Polyreactive anti-DNA monoclonal antibodies and a derived peptide as vectors for the intracytoplasmic and intranuclear translocation of macromolecules

ALEXANDRE AVRAMEAS†‡, THÉRÈSE TERNYCK†‡§, FARIDABADIO NÁTO§, GÉRARD BUTTIN*, and STRATIS AVRAMEAS§

Unités de *Génétique Somatique and §d’Immunocytochimie, Département d’Immunologie, †Hybridolab, Département de Biotechnologie, Institut Pasteur, 75015 Paris, France

Communicated by Fotis C. Kafatos, European Molecular Biology Laboratory, Heidelberg, Germany, February 23, 1998 (received for review September 19, 1997)

ABSTRACT Naturally occurring polyreactive anti-DNA mAbs derived from a nonimmunized (NZB × NZW)F₁ mouse with spontaneous lupus erythematosus penetrated and accumulated in the nuclei of a variety of cultured cells. These mAbs and their F(ab’)2 and Fab’ fragments, covalently coupled to fluorescein, peroxidase, or a 15-mer polynucleotide, also translocated to the cell nuclei. A 30-mer acid peptide corresponding to the combined sequences of the complementary-determining regions 2 and 3 of the heavy chain variable region of one mAb was able to penetrate into the cytoplasm and nucleus of cells of several lines. This peptide recognized DNA and was strongly polyreactive. Streptavidin-peroxidase conjugates complexed with the N-biotinylated peptide were rapidly translocated into cells. Similarly, peroxidase or anti-peroxidase polyclonal antibodies covalently coupled to the N-cysteinylated peptide through an heterobifunctional maleimide cross-linker were also rapidly internalized and frequently accumulated in nuclei. The peptide carrying 19 lysine residues at its N-terminal was highly effective in transfecting 3T3 cells with a plasmid containing the luciferase gene. Thus, penetrating mAbs and derived peptides are versatile vectors for the intracellular delivery of proteins and genes.

A long time ago, it was reported that human IgG from systemic lupus erythematosus patients with high titers directed against nuclear ribonucleoproteins and/or DNA were able to penetrate into living cells and to reach the nucleus (1). More recent studies of murine anti-DNA autoantibodies confirmed these observations and disclosed that different penetrating antibodies exhibited diverse behaviors and characteristics (2–7). In this study, we prepared several penetrating IgG anti-DNA mAbs from the spleen of a (NZB × NZW)F₁ lupus mouse and examined their specificities and their abilities to act as vectors of hapten, proteins, polynucleotides, and plasmids.

MATERIALS AND METHODS

Mice and Cell Lines. (NZB × NZW)F₁ hybrids and BALB/c mice were bred in the Institut Pasteur animal facilities. Cells used were from different species and from various tissues as follows: PtK2 (Potoroo kidney fibroblasts) or CCL-39 (hamster lung), 3T3 (mouse embryo fibroblasts), and HEp-2 (human larynx carcinoma). All cells were from the American Type Culture Collection and were cultured in RPMI 1640 medium (or in DMEM for CCL-39) containing 10% heat-inactivated fetal bovine serum and supplemented with 1-glutamine, sodium pyruvate, nonessential amino acids, and antibiotics (complete culture medium) at 37°C in a humidified atmosphere of 5% CO2/95% air.

mAbs. Spleen cells from a 9-month-old nonimmunized (NZB × NZW)F₁ mouse were fused with P3.X63Ag8 myeloma cells by the method of Köhler and Milstein (8), and hybridomas were selected in hypoxanthine/azaserine medium. Supernatants were tested by ELISA on double-stranded (ds) DNA-coated plates with β-galactosidase-labeled anti-Fcγ conjugate prepared from sheep antiserum (9). Isotypes were determined by using anti-IgG1-, -IgG2a-, -IgG2b-, and -IgG3-alkaline phosphatase conjugates (Southern Biotechnology Associates, Birmingham, AL). Anti-DNA-positive hybridomas were cloned and expanded, and cell culture supernatants were tested for the ability of their IgG to penetrate into living cells.

