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An Antibody That Inhibits the Binding of Diphtheria Toxin to Cells Revealed the Association of a 27-kDa Membrane Protein with the Diphtheria Toxin Receptor*

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A monoclonal antibody that blocks the binding of diphtheria toxin to Vero cells was isolated by immunizing mice with Vero cell membrane. The antibody inhibits the binding of diphtheria toxin and also CRM197, a mutant form of diphtheria toxin, to Vero cells, and consequently inhibits the cytotoxicity of diphtheria toxin. This antibody does not directly react with the receptor molecule of diphtheria toxin (DTR14.5). Immunoprecipitation and immunoblotting studies revealed that this antibody binds to a novel membrane protein of 27 kDa (DRAP27). When diphtheria toxin receptor was passed through an affinity column made with this antibody, the receptor was trapped only in the presence of DRAP27. These results indicate that DRAP27 and DTR14.5 closely associate in Vero cell membrane and that the inhibition of the binding of diphtheria toxin to the receptor is due to the binding of the antibody to the DRAP27 molecule. Binding studies using ^{125}I-labeled antibody showed that there are many more molecules of DRAP27 on the cell surface than diphtheria toxin-binding sites. However, there is a correlation between the sensitivity of a cell line to diphtheria toxin and the number of DRAP27 molecules on the cell surface, suggesting that DRAP27 is involved in the entry of diphtheria toxin into the target cell.

Diphtheria toxin inhibits protein synthesis of eukaryotic cells by catalyzing the ADP-ribosylation of elongation factor-2, which results in its inactivation (1–4). The first step of intoxication by diphtheria toxin is binding of the toxin to a susceptible cell (5, 6). The toxin molecules bound to the cell surface are internalized by endocytosis (7), and their A fragments, at least, enter the cytoplasm to exert their effect. Intravesicular low pH is required for the entry of the toxin into the cytoplasm (8–12), and V type H$^+$-ATPase is involved in this step (13). Cells from a number of mammals, including humans and monkeys, are sensitive to diphtheria toxin, but mouse and rat cells are insensitive (14). The difference in sensitivity between species is primarily determined by the presence or absence of a specific receptor for diphtheria toxin on the cell surface (15–18). However, other biochemically undefined factor(s) also affect the sensitivity (19, 20).

Vero cells, derived from monkey kidney, are one of the cell lines most sensitive to diphtheria toxin. Recent biochemical studies on diphtheria toxin receptor have been facilitated by

the use of Vero cells. By immunoprecipitation of material chemically cross-linked to diphtheria toxin with anti-diphtheria toxin antibody, 10–20-kDa proteins were shown to be candidates for the diphtheria toxin receptor (5). Using a partially purified diphtheria toxin receptor fraction, we demonstrated that a 14.5-kDa protein (DTR14.5) in Vero cell membrane is the diphtheria toxin receptor or at least a diphtheria toxin-binding molecule in the receptor complex (6). Moreover, in the accompanying paper (21), we described the purification of DTR14.5 from Vero cell membrane. We also suggested that DTR14.5 forms a complex with itself of another protein.

For further analysis of the diphtheria toxin receptor and related proteins, we isolated monoclonal antibodies to those molecules. We describe here a monoclonal antibody that inhibits the binding of diphtheria toxin to the receptor. This antibody revealed the association of diphtheria toxin receptor with a novel 27-kDa protein that we call DRAP27 (diphtheria toxin receptor-associated protein).

MATERIALS AND METHODS

Buffers—PBS(−): 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.2; PBS(+): 137 mM NaCl, 2.7 mM KCl, 0.2 mM CaCl$_2$, 0.2 mM MgCl$_2$, 10 mM phosphate buffer, pH 7.2; Buffer B: 10 mM phosphate buffer, 30 mM octyl β-D-glucoside, pH 7.2; Buffer C: 150 mM NaCl, 10 mM phosphate buffer, 20 μg/ml antipain, 20 μg/ml leupeptin, 10 μg/ml chymostatin, pH 7.2; Buffer E: 0.3 mM NaCl, 10 mM MES,[1] 30 mM octyl β-D-glucoside, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) at pH 6.0; Buffer F: 10 mM phosphate buffer, 30 mM octyl β-D-glucoside, 0.15 mM NaCl, 0.2 mM CaCl$_2$, 0.2 mM MgCl$_2$, 0.2 mM PMSF, 1 mg/ml BSA, pH 7.0; Buffer P(7.0): 50 mM Tris buffer, 15 mM octyl β-D-glucoside, 0.15 mM NaCl, 10 mM KCl, 0.2 mM CaCl$_2$, 0.2 mM MgCl$_2$, 0.2 mM PMSF, 3 mM NaN$_3$, 1 mg/ml ovalbumin, 1 mg/ml gelatin, pH 7.0; Buffer P(6.0): same as Buffer P(7.0) except for 50 mM MES in place of 50 mM Tris, pH 6.0; TTBS: 10 mM Tris buffer, 0.3 M NaCl, 0.05% Tween 20, pH 7.5; TMBS: 20 mM MES, 0.3 M NaCl, 0.05% Tween 20, 0.2 mM CaCl$_2$, pH 6.0; affinity buffer: 10 mM Hepes buffer, 30 mM octyl β-D-glucoside, 0.3 M NaCl, 10 mM KCl, 0.2 mM CaCl$_2$, 0.2 mM MgCl$_2$, 0.2 mM PMSF, 3 mM NaN$_3$, pH 7.0.

