THE PRODUCTION AND CHARACTERIZATION OF MURINE MONOCLONAL ANTIBODIES TO A DNA RECEPTOR ON HUMAN LEUKOCYTES

ROBERT M. BENNET, STEVEN H. HEFENEIDER, ANTONY BAKKE, MARCIE MERRITT, COLIN A. SMITH, DAN MOURICH, AND MICHAEL C. HEINRICH

From the Department of Medicine, The Oregon Health Sciences University, Portland, OR 97201

Two murine mAb have been generated with a reactivity toward a 30,000 m.w. DNA binding protein found on the cell surface of human leukocytes; mAb 12A has an IgG1/k isotype, and mAb 24T has an IgG2b/k isotype. Both react with the DNA binding domain or adjacent region of the putative DNA receptor and inhibit the binding of [3H]DNA to PBMC at concentrations as low as 100 ng/ml. Stoichiometric studies indicate that both mAb react with monocytes and T cells with a kDa of 10^(-7) M; about 0.5 x 10^8 molecules bind per cell at saturation. Flow cytometry indicated that 67% of lymphocytes and 98% of monocytes bore the DNA receptor. Dual labeling studies showed that 90% of B cells and 50% of T cells express the receptor; 50% of CD4 T cells are receptor positive. Immunomatrixes constructed with both mAb 12A and 24T allowed the receptor to be purified to a high degree of purity. A single protein of M, 30,000 was readily observed after SDS-PAGE and silver staining of the gel; after electrophoretic transfer of nitrocellulose this protein was shown to be a DNA binding molecule by use of a probe of biotin labeled DNA. These experiments provide further evidence to support the existence of a specific DNA receptor on human leukocytes; the availability of mAb to the receptor should be useful in its further characterization.

The existence of a cell surface receptor for DNA has been inferred from stoichiometric studies (1), the demonstration of a DNA binding molecule on cell membrane preparations (1-3), and the finding of anti-receptor antibodies in patients with SLE and kindred disorders (4, 5). Human leukocytes, but not erythrocytes, have been shown to bind λ-phage DNA in a saturable manner consistent with a ligand receptor interaction (1). It is hypothesized that the function of the receptor is related to a salvage pathway for nucleotides derived from effete DNA, as surface bound DNA is internalized and degraded to oligonucleotides of <12 basepairs. Receptor molecules are degraded concommitantly, and active ribosomal protein translation is required for receptor re-expression at the cell surface (1). A DNA binding protein of M, 30,000 has been demonstrated on cell surfaces using a probe of biotytulated DNA (1, 2). The recent finding of anti-receptor antibodies in SLE and kindred disorders (3, 4) suggests that the DNA receptor may be an important Ag in the repertoire of autoimmune responses.

Herein we describe the production and characterization of mAb to the DNA binding domain of the DNA receptor. These mAb provide additional evidence for the existence of a unique receptor for DNA and should be useful in its further characterization.

MATERIALS AND METHODS

Isolation of partially purified receptor. The Ag used in the production of murine hybridomas was a partially purified extract of solubilized cell membrane proteins obtained from human PBMC. Cell membranes from 2 x 10^9 PBMC were isolated as previously described (6) and electrophoretically separated by SDS-PAGE using two gels without individual troughs. A strip of gel, 1 inch in width, which corresponded to proteins migrating to the 30,000 region, was excised and crushed up in elution buffer (0.05 M Tris-HCl, pH 7.9, 0.1% SDS, 5.0 mM dithiothreitol, 0.1 mg/ml BSA, 0.2 M NaCl). Proteins were extracted by rocking the suspension for 48 h at 4°C. After centrifugation, the cleared supernatant was transferred to a 30 ml pre-siliconized tube, and ×3 volume of acetone at -20°C was added; after incubation on ice-ethanol for 30 min, the precipitate was collected by centrifugation. The precipitate was washed once with 80% acetone and 20% dilution buffer (0.05 M Tris-HCl, pH 7.9, 20% glycerol, 0.1 mg/ml BSA, 0.15 M NaCl, 1 mM dithiothreitol, 0.1 mM EDTA) and then re-solubilized in 2 ml of 6 M guanidine hydrochloride dissolved in dilution buffer. After incubation at room temperature for 20 min, the mixture was made up to 10 ml with dilution buffer and incubated for 60 min at room temperature. Thereafter gradual renaturation of the proteins was achieved by dialysis for 24 h against PBS containing 0.1% SDS. Verification that the solubilized proteins contained the DNA receptor was achieved by immobilizing the Ag on nitrocellulose (Bio-Rad Laboratories, Richmond, CA) and using a dot-blot assay using a probe of biotinated DNA (2).