Penetration of Antibodies into Cells. Cell monolayers were obtained by seeding 2–5 × 10⁴ cells in 0.5 ml of complete medium on round microscopic coverslips deposited in 24-well tissue culture plates. One to 2 days after culture initiation, the medium was replaced by undiluted hybridoma-positive supernatants or purified mAbs diluted in complete medium, and cultures were allowed to proceed for 2–24 h. Cells were washed with PBS, either fixed for 15 min in ethanol at −20°C and dried or fixed in 2% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min, washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 2 min. Intracellular mAb was visualized by incubating fixed cells with horseradish peroxidase (PO)-coupled anti-mouse IgG Fab (10 μg/ml) prepared from sheep antiserum (9) or fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG antibodies (Southern Biotechnology Associates) (5 μg/ml) for 60 min followed by several washes in PBS. PO deposits were visualized by using the metal-enhanced diaminobenzidine substrate kit (ME-DAB; Pierce). To evaluate the amounts of mAb internalized after various incubation times, cells were trypsinized, washed, counted, and lyzed in 0.1 M Tris HCl (pH 8) containing 1% Nonidet P-40. The amount of mAb in lysates was evaluated by ELISA using anti-IgG2a-alkaline phosphatase conjugate. Enzyme activity was determined by using p-nitrophenyl phosphate (Sigma).

Purification, Fragmentation, and Labeling of mAbs. IgG mAbs were isolated by using a protein A-Sepharose column (10). The reactivities of purified mAbs with dsDNA and other double-stranded DNA; ME-DAB, metal-enhanced diaminobenzidine; FITC, fluorescein isothiocyanate; V₁β, Ig heavy chain variable region; PO, horseradish peroxidase; PEI, polyethylenimine; RLU, relative light units. Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF004398–AF004402).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1998 by The National Academy of Sciences 0027-8424/yy© 1998 by The National Academy of Sciences 0027-8424/yy

PNAS is available online at http://www.pnas.org.
Table 1. Sequence of the peptides derived from F4.1 mAb

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Region</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>CDR2</td>
<td>VAYISRGGVSTYYSDTVKGRTF</td>
</tr>
<tr>
<td>P2</td>
<td>CDR3</td>
<td>AROKYNKRAMDY</td>
</tr>
<tr>
<td>P3</td>
<td>CDR2–CDR3</td>
<td>VAYISRGGVSTYYSDTVKGRTFRQOKYNKRA</td>
</tr>
</tbody>
</table>
there was no correlation between the avidity of a mAb for DNA and its penetration capacity (Table 2). Hybridomas producing three mAbs (J20.8, F4.1, and F14.6) were bulk-cultured, and mAbs were purified and further analyzed. The purified mAbs were previously found, using phage-display peptide libraries, to recognize different peptides (18). These mAbs, used at concentrations from 10 to 50 μg/ml, penetrated all cell lines examined in a time-dependent manner. Each mAb exhibited different kinetic for intracellular localizations; F(ab')2 and Fab' fragments internalized like intact IgG molecules. At the above concentrations, compared with controls, no increase of cell death was noted as assessed by trypan blue exclusion. No change in cell morphology was observed by using either the tetrazolium salt procedure or [3H]thymidine incorporation (data not shown). However, F4.1 translocation, which occurs at 37°C but not at 4°C (Fig. 1), was often accompanied by morphological changes and some dead cells were seen.

Intracellular Targeting of Haptens and Macromolecules by Anti-DNA mAbs. FITC-conjugated mAbs, F(ab')2, and Fab' fragments penetrated cells as well as unlabeled molecules did. Confocal microscopy analysis of fluorescent cell preparations incubated for 4 h showed primarily a nuclear localization (data not shown).