Diphtheria Toxin and Related Proteins—Diphtheria toxin and CRM197 were produced as described previously (22). The nicked form of CRM197 was prepared by treatment with trypsin (23).

Production and Purification of Monoclonal Antibodies—Eight-week-old BALB/c mice were immunized by subcutaneous injection of 5 ↑g of Vero cell membrane fraction prepared by an alkali extraction method (6). Spleen cells from the immunized mice were fused with mouse myeloma cells X63-Ag8-653 by polyethylene glycol (24,

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[1] The abbreviations used are: MES, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NMEM, minimum Eagle's medium containing nonessential amino acids.

25), and the fused cells were cultured in Dulbecco's modification of Eagle's medium containing 15% fetal calf serum, 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT medium). Hybridomas selected in HAT medium were screened for the ability to inhibit the binding of ^{125}I-CRM197 to Vero cell monolayers (the details are described below). The cloned hybridoma cells were injected into the peritoneum of BALB/c mice pretreated with 2,6,10,14-tetramethylpentadecane, and about 2 weeks later, ascitic fluid was collected. The antibody was precipitated with 50% saturated ammonium sulfate, dissolved in 20 mM Tris-HCl, 30 mM NaCl, pH 8.0, and dialyzed against the same buffer. Then the antibody solution was applied to a DEAE-Sephacel column equilibrated with the above buffer and eluted with NaCl gradient. Each fraction was tested for inhibition ^{125}I-CRM197 binding, and the fractions containing the activity were collected. For assay of the binding of ^{125}I-labeled antibody to cells, this antibody fraction was further purified by gel filtration with Sephacryl S-300 and by high performance liquid chromatography using a PL-SAX ion exchange column (Polymer Laboratories Ltd.).

Assay of Antibodies for Inhibition of the Binding of ^{125}I-CRM197 to Vero Cells—Nicked CRM197 was labeled with Na^{125}I using Enzymobeads (Bio-Rad) as described (28). The specific activity of CRM197 used for this assay was 1.5–2 × 10^7 cpm/μg. Vero cells were seeded in 12-well multiplates at 2 × 10^5 cells/well and cultured overnight at 37 °C with NMEM (minimum Eagle's medium containing nonessential amino acids) and 10% calf serum. The medium was replaced with Hepes-buffered NMEM, pH 7.1, containing 10% calf serum. Antibody samples were added to the cells, and the cells were incubated for 2 h at 4 °C. ^{125}I-CRM197 was added at 50 ng/ml, and the cells were incubated for 4 h at 4 °C. Cells were washed three times with PBS(+) and lysed with 1 ml of 0.1 N NaOH, and the radioactivity of the lysate was counted with a γ-counter. Nonspecific binding of ^{125}I-CRM197 was assessed in the presence of a 1,000-fold excess of unlabeled diphtheria toxin.

Cytotoxicity of Diphtheria Toxin—Cytotoxicity of diphtheria toxin was measured by assaying the rate of protein synthesis in cultured cells as described previously (27).

Purification of Diphtheria Toxin Receptor—The purification procedure was essentially the same as the method described in the accompanying paper (21). The Vero cell membrane fraction was obtained by an alkali extraction method (6). The membrane fraction was solubilized under the following conditions: 6–7 mg of protein/ml, 60 mM octyl β-D-glucoside, 0.4 M NaCl, and 10 mM phosphate-buffer at pH 7.2, incubated on ice for 60 min. The undissolved materials were removed by centrifugation at 100,000 × g for 60 min. The clear supernatant was used as the solubilized membrane fraction.

The solubilized membrane fraction was diluted with Buffer B to adjust the NaCl concentration to 0.19 M and applied to a CM-Sepharose column equilibrated with Buffer B. The column was washed with Buffer B containing 0.19 M NaCl and eluted with a linear gradient of 0.19–1 M NaCl in Buffer B. The CRM197-binding activity in each fraction was determined by the acetone-precipitation method described in the accompanying paper (21). The main fractions containing the CRM197-binding activity were combined and used as the CM fraction.

The CM fraction was further purified by affinity chromatography. The CM fraction was applied to a CRM197-conjugated Sepharose 4B column equilibrated with Buffer E. After washing with Buffer E, the column was eluted with 0.2 M NaCl, 30 mM octyl β-D-glucoside, 0.2 mM PMSF, and 0.1 M glycine HCl buffer at pH 2.5. Each eluted fraction was neutralized with a quarter-volume of 0.5 M phosphate buffer at pH 7.0. The CRM197-binding activity was measured, and the fractions with binding activity were collected and used as the diphtheria toxin affinity fraction.

Purification of DRAP27—The flow-through fraction of the solubilized Vero cell membrane from CM-Sepharose chromatography was applied to an antibody 007-conjugated Sepharose 4B column equilibrated with affinity buffer. The column was washed with this buffer and then was eluted with 50 mM sodium acetate HCl buffer, 0.15 M NaCl, 30 mM octyl β-D-glucoside, 0.2 mM CaCl$_2$, 0.2 mM MgCl$_2$, pH 3.0. Each eluted fraction was neutralized with 5% volume of 1 M NaHCO$_3$ to pH 7.0.