Immunization and cell fusion. Female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) 8 to 12 wk of age were immunized with the partially purified DNA preparation. Each of five animals received 20 to 50 μg of material in 0.4 ml of CFA intradermally to the hind legs. A series of five additional weekly injections was given to each animal using 20 to 50 μg of material in IFA. Sera were collected and tested individually for binding to the immunizing Ag (200 μl, 60 μg/ml) immobilized on nitrocellulose in a dot-blot assay employing goat-anti-mouse IgG conjugated to horse-radish peroxidase (Bio-Rad) as the second antibody; sera collected before immunization served as a negative control. Each of the animals demonstrated significant binding in this assay. The animal found to have the highest reactivity on the dot-blot assay received an additional 40 μg of immunogen in PBS, administered i.v. The animal was killed 4 days later and the spleen was removed and used for fusion with the murine SP/2 cell line (7). Selected hybridomas were inoculated i.p. (1 to 2 x 10^6 cells/animal) into BALB/c mice with pristane-induced ascites; IgG was isolated by ion exchange chromatography on DEAE cellulose (Whatman DE-52) followed by elution from a column of Protein A Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ).

Isolation of leukocyte subpopulations. Human buffy coats were obtained from the local Red Cross and the PBMC fraction was...
separated with Ficoll-Hypaque density centrifugation. The PBMC were separated into populations enriched for monocytes and T cells by the standard techniques of adherence to plastic dishes and rosetting with SRBC. These populations were assessed for viability at the beginning and end of experiments by trypan blue exclusion: in all experiments viability was 85%.

Inhibition of the binding of DNA to PBMC by mAb and displace
ment assays. MonoAb-ID EIA kit (Zymed Laboratories, San Francisco,
CA) was used according to the manufacturers instructions. The ascites
derived mAb 24T was conjugated with FITC and over-conjugated antibodies were removed by DEAE chromatography. The fluores
cine/protein molar ratio was 3.5. To maximize receptor expression
the purified preparation of cell membranes from PBMC was prepared as
previously described (1). Membrane proteins were solubilized by
membrane proteins were solubilized by

purification resulted in the production of antibodies reactive
with Ag in this preparation. One immune animal was killed and its spleen cells fused with the murine SP/2 myeloma cell line. After 10 days in culture more than 85% of the microcultures exhibited hybrid cell growth. A 10-μl aliquot of supernatant was removed from each culture and tested using a dot-blot procedure using partially purified DNA receptor immobilized on nitrocellulose. Of the 350 supernatants screened, 90 demonstrated significant reactivity in the dot-blot assay. These hydrids were transferred to 1 ml cultures and allowed to reach 75 to 90% confluence and the supernatants screened for their ability to inhibit the binding of [3H] DNA to human PBMC. Of these 90 hybrids only 12 demonstrated significant inhibition of DNA binding. Two of these hybrids, which produced the highest level of inhibition, were weaned to HAT-free medium and were subcloned twice at 1 cell/well. These two hybrids were designated 12A and 24T. They were isotyped using the Mono Ab-ID EIA kit (Zymed Laboratories, San Francisco, CA) according to the manufacturers instructions. 12A was IgG1/x and 24T was IgG2b/x.