The intracellular penetration of PO-conjugated IgG mAbs, F(ab')2, and Fab' fragments was examined on PtK2 and HEP-2 cells incubated for 16 h with the conjugates. PO-F(ab')2; and PO-Fab' generated intense signals mostly localized in the nucleus but sometimes also in the cytoplasm (Fig. 2A). After a 2-h incubation of PtK2 with Fab'-PO conjugate, 1–2 × 10⁶ molecules of conjugate per cell were found to be internalized. No PO deposits were detected when cell preparations were incubated under the same conditions with nonpenetrating F(ab')2; mAbs coupled to PO, free PO, or unlabeled penetrating F(ab')2; mAbs plus PO.

Table 2. Reactivities assessed by ELISA of penetrating and nonpenetrating mAbs with a panel of antigens

<table>
<thead>
<tr>
<th>mAbs</th>
<th>dsDNA</th>
<th>ssDNA</th>
<th>Histone</th>
<th>DNase</th>
<th>Actin</th>
<th>Myosin</th>
<th>Tubulin</th>
<th>Ars</th>
<th>Ph-Ox</th>
<th>TNP</th>
<th>Kd (×10⁻⁹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2.4 (+)</td>
<td>0.070*</td>
<td>3.5</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>5.6</td>
<td>3</td>
<td>1.3</td>
<td>1.2</td>
<td>5</td>
<td>90</td>
</tr>
<tr>
<td>C12.24 (+)</td>
<td>0.022</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>Neg.</td>
<td>1.2</td>
<td>3</td>
<td>Neg.</td>
<td>80</td>
</tr>
<tr>
<td>F4.1 (+)</td>
<td>0.040</td>
<td>2</td>
<td>5</td>
<td>15</td>
<td>1.8</td>
<td>5</td>
<td>6</td>
<td>1.2</td>
<td>3</td>
<td>20</td>
<td>90</td>
</tr>
<tr>
<td>J20.8 (+)</td>
<td>0.066</td>
<td>60</td>
<td>16</td>
<td>30</td>
<td>16</td>
<td>2.5</td>
<td>3.5</td>
<td>1.8</td>
<td>5</td>
<td>30</td>
<td>350</td>
</tr>
<tr>
<td>G14 (+)</td>
<td>0.066</td>
<td>2.6</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>1.5</td>
<td>2</td>
<td>0.4</td>
<td>1</td>
<td>4</td>
<td>770</td>
</tr>
<tr>
<td>F14.6 (+)</td>
<td>0.060</td>
<td>0.8</td>
<td>0.6</td>
<td>10</td>
<td>0.6</td>
<td>0.2</td>
<td>1.8</td>
<td>0.5</td>
<td>0.45</td>
<td>3</td>
<td>150</td>
</tr>
<tr>
<td>H9.3 (-)</td>
<td>0.140</td>
<td>Neg.</td>
<td>30</td>
<td>Neg.</td>
<td>30</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
<td>1,500</td>
</tr>
</tbody>
</table>

+ Pierceant mAb; −, nonpeneating mAb; Neg., negative; ssDNA, single-stranded DNA; Ars., arsonate; Ph-Ox, phenyloxazolone; TNP, trinitrophenyl.

*Reactivity is expressed as the mAb concentration in μg/ml giving an OD414 of 0.200 in 2 h.

When either FITC- or PO-coupled antibodies were translocated, the percentage of positive cells varied from 5 to 100%.

To evaluate the intracellular penetration of Fab' conjugated to fluorescein-polynucleotide, 5 × 10⁵ thymocytes or spleen cells from a 6-week-old BALB/c mouse were incubated in 250 μl of complete medium containing 13 μg of Fab' conjugated to fluorescein-polynucleotide. After 3 h, cells were washed and fixed with 1% formaldehyde. Examined by flow cytometry, 4.5% of thymocytes and 15% of spleen cells were labeled. Analysis by confocal microscopy revealed that the fluorescein was localized in the nucleus (Fig. 3).

The ability of mAbs J20.8, F4.1, and F14.6 to transport the plasmid pHu Vim 830-T/t were examined. Plasmid/mAb ratios were determined that avoided plasmid immunoprecipitation but allowed penetration into the nucleus. Although all three mAbs were able to transport the gene, wide variability was noted from one experiment to another, and rarely more than 0.1% of the cells expressed the transfected gene (data not shown).