Immunoprecipitation with Monoclonal Antibody or Diphtheria Toxin—Vero cell membrane was radioiodinated with Bolton-Hunter reagent in 50 mM borate buffer, pH 9.1. The labeled membrane sample (5 × 10^7 cpm) was incubated at 4 °C for 12 h with antibody 007 or BSA at the concentration of 200 μg/ml in 10 mM phosphate buffer, 0.3 M NaCl, 0.2 mM PMSF, pH 7.0, with gentle shaking. Then the

membrane was solubilized with 60 mM octyl β-D-glucoside at 4 °C for 1 h, and the insoluble materials were removed by centrifugation. Sepharose beads conjugated with goat anti-mouse IgG antibody were added to the solubilized material, and the mixture was incubated at 4 °C for 8 h with gentle shaking. After washing with buffer containing 50 mM Tris, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.01% SDS, 20 μg/ml leupeptin, 20 μg/ml antipain, pH 7.0, the material bound to the gels was subjected to SDS-PAGE (28). The gel was fixed, dried, and autoradiographed.

The CM fraction of diphtheria toxin receptor was labeled with Bolton-Hunter reagent. The labeled material (1.5 × 10^7 cpm) was incubated either with 5 μg/ml antibody 007 in Buffer P(7.0) or with 0.3 μg/ml diphtheria toxin in Buffer P(6.0). Sepharose beads conjugated with goat anti-mouse IgG antibody or with horse anti-diphtheria toxin antibody was added, and each mixture was incubated at 4 °C for 8 h with gentle shaking. The Sepharose beads were washed with the appropriate Buffer P containing 0.01% SDS, and the material bound to the gels was analyzed by SDS-PAGE and autoradiography.

Western Blot and ^{125}I-CRM197 Blot Analysis—The solubilized Vero cell membrane, CM fraction, and affinity fraction of diphtheria toxin receptor were incubated with SDS-gel sample buffer at room temperature for 12 h without reducing agent. Four sets of these protein samples were run on 15% SDS gels and then transferred to nitrocellulose filters (Schleider & Schuell BAS 85) by electroblotting. The filters were treated with blocking solution (PBS(−) containing 1% gelatin) for 12 h at 24 °C. Then, two of the filters were used for analysis of antibody 007 binding; one of the filters was treated with 100 ng/ml of ^{125}I-antibody 007 in TTBS containing 1 mg/ml BSA and 0.1% gelatin and the other with ^{125}I-antibody 007 plus 1,000 times more unlabeled 007 antibody. The remaining two filters were treated with ^{125}I-CRM197 with or without excess unlabeled CRM197 in TMBS containing 1 mg/ml BSA and 0.1% gelatin. After incubation at 4 °C for 12 h, the filters were washed, dried, and autoradiographed.

Adsorption Assay of Membrane Proteins to an Antibody 007 Affinity Column—Antibody 007 was conjugated to CNBr-activated Sepharose beads (1.5 mg of antibody/ml of gel). The gel (500 μl) was packed in a small column equilibrated with affinity buffer. Three kinds of samples containing diphtheria toxin receptor were applied to the column: 1) solubilized Vero cell membrane, 2) the CM fraction containing DTR14.5, and 3) a mixture of the CM fraction and purified DRAP27. In order to adsorb completely, the sample was recycled for 12 h at 4 °C. After the column was washed with 15 ml of affinity buffer, material bound to the column was eluted with 0.2 M glycine HCl, pH 3.0, 30 mM octyl β-D-glucoside, 0.2 mM CaCl$_2$, 0.2 mM MgCl$_2$. The CRM197-binding activity in the eluted fractions was measured by the acetone precipitation assay (21).

Binding of ^{125}I-labeled Antibody 007 to Cells—Purified antibody 007 was radioiodinated with Na^{125}I using Enzymobeads (1.0 × 10^7 cpm/μg). Various concentrations of ^{125}I-labeled antibody 007 (0.05–3 μg/ml) were added to cell monolayers (2 × 10^5 cells), and the cells were incubated for 12 h at 4 °C in the presence or absence of 300 μg/ml of unlabeled antibody 007. Cells were washed with PBS(+), lysed with 0.1 N NaOH, and the radioactivity in the lysate was counted in a γ-counter. The specific binding of antibody 007 was calculated by subtracting the counts obtained with cells incubated with unlabeled antibody 007 from those obtained with ^{125}I-antibody 007 alone.

RESULTS

Isolation of a Monoclonal Antibody That Inhibits the Binding of Diphtheria Toxin to Cells—In order to obtain a monoclonal antibody that recognizes the diphtheria toxin receptor or a related protein, we immunized mice with Vero cell membrane. The Vero cell line, derived from monkey kidney, is highly sensitive to diphtheria toxin and contains a relatively high number of diphtheria toxin receptors on the cell surface (14). The culture media of the hybridoma clones were screened for their ability to inhibit ^{125}I-CRM197 binding to Vero cells. About 1,200 HAT-positive hybridoma clones were screened and one clone secreted an IgG class antibody with the desired character. This antibody, referred to as antibody 007, was produced in the abdomens of mice, purified, and used for further studies.

