molecule such as RNA polymerase, can then become immunogenic. Conversely, the interaction between an antibody and nucleosomes associated with various proteins could lead to the recognition of a variety of other structures, as observed in SLE. A number of observations support this model: the presence in SLE sera of antibodies directed against a variety of molecules more or less directly related to nucleosomes (21–23); the recognition of a 94-kDa protein by human SLE sera that have been purified on a DNA-cellulose column, provided that DNA–histone complexes are added. It can be predicted that anti-histone antibodies present in sera of SLE patients could bind to the 94-kDa cell-surface protein via nucleosome complexes. We also suggest that anti-DNA antibodies are pathogenic only when they are bound to nucleosomes. Such complexed antibodies bind to a 94-kDa cell-surface protein, which could play the role of target molecule in SLE. This hypothesis is supported by the detection of small fragments of DNA in the immune complexes from kidneys of SLE patients (24) and the observation that histones can provoke immune complex formation in the glomerular capillary wall (25). In our model, there is no internalization of nucleosomes in the absence of antibodies, nor are the antibodies pathogenic in the absence of nucleosomes.

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- Arana, R. & Seligman, M. (1967) *J. Clin. Invest.* **46**, 1867–1882.
- Dixon, F. J., Theophilopoulos, A. G., Izui, S. & McConahey, I. J. (1980) in *Progress in Immunology*, eds. Fougereau, M. & Dausset, J. (Academic, New York), pp. 959–995.
- Lafer, E. M., Rauch, J., Andrzejewski, C., Mudd, C., Furie, B., Schwartz, R. J. & Stollar, B. D. (1981) *J. Exp. Med.* **153**, 897–909.
- Faaber, P., Riike, M., Van de Putte, L. B. A., Capel, P. J. A. & Berden, H. M. (1986) *J. Clin. Invest.* **77**, 1824–1827.
- Tron, F., Charron, D., Bach, J. F. & Talal, N. (1980) *J. Immunol.* **125**, 2805–2809.
- Jacob, L. & Tron, F. (1982) *J. Immunol.* **128**, 895–898.
- Jacob, L., Kerjaschki, D., Viard, J. P., Sahuquillo-Merino, C., Lety, M. A., Bach, J. F. & Louvard, D. (1988) *Arthritis Rheum.* **31**, Suppl., 107 (abstr.).
- Jacob, L., Tron, F., Bach, J. F. & Louvard, D. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3843–3845.
- Jacob, L., Lety, M. A., Bach, J. F. & Louvard, D. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6970–6974.
- Rekvig, O. P. & Hannestad, K. (1980) *J. Exp. Med.* **152**, 1720–1733.
- Holers, V. M. & Kotzin, B. L. (1985) *J. Clin. Invest.* **76**, 991–998.
- Bennett, R. M., Kotzin, B. L. & Merritt, M. J. (1987) *J. Exp. Med.* **166**, 850–863.
- Gazdar, A. F., Chick, W. L., Oie, H. K., Sims, H. L., King, D. L., Weir, G. C. & Lauris, V. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3519–3523.
- Tron, F., Jacob, L. & Bach, J. F. (1984) *Eur. J. Immunol.* **14**, 283–286.
- Gastinel, L. N., Pléau, J. M., Dardenne, M., Sasaki, A., Bricas, E., Morgat, J. L. & Bach, J. F. (1982) *Biochim. Biophys. Acta* **684**, 117–126.
- Bordier, J. (1981) *J. Biol. Chem.* **256**, 1604–1607.
- Lutter, C. (1978) *J. Mol. Biol.* **124**, 391–420.
- Kotzin, B. L., Lafferty, J. A., Portanova, J. P., Rubin, R. L. & Tan, E. M. (1984) *J. Immunol.* **133**, 2554–2557.
- Hardin, J. & Thomas, J. O. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7410–7414.
- Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W. & Schneider, W. J. (1985) *Annu. Rev. Cell. Biol.* **1**, 1–39.
- Lerner, M. R. & Steitz, J. A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5495–5497.
- Takasaki, Y., Deng, J. S. & Tan, E. M. (1981) *J. Exp. Med.* **154**, 1899–1909.
- Stetler, D. A., Sipes, D. & Jacob, S. (1985) *J. Exp. Med.* **162**, 1760–1770.
- Sano, H. & Morimoto, C. (1981) *J. Immunol.* **126**, 538–539.
- Schmiedeke, T., Stoeckl, F., Batsford, S. & Vogt, A. (1987) *Kidney Int.* **79**, 177A (abstr.).