Intracellular Targeting of Vh Peptides of Anti-DNA mAbs. The nucleotide sequences of heavy and light chains of three penetrating and two nonpenetrating anti-DNA mAbs were determined and their amino acid sequences were deduced (Fig. 4). The penetrating mAbs (J20.8, F4.1, and F14.6) possessed highly homologous Vh CDR2 sequences, including one arginine and one lysine residue, and distinct CDR3 regions, all containing arginine, a property typical of anti-DNA antibodies (19). The two nonpenetrating mAbs (H9.3 and A2.1) exhibited no CDR2 sequence homology with the penetrating mAbs. The CDR3 of one of them (A2.1) had two arginines, and the other one did not include arginine or lysine residues.

The biotinylated peptides P1, P2, and P3 shown in Table 1 and corresponding to CDR2 and or CDR3 sequences of the penetrating mAb F4.1 were prepared and tested immunocytochemically for their capacities to penetrate into PtK2, HEP2, and CCL-39 cells. No penetration was detected in the three cell lines with P1, at all concentrations examined. With P2 at 20 μg/ml, a weak but definite labeling in the cytoplasm of most of the cells was observed. With P3 at 5–20 μg/ml, cytoplasmic positivity was noted in most of the cells, and intense nuclear labeling of PtK2 cells was also seen (Fig. 2B). Maximal penetration seemed to be achieved by 1 h, because no intensification was observed up to 5 h, after which time label intensity seemed to decrease.

To compare the reactivities of biotinylated P1, P2, and P3 with various antigens used in the present study (see Table 2), parallel ELISAs were performed. Of the three peptides, only P3 reacted strongly with DNA and various other antigens, although less intensely than F4.1. P2 bound weakly to a few antigens but never with DNA. P1 was always negative.

To determine whether the peptides could be used as vectors to deliver macromolecules intracellularly, complexes were prepared with biotinylated peptides and streptavidin-PO conjugate at various ratios and tested on PtK2, HEP2, and
CCL-39 cells. Complexes prepared with 1.4 μg of biotinylated P2 or P3 and 10 μg of streptavidin-PO gave the most satisfactory and reproducible results. After 1 h of incubation, P2 complexes generated a weak cytoplasmic positivity. Biotinylated P3 and streptavidin-PO complexes generated intense nuclear signals after 1 h (Fig. 2C) that were still visible 6 h later. A less intense but strong signal was noted in the cytoplasm and nuclei of almost all cells incubated for 1 h with either PO at 12–50 μg/ml or anti-PO antibodies directly coupled to P3 at 12–50 μg/ml. Measurable quantities of internalized enzymes were noted only when P3 was used as the vector. Incubation of PtK2 cells for 2 h with P3 complexed to streptavidin-PO or covalently linked to PO resulted, respectively, in the internalization of 2 × 10^5 to 8 × 10^6 molecules of streptavidin-PO and 5 × 10^5 to 5 × 10^6 of PO per cell. Values obtained with P1 and P2 were within the background level.

Transfection of cells with pCMVL-K<sub>19</sub>-P3 complexes resulted in a mean luciferase expression of 5 × 10<sup>4</sup> ± 1 × 10<sup>4</sup> RLU/mg of protein, values higher (two to three orders of magnitude) than those obtained with complexes of 19-residue polylysine alone (Fig. 5). The efficacy of transfection was of the same order of magnitude as that obtained by using the PEI procedure (20).

**DISCUSSION**

The main goal of this study was to examine the possibility of using anti-DNA mAbs, obtained from a 9-month-old (NZB × NZW)F<sub>1</sub> mouse, as vectors for the intracellular transport of haptens and macromolecules. Many hybridoma clones indeed secreted penetrating IgG anti-DNA mAbs. We analyzed several of them of the IgG2a isotype and studied in greater detail three of them (J20.8, F4.1, and F14.6). These antibodies and their (Fab′)<sub>2</sub> and Fab′ fragments could penetrate into various types of living cells, enter the nucleus, and accumulate there. Under the conditions used, such penetration did not appreciably alter cell viability or metabolism. Each mAb exhibited distinct kinetics of intracellular transit for a given cell line.