When the purified antibody was added to Vero cell cultures, the binding of ^{125}I-CRM197 was blocked in a dose-dependent

manner (Fig. 1). Since the antibody inhibited the binding of native diphtheria toxin, the inhibitory effect is not specific for CRM197 (data not shown). However, CRM197 was more suitable for screening hybridoma clones, as the culture fluid of hybridoma cells often inhibited the binding of diphtheria toxin to Vero cells. This inhibitory activity was not derived from antibody and was probably due to the nucleotide-like substance we described previously (6). The binding of CRM197 to Vero cells was not affected by this inhibitor.

The effect of the antibody on the cytotoxicity of diphtheria toxin was also determined by assaying the rate of cellular protein synthesis (Fig. 2). Without the antibody, diphtheria toxin reduced protein synthesis to about 21% of the control. In the presence of the antibody at 10 or 100 μg/ml, protein
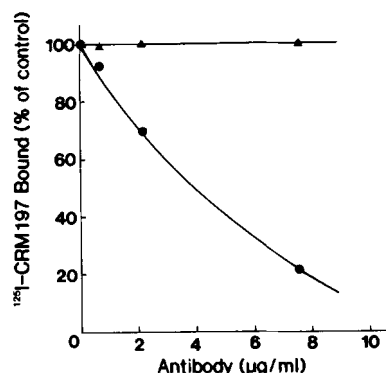

FIG. 1. **Inhibition of ^{125}I-CRM197 binding to Vero cells by antibody 007.** Vero cells were incubated with various amounts of antibody 007 (●) or control (anti-human insulin) monoclonal antibody (▲) for 2 h at 4 °C. Then, ^{125}I-CRM197 was added to the cells, which were incubated for 4 h at 4 °C. The cells were washed with PBS(+) and lysed with 0.1 N NaOH, and the radioactivity of the lysate was counted. The specific binding of ^{125}I-CRM197 was calculated under "Materials and Methods." The values are expressed as percent of specific binding for control without antibody.
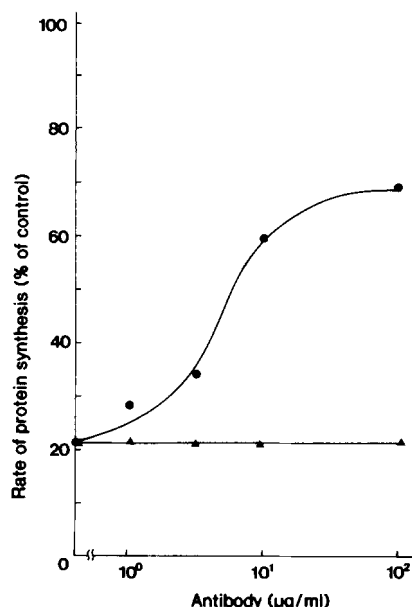

FIG. 2. **Inhibition of diphtheria toxin cytotoxicity by antibody 007.** Vero cells were incubated with various amounts of antibody 007 (●) or control (anti-human insulin) monoclonal antibody (▲) for 10 min at 24 °C. Diphtheria toxin (1 ng/ml) was added, and the cells were incubated for 2 h at 37 °C. Then, the cells were labeled with [^{3}H]leucine for 1 h at 37 °C to measure the rate of protein synthesis. The values are expressed as percent of protein synthesis for control without antibody or diphtheria toxin.

synthesis was higher, about 60 or 70% of control, respectively. The inhibitory effect of this antibody for diphtheria toxin cytotoxicity was also observed using HEL cells derived from human embryonic lung, and FL cells derived from human amnion.

This monoclonal antibody was obtained by immunization with Vero cell membrane, and there was no reason to suspect that diphtheria toxin molecules might be present in the membrane fraction. Thus, it was unlikely that this antibody would react directly with diphtheria toxin and inhibit its binding. However, to eliminate this possibility, we carried out two experiments. One was a binding assay using a diphtheria toxin-coated enzyme-linked immunosorbent assay plate. Antibody 007 was added to the plate, and the bound antibody was detected by enzyme-linked second antibody. No significant binding of antibody 007 to diphtheria toxin plates was observed (data not shown). The other experiment was a Western blot of diphtheria toxin probed with this antibody. As shown in Fig. 4A, lane 4, no binding of this antibody to diphtheria toxin was observed. Therefore, inhibition of cytotoxicity and the binding of diphtheria toxin are not due to the binding of this antibody directly to diphtheria toxin.

The Antibody Recognizes a 27-kDa Protein (DRAP27)— Recently, diphtheria toxin receptor has been purified from Vero cells. The 14.5-kDa protein (DTR14.5) is the major form of the diphtheria toxin-binding molecule in Vero cell membrane (6, 21). In the early phase of this work, we thought that antibody 007 might inhibit the binding of diphtheria toxin to Vero cell by interaction with DTR14.5. To identify the molecule recognized by antibody 007, we carried out immunoprecipitation of Vero cell membrane components. The radioiodinated membrane was incubated with antibody 007 and then solubilized with octyl β-D-glucoside. Sepharose beads conjugated with anti-mouse IgG were added, and the precipitated material was subjected to SDS-PAGE and visualized by autoradiography. As shown in Fig. 3A, only one 27-kDa protein was precipitated (DRAP27). In some preparations, immunoprecipitates gave broader bands ranging between 24 and 27 kDa. However, neither the 14.5-kDa protein (DTR14.5) nor the related 20-kDa diphtheria toxin-binding molecules (5) were precipitated in any preparations.