Reactivity of mAb to DNA. mAb 12A and 24T, derived from inoculated ascitic fluid, both demonstrated inhibition of [3H]DNA binding to PBMC, as determined by the initial screening of the hybridomas. When increasing dilutions of mAbs were incubated with PBMC, significant inhibition of [3H]DNA binding was seen down to a dilution of 3 × 10⁻⁴ (Fig. 1). mAb 12A rapidly inhibited [3H]DNA binding to its receptor (Fig. 2). When PBMC were incubated with either mAb for 10 min at 4°C, subsequent [3H]DNA binding was inhibited for 18 h, but cell viability was 85% (data not shown). Ascites fluid from non-immunized BALB/c mice did not influence [3H]DNA binding.

Stoichiometry of mAb binding to monocytes and T cells. To obtain an estimate of the affinity of the mAb for the DNA receptor and the number of receptor sites, a radiolabeled ligand study was performed. It is seen that the binding of ascites derived mAb 12A to monocytes is

Abbreviation used in this paper: PE, phycoerythrin.
Figure 1. Inhibition of DNA binding to human PBMC by mAb 12A and 24T at different dilutions. mAb were incubated with 2 x 10^6 PBMC, 4°C, 30 min; after washing three times with HBSS the ability to bind [^3H]DNA was assessed. Results are expressed as the mean binding of three replicate wells. Maximal binding of[^3H]DNA was assessed without the addition of mAb and with the addition of an irrelevant mAb, giving counts of 22,705 ± 1033 and 20,614 ± 983/min, respectively. The original concentrations of the mAb were 3 mg/ml. This experiment is representative of six similar experiments done over a 2-mo period.

Figure 2. Kinetics of loss of functional receptor from the surface of human PBMC exposed to mAb 12A. Cells were exposed to mAb 12A (conc. 100 μg/ml) for various times periods, before assessing binding of[^3H]DNA. Time points refer to timing of addition of[^3H]DNA to incubation mixture. Maximal[^3H]DNA binding was assessed without the addition of mAb (24.3 ± 861 cpm). Results are expressed as the percentage of maximal binding plotted against time. Each point represents the mean binding in three replicate wells. Figure 2 is representative of two identical experiments.

Flow cytometry. Using ascites-derived 24T and electronically "gating" on either lymphocytes or monocytes based on forward and 90° light scatter, 67% of lymphocytes and 95% of monocytes were found to bear receptors for DNA (Fig. 4). Dual labeling studies were used to dissect the gated lymphocyte population; they showed that 90% of B cells (CD19 positive), and 50% of T cells (CD 2 positive) express DNA receptors (Fig. 5). Within T lymphocyte subsets, 50% of CD4+ and CD8+ cells bear receptors. Finally, 98% of "gated," My 4-positive monocytes are 24T positive. These flow cytometry results are representative of six separate studies done over a period of 4 months. Interestingly, a relatively high concentration of

TABLE I

<table>
<thead>
<tr>
<th></th>
<th>Monocytes</th>
<th>T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb 12A</td>
<td>1.01</td>
<td>1.51</td>
</tr>
<tr>
<td>mAb 24T</td>
<td>1.69</td>
<td>1.52</td>
</tr>
</tbody>
</table>

 interpretations of Scatchard's linear transformation; the Kd and the maximum number of molecules bound per cell (Bmax) are shown for each cell.
mAb 24T was required to generate the rather low intensity staining shown in Figures 4 and 5. mAb 24T exhibits progressive internalization, as assessed by acid washes, starting at about 10 min; this process is not very sensitive to inhibition by azide at 4°C. We surmise that quenching of non-specific fluorescence determined by means of a FITC labeled irrelevant murine IgG of the same isotype.