These results confirm data previously reported for other anti-DNA antibodies (1–7). Remarkably, we found that, among the various anti-DNA antibodies from the lupus mouse, only polyreactive antibodies could penetrate into the cells. Because these polyreactive antibodies seem to possess conserved CDR1 and CDR2 and similar CDR3 sequences, the penetrating antibodies appear to represent clones of natural polyreactive autoantibodies that escape normal control and expand in lupus disease. Polyreactive anti-DNA antibodies, compared with monoreactive ones, should possess increased capabilities to bind to cell membranes. Indeed, flow cytometry analysis of our polyreactive anti-DNA mAbs showed that most, but not all, bound to various degrees to the surface of mouse spleen cells in suspension (data not shown). This finding is consistent with the results of previous work with other penetrating autoantibodies (1, 5–7), suggesting that internalization of anti-DNA mAbs is mediated by their binding to cell surface structures.

Penetrating anti-DNA mAbs and/or their (Fab′)<sub>2</sub> or Fab′ fragments were effective vectors for the intracellular transport of haptens and macromolecules covalently linked to them. Thus fluorescentin (M<sub>r</sub> = 389), a 15-mer oligonucleotide (M<sub>r</sub> = 5,000), and PO (M<sub>r</sub> = 40,000) could be transported through the cytoplasm and into the nuclei of the various cell types examined. Furthermore, incubation of PtK2 cells with complexes formed between penetrating anti-DNA mAbs and a plasmid coding for the tumor antigen of simian virus 40 resulted in the nuclear expression of the tumor antigen but in no more than 0.1% of the cells. Transfection supplies evidence, independent of immunohistochemical observations, for anti-DNA mAb-mediated internalization of nucleic acids. In these experiments, the percentages of cells containing internalized mAbs detected immunocytochemically ranged, as for penetrating mAbs alone, from 5 to 100%. It will be of special interest to determine whether the limiting factor in these transfections is misrouting of most internalized plasmids or strong hindrance to the internalization of most complexes, arising from the structural alteration imposed on mAbs by plasmid binding. The observation made with site-directed mutants of a penetrating anti-DNA mAb (5), that the same residues required for binding DNA are necessary for penetration of this mAb, would agree with the latter hypothesis.

Our initial working hypothesis was that penetrating anti-DNA mAbs carry common peptides involved in their translocating abilities, which, if proven true, could be used as more effective vectors than whole antibodies. To explore this possibility, we determined the nucleotide sequences of the heavy and light chains of three penetrating mAbs. Highly homologous V<sub>H</sub> CDR2 sequences were noted. The synthetic peptide P1, corresponding to the CDR2 of F4.1, was unable to translocate into cells or to bind to antigens. In contrast, peptide P2, which corresponds to the V<sub>H</sub> CDR3 and, even more so, peptide P3 (P1 linked to P2) gave positive results. Only P3 was found by ELISA to be highly polyreactive and to bind strongly.
with DNA and various self and nonself antigens. Although weak binding to a few antigens, but never with DNA, was noted with P2, only P3 readily and abundantly penetrated into living cells, but small amounts of intracellular P2 could always be detected. These results suggest that the penetrating ability of F4.1 anti-DNA mAbs relies on CDR3 but is only manifested when CDR2 is associated with it. Further studies are needed to determine whether efficient penetration of CDR3 requires its linking to this specific CDR2 to achieve anti-DNA binding and increased auto- and polyreactivity or whether its association with an unrelated CDR2 would be sufficient.

Only P3 was found to be an effective vector, capable of translocating in a short time across plasma and nuclear membranes up to 5 × 10^6 protein macromolecules (from 40 to 200 kDa) to which it was linked covalently or not. However, although P3 at high concentrations was able to condense plasmids, it was ineffective in gene delivery. The addition of 19-kDa P2, only P3 readily and abundantly penetrated into living cells, but small amounts of intracellular P2 could always be detected. These results suggest that the penetrating ability of F4.1 anti-DNA mAbs relies on CDR3 but is only manifested when CDR2 is associated with it. Further studies are needed to determine whether efficient penetration of CDR3 requires its linking to this specific CDR2 to achieve anti-DNA binding and increased auto- and polyreactivity or whether its association with an unrelated CDR2 would be sufficient.