There was a possibility that antibody 007 binds DTR14.5 as well as DRAP27, but DTR14.5 could not be detected because of its lower abundance. To clarify this point, we carried out immunoprecipitation using a more concentrated diphtheria toxin receptor fraction. The diphtheria toxin receptor fraction purified by CM-Sepharose was radioiodinated with Bolton-Hunter reagent and incubated with antibody 007 and immobilized second antibody. The same material was also treated with diphtheria toxin and anti-diphtheria toxin antibody as described previously (6). As shown in Fig. 3B, DTR14.5 was precipitated by diphtheria toxin but not by antibody 007.

The molecule recognized by antibody 007 was also analyzed by Western blotting. Vero cell membrane or partially purified diphtheria toxin receptor fractions were electrophoresed in SDS gels and transferred to a nitrocellulose filter. One set of filters was probed with ^{125}I-antibody 007, the other with ^{125}I-CRM197. The filter probed with ^{125}I-CRM197 showed the 14.5 kDa band in the lanes of purified diphtheria toxin receptor samples (Fig. 4B, lanes 2 and 3). On the filter probed with ^{125}I-antibody 007, the 27-kDa band was clearly observed in the crude membrane sample (Fig. 4A, lane 1), but the 14.5 kDa band was not labeled in the crude membrane sample or purified diphtheria toxin receptor lanes. The specific activities of the ^{125}I-antibody 007 and ^{125}I-CRM197 used for this exper-

A

1   2

75—

50—

39—

27—

17—

B

1   2   3   4

92.5—
69—

46—

30—

14.3—
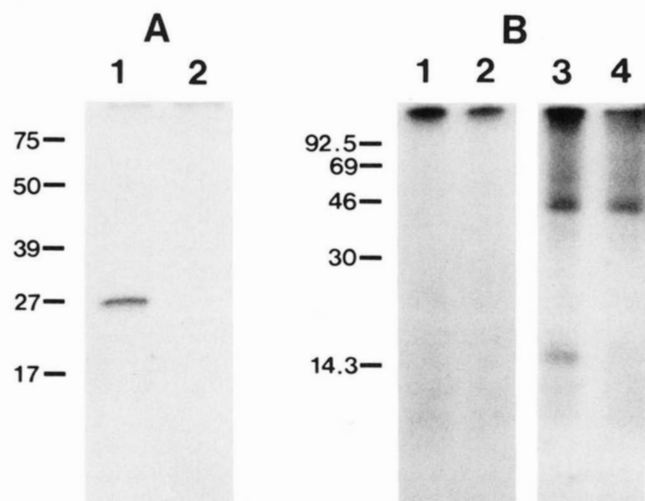
FIG. 3. **Immunoprecipitates with antibody 007.** *A*, Vero cell membrane fraction was radioiodinated and incubated with antibody 007 (*lane 1*) or irrelevant (anti-human insulin) monoclonal antibody (*lane 2*) for 12 h at 4 °C. The mixture was solubilized, precipitated with immobilized anti-mouse IgG antibody, and analyzed by SDS-PAGE. *B*, CM fraction of diphtheria toxin receptor from Vero cell was radioiodinated and divided. Samples were incubated with antibody 007 (*lane 1*) or irrelevant antibody (*lane 2*) for 12 h at 4 °C, and then precipitated with immobilized anti-mouse IgG antibody. Other samples were incubated with diphtheria toxin (*lane 3*) or BSA (*lane 4*) for 12 h at 4 °C, and precipitated with immobilized antidiphtheria toxin antibody. The precipitated molecules were analyzed by SDS-PAGE (15% gel).

A

1   2   3   4

130—
75—
50—

39—

27—

17—

B

2   3
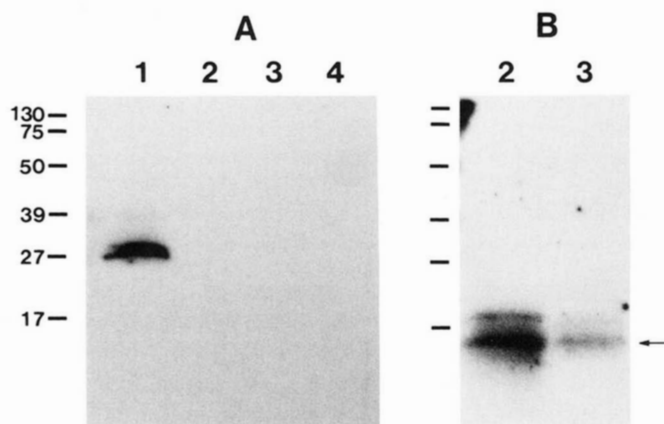
FIG. 4. **Analysis of diphtheria toxin receptor fractions by blotting with ^{125}I-antibody 007 or ^{125}I-CRM197.** Samples from each step of the diphtheria toxin receptor purification were run on SDS-PAGE (15% gel) and transferred to nitrocellulose filters. Filters were probed with 100 ng/ml of ^{125}I-007 antibody (*A*) or ^{125}I-CRM197 (*B*). Then the filters were washed, dried, and autoradiographed. (*Lane 1*, solubilized membrane fraction; *lanes 2*, CM fraction; *lanes 3*, affinity fraction; *lane 4*, diphtheria toxin.) Only the 27 kDa band is detected when ^{125}I-antibody 007 was used as a probe.

iment were similar (1.9×10^7 cpm/μg for diphtheria toxin and 1.7×10^7 cpm/μg for antibody 007). From these results, we concluded that antibody 007 recognizes DRAP27 and does not recognize DTR14.5. Therefore, the antibody must inhibit the binding of diphtheria toxin to diphtheria toxin receptor by its binding to DRAP27. Figs. 3 and 4 also show that DRAP27 was not co-eluted with diphtheria toxin receptor from the CM-Sepharose column.