Isolation of DNA binding protein by immunomatrix. The above results are supportive evidence that mAb 12A and 24T are reactive with the DNA receptor on human leukocytes, but they do not definitively prove that both DNA and the mAb react with the same surface molecule. To determine whether the molecule recognized by mAb 12A and 24T was identical to the receptor for DNA, immunomatrices of 12A and 24T were constructed, and the reactive molecule from solubilized cell membrane preparations was isolated by the method of affinity chromatography. After washing the immunomatrix extensively with a starting buffer of pH 7.0, the pH was successively decreased by 1 pH unit, the proteins specifically bound to the immunomatrix were eluted at a pH of 4.0. Analysis on SDS-PAGE showed a single band migrating at M, 30,000—proteins eluted from the mAb 12A immunomatrix are in lane B, and from the mAb 24T immunomatrix in lane C; lane D shows the electrophoretically resolved proteins from the original membrane preparation; lane A shows m.w. markers. The gel was visualized after silver staining.

Figure 6. Isolation of purified DNA receptor by affinity chromatography. Immunomatrices of mAb 12A and 24T were constructed by using a bifunctional linker to irreversibly attach antibodies to protein A Sepharose CL-4B. The solubilized proteins from a purified cell membrane preparation of human PBMC were applied to the immunomatrices; the DNA receptor eluted at a pH of 4.0. Analysis on SDS-PAGE showed a single band migrating at M, 30,000—proteins eluted from the mAb 12A immunomatrix are in lane B, and from the mAb 24T immunomatrix in lane C, lane D shows the electrophoretically resolved proteins from the original membrane preparation; lane A shows m.w. markers. The gel was visualized after silver staining.

Figure 5. DNAR expression on PBMC, as assessed by dual fluorescence labelling using FITC-conjugated mAb 24T and PE conjugated mAb CD2, CD19, CD4, and MY4. A to C were created by gating on lymphocytes; D was gated on monocytes. A, 50% of T cells (CD4+) express DNA receptors and this is equally distributed between CD4+ (B) and CD8+ (data not shown) cells. However, 90% of CD 19+ B cells (C) and 98% of My4+ gated monocytes (D) bear DNA receptors. The arrows delineate the boundaries of non-specific fluorescence determined by means of a FITC labeled irrelevant murine IgG of the same isotype.

The proteins obtained from the acid wash of the immunomatrix were isolated by the method of affinity chromatography. After washing the immunomatrix extensively with a starting buffer of pH 7.0, the pH was successively decreased by 1 pH unit, the proteins specifically bound to the immunomatrix were eluted at a pH of 4.0. The proteins obtained from the acid wash of the immunomatrix were electrophoretically separated using reducing conditions on SDS-PAGE, a single band was visualized after silver staining of the gels; both mAb 12A and 24T affinity-purified proteins exhibited a relative M, 30,000 (Fig. 6). To determine whether the 30,000 protein was identical to the DNA receptor (which also has a M, of 30,000), the proteins from unstained SDS-PAGE gels were electrophoretically transferred to a nitrocellulose sheet and reacted with a probe of biotin labeled DNA. It is seen that mAb 12A and 24T both react with a DNA-binding protein of relative M, 30,000 (Fig. 7). These results provide confirmatory evidence that mAb 12A and 24T are specific for the DNA receptor.

Reactivity with histones and DNA. As there is experimental evidence for the presence of histones on cell membranes (5, 10), it was of relevance to test whether our mAb were reacting with these molecules; no reactivity was seen in an ELISA. Jacob et al. (11) have recently described a mAb to DNA that exhibits a cross-reactivity with a cell membrane molecule, it was therefore pertinent to see whether our mAb demonstrated the converse, namely a reactivity with DNA; no such reactivity was seen in an ELISA.

DISCUSSION

The question of whether there is a specific receptor for DNA on human cells is of relevance to molecular biologists interested in the mechanisms of transfection, investigators interested in the etiopathogenesis of autoimmunity in patients with SLE, and biochemists concerned with salvage pathways for effete DNA. We embarked on the current project as a means of defining the DNA receptor with greater precision by generating mAb to probe its functional and structural characteristics.