Only P3 was found to be an effective vector, capable of translocating in a short time across plasma and nuclear membranes up to 5 × 10^6 protein macromolecules (from 40 to 200 kDa) to which it was linked covalently or not. However, although P3 at high concentrations was able to condense plasmids, it was ineffective in gene delivery. The addition of 19-kDa P2, only P3 readily and abundantly penetrated into living cells, but small amounts of intracellular P2 could always be detected. These results suggest that the penetrating ability of F4.1 anti-DNA mAbs relies on CDR3 but is only manifested when CDR2 is associated with it. Further studies are needed to determine whether efficient penetration of CDR3 requires its linking to this specific CDR2 to achieve anti-DNA binding and increased auto- and polyreactivity or whether its association with an unrelated CDR2 would be sufficient.

Only P3 was found to be an effective vector, capable of translocating in a short time across plasma and nuclear membranes up to 5 × 10^6 protein macromolecules (from 40 to 200 kDa) to which it was linked covalently or not. However, although P3 at high concentrations was able to condense plasmids, it was ineffective in gene delivery. The addition of 19-kDa P2, only P3 readily and abundantly penetrated into living cells, but small amounts of intracellular P2 could always be detected. These results suggest that the penetrating ability of F4.1 anti-DNA mAbs relies on CDR3 but is only manifested when CDR2 is associated with it. Further studies are needed to determine whether efficient penetration of CDR3 requires its linking to this specific CDR2 to achieve anti-DNA binding and increased auto- and polyreactivity or whether its association with an unrelated CDR2 would be sufficient.

Only P3 was found to be an effective vector, capable of translocating in a short time across plasma and nuclear membranes up to 5 × 10^6 protein macromolecules (from 40 to 200 kDa) to which it was linked covalently or not. However, although P3 at high concentrations was able to condense plasmids, it was ineffective in gene delivery. The addition of 19-kDa P2, only P3 readily and abundantly penetrated into living cells, but small amounts of intracellular P2 could always be detected. These results suggest that the penetrating ability of F4.1 anti-DNA mAbs relies on CDR3 but is only manifested when CDR2 is associated with it. Further studies are needed to determine whether efficient penetration of CDR3 requires its linking to this specific CDR2 to achieve anti-DNA binding and increased auto- and polyreactivity or whether its association with an unrelated CDR2 would be sufficient.

Only P3 was found to be an effective vector, capable of translocating in a short time across plasma and nuclear membranes up to 5 × 10^6 protein macromolecules (from 40 to 200 kDa) to which it was linked covalently or not. However, although P3 at high concentrations was able to condense plasmids, it was ineffective in gene delivery. The addition of 19-kDa P2, only P3 readily and abundantly penetrated into living cells, but small amounts of intracellular P2 could always be detected. These results suggest that the penetrating ability of F4.1 anti-DNA mAbs relies on CDR3 but is only manifested when CDR2 is associated with it. Further studies are needed to determine whether efficient penetration of CDR3 requires its linking to this specific CDR2 to achieve anti-DNA binding and increased auto- and polyreactivity or whether its association with an unrelated CDR2 would be sufficient.
We thank Dr. J.-C. Mazie (Institut Pasteur) for his interest in this project and Dr. P. Lafaye (Institut Pasteur) for his help in gene sequencing. We are grateful to Dr. P. Vicart, Institut Pasteur, for providing the pHu Vim 830-T/t plasmid and the hybridoma secreting anti-tumor antigen antibody, and to Dr. U. Hazan (Institut Cochin de Génétique Moléculaire) for the gift of the pCMVL plasmid. A. Avrameas received a fellowship from Centre Européen de Bioprospective, Rouen, France.