Effect of Antibody 007 on the Binding of CRM197 to Various Membrane Fractions Containing Diphtheria Toxin Receptor— If antibody 007 inhibits the binding of diphtheria toxin to the

receptor by binding to DRAP27, the antibody should not inhibit the binding of diphtheria toxin to diphtheria toxin receptor in fractions that do not contain DRAP27. We examined the effect of antibody 007 on ^{125}I-CRM197 binding using three kinds of receptor samples: Vero cell membrane (membrane fraction), acetone-precipitated material from solubilized membrane, and acetone-precipitated material from the CM fraction containing diphtheria toxin receptor. The CM fraction was free of DRAP27, which was confirmed by immunoblotting using antibody 007 as shown in Fig. 4. Parallel experiments were also performed using intact Vero cells. As shown in Fig. 5, antibody 007 at 30 μg/ml almost completely inhibited the binding of CRM197 to intact monolayer cells, whereas it did not inhibit the binding to the CM fraction. It was interesting that the antibody did not inhibit the ^{125}I-CRM197 binding to the acetone-precipitated solubilized membrane, although DRAP27 was present in this fraction. Furthermore, antibody 007 was less effective in blocking the binding to the membrane fraction than to intact Vero cells. These results indicate that the native membrane constitution is required for the inhibitory effect of the antibody.

Association of DRAP27 to DTR14.5—Antibody 007 inhibits the binding of diphtheria toxin to the diphtheria toxin receptor. However, this antibody does not react with the 14.5-kDa diphtheria toxin receptor, it reacts with DRAP27. It is likely that DRAP27 noncovalently associates with the DTR14.5 in the membrane. To examine this possibility, we carried out the following experiments. Diphtheria toxin receptor fractions, containing or not containing DRAP27, were applied to an antibody 007 affinity column, and it was determined whether diphtheria toxin binding activity was bound to the column. If DRAP27 associates with DTR14.5, diphtheria toxin receptor is expected to be adsorbed to the column together with DRAP27. As shown in Fig. 6, when solubilized Vero cell membrane fraction, which contained both DRAP27 and DTR14.5, was applied to an antibody 007 column, diphtheria toxin-binding activity was trapped by the column and was eluted with DRAP27 by low pH buffer. When diphtheria toxin receptor fraction purified by CM Sepharose, which did
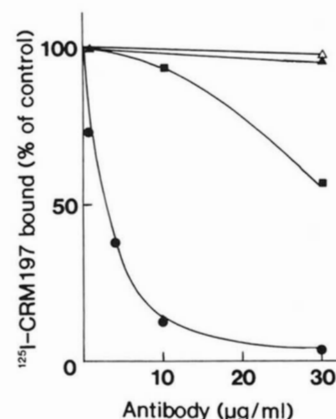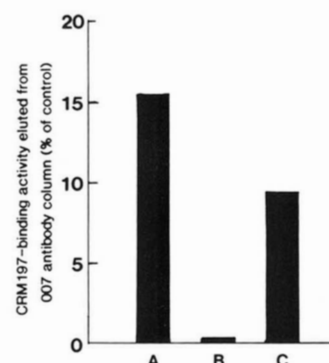
FIG. 5. **Comparison of the effect of antibody 007 on the binding of ^{125}I-CRM197 to various diphtheria toxin receptor fractions.** Vero cells or membrane fractions were incubated with various amounts of antibody 007 at 4 °C for 2 h, and 30 ng/ml ^{125}I-CRM197 was added to the mixture. After incubation at 4 °C for 4 h, specific binding was determined under "Materials and Methods." The solubilized membrane or CM fraction of diphtheria toxin receptor was precipitated by the acetone precipitation method as described (21) and suspended with Hepes-NMEM, and the effect of the antibody for the binding of ^{125}I-CRM197 was determined. The data was expressed as percent of control value without antibody for each sample. ●, Vero cell; ■, alkali membrane; ▲, solubilized membrane; △, CM fraction.