The association of DNA with cell surfaces, both in prokaryotic and eukaryotic organisms, has been the subject of numerous studies and two extensive reviews (10, 13). In general this topic has evoked little general interest as it has been assumed that it represents a non-specific interaction of a highly charged molecule with an external membrane. We have presented evidence that the interaction of DNA with human leukocytes is compatible with a ligand receptor relationship in terms of saturability, affinity, competition studies, and the demonstration of a
DNA binding molecule on cell membranes (1). Furthermore, there are several independent reports that support the notion of a specific DNA receptor: 1) Rogers and coworkers (14, 15) described the in vitro release of DNA by cultured lymphocytes and noted that the excreted DNA was taken up by other lymphocytes and capped; 2) Bankhurst and Williams (16) described the occurrence of DNA binding B lymphocytes in both normals and patients with SLE. They speculated that the DNA was binding to B lymphocytes expressing cell surface anti-DNA IgG: 3) Okudaira et al. (17) have recently reported that a murine anti-DNA mAb binds to cell surface DNA on mouse thymocytes and human T cells, and becomes internalized in a manner consistent with receptor-mediated endocytosis of DNA; and 4) Abdou et al. (18) have shown that SLE sera could suppress the binding of [3H]DNA to B cells. They hypothesized that this resulted from anti-idotypic antibodies blocking the paratope of anti-DNA antibodies, however these results could also be interpreted as evidence for anti-receptor antibodies. We have described a humoral factor in the sera from patients with SLE and similar disorders that inhibits the binding and internalization of DNA by human PBMC (1). More recently we have shown that this inhibition is mediated by at least three antibody systems—anti-DNA, anti-histone, and anti-DNA receptor (5).

Herein we provide further evidence for the existence of a specific cell surface receptor for DNA. We have produced two mAb that exhibit the properties predicted for a specific interaction with a DNA receptor: 1) they inhibit the binding of [3H]DNA to PBMC, suggesting that they are reactive with the DNA binding domain of the receptor or an adjacent area; 2) they interact with the receptor with moderate affinity ($K \sim 1 \times 10^{-7}$ M); 3) some $5 \times 10^{6}$ molecules of mAb bind per cell, indicating that the receptor is a relatively abundant molecule; 4) construction of an immunomatrix incorporating the mAb allows the DNA receptor to be purified to near homogeneity (as assessed by SDS-PAGE), the affinity purified protein migrates as a single band on SDS-PAGE and is easily visualized on silver staining of the gel; 5) after isolation by affinity chromatography, subsequent SDS-PAGE and Western blotting reveals a single DNA binding molecule of relative $M$, 30,000; and 6) flow cytometry studies support our previous observations indicating that the DNA receptor is expressed on monocytes, B cells, and T cells (1).

It is of note that the number of mAb molecules binding per cell is about $5 \times 10^{6}$, whereas our previous study using $\lambda$-phage DNA gave a figure of $10^{6}$ (1). This apparent discrepancy is probably a result of the large size of $\lambda$-phage DNA (~$33 \times 10^{6}$) allowing for each molecule to bind to several receptors; indeed when we used a DNA of lower $M$, ($5 \times 10^{6}$) the number of DNA molecules bound increased to ~$4 \times 10^{6}$ (1). We surmise that the figure of $5 \times 10^{6}$ most accurately reflects the number of receptors per cell.

These two mAb should provide useful reagents for the further characterization of the DNA receptor, as they provide a simple strategy for its isolation to a high degree of purity. Jerne’s network hypothesis predicts that antibodies reactive with the ligand binding domain of a receptor will be reactive with an idiotope on antibodies to the ligand. We have presented preliminary evidence that a subset of anti-idotypic anti-DNA antibodies have anti-receptor activity (19); these two mAb may prove useful in exploring idiotypic mimicry of the DNA receptor.

## REFERENCES