A

B
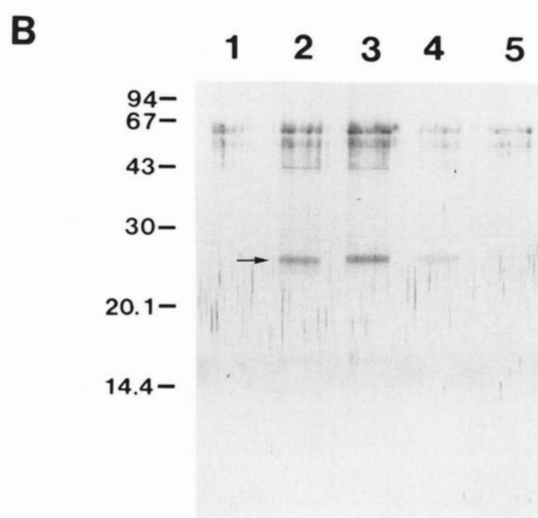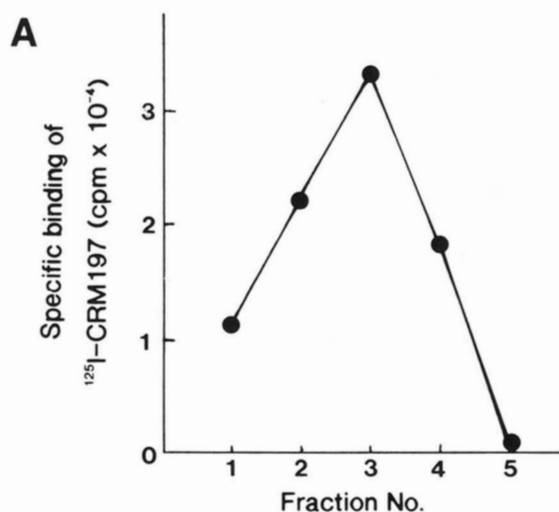
FIG. 6. **Co-elution of the CRM197-binding activity with DRAP27 from an antibody 007 affinity column.** The solubilized membrane fraction was applied to an antibody 007 affinity column. After the column was washed with affinity buffer, the material bound to the column was eluted with elution buffer. Eluted fractions were assayed for CRM197-binding activity (*A*) or analyzed by SDS-PAGE (15%) with silver staining (*B*). The 27 kDa bands are shown by the *arrows*. The CRM197-binding activity was co-eluted with DRAP27.

FIG. 7. **Trapping of the CRM197-binding activity on an antibody 007 column in the presence of DRAP27.** Diphtheria toxin receptor fractions were applied to an antibody 007 column, which was washed and eluted with elution buffer. CRM197-binding activities of eluted fractions were measured. The results were expressed as a percent of the initial activity applied to the column. *A*, solubilized membrane; *B*, CM fraction; *C*, CM fraction + DRAP27.
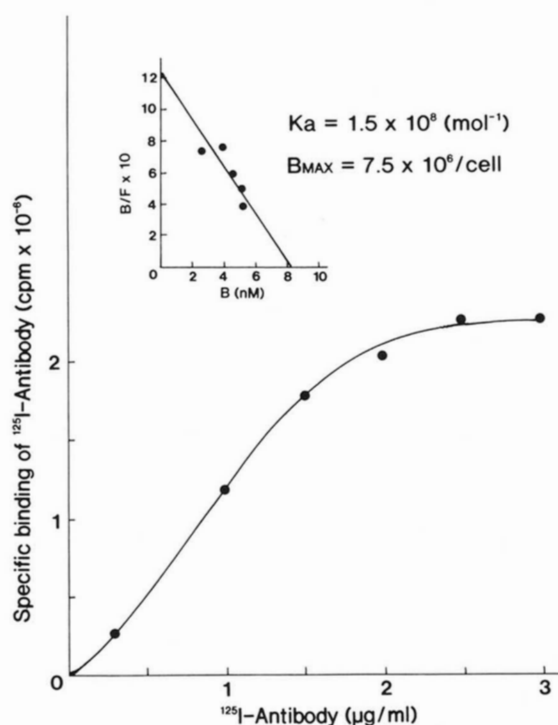
FIG. 8. **Binding of ^{125}I-antibody 007 to Vero cells.** Vero cells were incubated with various amounts of ^{125}I-antibody 007 in the presence or absence of 300 μg/ml of unlabeled antibody 007. Then the cells were washed with PBS(+) and lysed with 0.1 N NaOH, and the radioactivity of the lysate was counted with a γ-counter. The data is expressed as specific binding, which was calculated by subtracting the nonspecific binding from total counts. *Inset*, Scatchard plot of the specific binding of ^{125}I-007 antibody to Vero cells.

not contain DRAP27, was applied to the column, diphtheria toxin-binding activity was not adsorbed (Fig. 7). However, when the purified DTR14.5 sample was mixed with DRAP27 and then the mixture was applied to the column, diphtheria toxin-binding activity was adsorbed. These results show that diphtheria toxin-binding activity was adsorbed to the antibody-conjugated column only in the presence of DRAP27 and strongly suggest that DRAP27 associates with DTR14.5. In the above experiments, the amount of the binding activity trapped to the column was only about 15% of total binding activity. This trapping efficiency was not improved by using a larger column, indicating that some fractions of DTR14.5 dissociated from DRAP27 under the experimental conditions. It should be noted that DRAP27 did not co-elute with DTR14.5 when DTR14.5 was purified from crude membrane fraction by CM-Sepharose chromatography. Therefore, the association of DRAP27 to DTR14.5 does not seem to be tight, at least in the presence of the detergent.

Correlation of the Amount of DRAP27 with Diphtheria

Toxin Sensitivity—As shown above, the binding of antibody 007 to DRAP27 results in the inhibition of the diphtheria toxin binding to diphtheria toxin receptor. Moreover, diphtheria toxin receptor associates with DRAP27. Thus, it is possible to speculate that DRAP27 also plays some role in the binding of the toxin. To further characterize DRAP27, we determined the number of DRAP27 molecules on the cell surface by measuring the binding of ^{125}I-labeled antibody 007. Fig. 8 shows the binding profile of ^{125}I-antibody 007 to Vero cells. Scatchard plot analysis showed that saturation occurred at about 7.5×10^6 sites/cell. If we assume that DRAP27 binds to the antibody at a ratio of 1:1, 7.5×10^6 molecules of DRAP27 are estimated to be present on the cell surface of

Association of 27-kDa Protein with Diphtheria Toxin Receptor

TABLE I

DRAP27 density on several cell lines

The cells were incubated with various concentrations of ^{125}I-antibody 007 for 12 h at 4 °C in the presence or absence of excess unlabeled antibody. The specific binding of antibody was calculated by subtracting the counts obtained with cells incubated with unlabeled antibody from that obtained with ^{125}I-antibody 007 alone. The maximum binding was calculated from the plateau of the binding curve. ND, not determined.

Cell line	No. of DRAP27/ cell	Binding sites of diphtheria toxin/cell[a]	Receptor density (relative values)[b]	Sensitivity[c]
				ng/ml
Vero	7.5×10^6	$0.5-1.5 \times 10^4$	100	2
HEL	1.0×10^5	ND	11.5	3
FL	7.8×10^4	ND	4.9	25
L	1.9×10^3	ND	0.05	2×10^4

[a] The data were referred from Mekada and Uchida (26).

[b] In the case of HEL, FL, and L cells, the binding site numbers of diphtheria toxin have not been determined because of a too low density of the receptor in these cell lines. The values listed here were estimated from the data obtained by the method using methylamine (17).

[c] Sensitivity was expressed as the concentration of toxin required to reduce the rate of protein synthesis to 50% of the control value (CD_{50}).

single Vero cell. This value is about 700-fold higher than the number of diphtheria toxin receptors (26). We also determined the saturation values for antibody 007 on other cell lines. The results are summarized in Table I. The table also shows the binding site numbers of diphtheria toxin and the relative sensitivities of these cell lines to the toxin. In all cell lines tested, the saturation values of each cell line for antibody 007 seemed to be much larger than for diphtheria toxin. It is interesting that there is a correlation between the sensitivity to diphtheria toxin and the number of DRAP27 molecules (binding sites for antibody 007). This correlation supports the notion that DRAP27 has some role in binding or entry of diphtheria toxin.

DISCUSSION

We described here the characteristics of a monoclonal antibody obtained by immunization with Vero cell membrane. This antibody completely inhibits the binding of diphtheria toxin and CRM197 to intact Vero cells and consequently inhibits the cytotoxicity of diphtheria toxin. A 27-kDa membrane protein (DRAP27) was specifically precipitated by this antibody. The antibody does not react with the 14.5-kDa diphtheria toxin receptor from Vero cells. Therefore, we concluded that the inhibitory effect of this antibody on the binding of diphtheria toxin to Vero cells is due to the binding of this antibody to DRAP27. This was supported by the fact that antibody 007 does not inhibit the binding of CRM197 to partially purified diphtheria toxin receptor fraction that does not contain DRAP27.

How does the antibody 007 inhibit the binding of diphtheria toxin to diphtheria toxin receptor? The fact that antibody 007 had little or no inhibitory effect on binding of CRM197 to isolated membrane or solubilized membrane is suggestive. Furthermore, as we showed, diphtheria toxin receptor associates with DRAP27. Therefore, it is likely that binding of the antibody to DRAP27 sterically hinders the binding of diphtheria toxin to the receptor. However, there are other possibilities, for example the antibody could cause the removal of DTR14.5, along with DRAP27, from the membrane of intact cells.

CRM197-binding activity was adsorbed to an antibody 007 affinity column only in the presence of DRAP27, indicating that diphtheria toxin receptor associates with, or have some affinity to, DRAP27. Since the DTR14.5 used for this experiment was not completely purified, it is possible that other factors mediate the association of DTR14.5 with DRAP27. To demonstrate the association of DTR14.5 with DRAP27 more directly, we attempted to co-precipitate DTR14.5 using antibody 007. In some preparations, DTR14.5 was specifically and clearly precipitated by antibody 007 in the presence of DRAP27, but the experiments were not highly reproducible. Treatment with detergent may weaken the association of the two molecules. Moreover, the molecular ratio of DRAP27 to diphtheria toxin receptor is about 700 in Vero cells. Thus, most of the DRAP27 may not be associated with diphtheria toxin receptor. The presence of this unassociated DRAP27 is probably another reason for the failure of co-precipitation.

We do not know whether DRAP27 is a protein that has already been defined. We only know that DRAP27 is a membrane protein and the antibody reacts to it at the extracellular surface. Does DRAP27 play some role in the binding and toxication step of diphtheria toxin? The purified DTR14.5 clearly binds to diphtheria toxin in the absence of any other proteins. Thus, the existence of DRAP27 does not seem to be essential for the binding activity of the diphtheria toxin receptor. However, the amount of DRAP27 on the surface of various cell lines correlates with their sensitivity to diphtheria toxin. Furthermore, we suggested previously that DTR14.5 forms a complex with other proteins (6). Thus, it is conceivable that DRAP27, or at least some subpopulation of DRAP27 molecules, is a component of the diphtheria toxin receptor complex. In the complex, DRAP27 could serve to stabilize DTR14.5, or more directly, it could be involved in the entry of diphtheria toxin into the cytoplasm. To clarify whether DRAP27 plays some role in the sensitivity to diphtheria toxin and to understand its function, the genetic approach will be useful. We are proceeding to clone the cDNA of DRAP27 using antibody 007.

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