Biological Activities and Pore Formation of *Clostridium perfringens* Beta Toxin in HL 60 Cells*

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*Clostridium perfringens* beta toxin is an important agent of necrotic enteritis. Of the 10 cell lines tested, only the HL 60 cell line was susceptible to beta toxin. The toxin induced swelling and lysis of the cell. Treatment of the cells with the toxin resulted in K⁺ efflux of the cells and Ca²⁺, Na⁺, and Cl⁻ influxes. These events reached a maximum just before the cells were lysed by the toxin. Incubation of the cells with the toxin showed the formation of toxin complexes of about 191 and 228 kDa, which were localized in the domains that fulfilled the criteria of lipid rafts. The complex of 228 kDa was observed until 30 min after incubation, and only the complex of 191 kDa remained after 60 min. Treatment of the cells with methyl-β-cyclodextrin or cholesterol oxidase blocked binding of the toxin to the rafts and the toxin-induced K⁺ efflux and swelling. The toxin-induced Ca²⁺ influx and morphological changes were inhibited by an increase in the hydrodynamic diameter of polyethylene glycols from 200 to 400 and markedly or completely inhibited by polyethylene glycol 600 and 1000. However, these polyethylene glycols had no effect on the toxin-induced K⁺ efflux. The toxin induced carboxyfluorescein release from phosphatidylcholine-cholesterol liposomes containing carboxyfluorescein and formed an oligomer with 228 kDa in a dose-dependent manner but did not form an oligomer with the 191-kDa complex. We conclude that the toxin acts on HL 60 cells by binding to lipid rafts and forming a functional oligomer with 228 kDa.

*Clostridium perfringens* beta toxin is known to be the primary pathogenic factor of necrotic enteritis in the type C strains that produce beta toxin. The toxin was purified from the supernatant fluid of a culture controlled at pH 7.5 using an anti-alpha toxin affinity column (1) or Toyopearl HW 60 column (2). Beta toxin possesses lethal, dermonecrotic, and presor activities (3). Recently, we also reported that the plasma extravasation induced by beta toxin in mouse skin is mediated via a mechanism involving tachykinin NK1 receptors (4).

By using an oligonucleotide probe designed on the basis of the N-terminal sequence of the purified toxin, the beta toxin gene was isolated from *C. perfringens* type B (5). The deduced amino acid sequence of beta toxin was found to have significant homology with that of *Staphylococcus aureus* alpha toxin (28% similarity), the A and B components of gamma toxin (22 and 28% similarity, respectively), and the S and F components of leukocidin (17 and 28%, respectively) (5). The alpha toxin is known to form an oligomer in biological membranes and artificial membranes (6–8). The alpha toxin is the prototype of a family of these toxins with membrane-damaging activity. On the basis of crystallographic findings of the alpha toxin, the structure of the oligomer of the alpha toxin is divided into four domains. First, the cap of the oligomer is composed of seven β-sandwiches and the N-terminal region. Second, the stem domain forms a transmembrane channel. Third, the rim domain protrudes from the underside of the oligomer, participates in some protomer-protomer interactions, and interacts with the lipid bilayer. Fourth, the triangle region participates in a crucial protomer-protomer interaction (9). Beta toxin has regions corresponding to the cap, the triangle region, the rim domain, and the stem domain of alpha toxin, according to the conserved amino acid sequences of the family (5).

Beta toxin has only one cysteine residue at position 265 (5). Furthermore, the toxin is inactivated by thiol group modifying reagents (10, 11). It therefore was speculated that the Cys residue is important in the lethal activity of the toxin. However, Steinhorsdottir et al. (12) reported that replacement of the Cys residue did not affect the toxin activity. Furthermore, we also reported that the replacement of Cys-265 with alanine did not affect the activity of beta toxin and resulted in thiol group reagents having no effect on the activity (13). The primary amino acid sequence surrounding Cys-265 in the C terminus of beta toxin (positions 255 to 276) is homologous to that at positions 245–267 in the C terminus of the alpha toxin (a conserved 11-amino acid sequence). Thus it appears that Cys-265 in beta toxin corresponds to Asp-255 in the alpha toxin. Walker and Bayley (14) reported that treatment of D254C and D255C (variant toxins of the alpha toxin) with sulfhydryl-modifying reagent, 4'-acetamido-4-((iodoacetylamino)stilbene-2,2'-disulphonate, resulted in a significant reduction or complete loss of binding, oligomer formation, and hemolytic activity, suggesting that the C terminus containing Asp-254 and -255 of the alpha toxin is implicated in binding to cells. Y266A and L268C surrounding Cys-265 in beta toxin showed no lethal activity and did not inhibit lethal activity of the toxin, suggesting that Tyr-266 and Leu-268 play a role in binding to the receptor (13). These observations suggest that the characteristics of beta toxin resemble those of the alpha toxin.

Steinhorsdottir et al. (15) showed that beta toxin formed oligomeric complexes on the membranes of human umbilical vein endothelial cells and induced the release of arachidonic acid and inositol from these cells. Shatursky et al. (16) hypothesized that the lethal action of beta toxin is based on the formation of cation-selective pores in the lipid bilayers. How-
ever, little is known about the precise mechanism of formation of oligomers of beta toxin and the relationship between the biological activities and oligomer formation of the toxin.

Direct evidence concerning the formation of the membrane pore and biological activities is lacking due to the absence of a susceptible cell line for in vitro studies of beta toxin activity. In this study, we attempted to find cell lines that are susceptible to beta toxin. We demonstrate the binding of the toxin to and oligomer formation of the toxin in biological membranes. Furthermore, we report here a relationship between the biological activities and oligomer formation of the toxin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Methyl-β-cyclohexetrin (MβCD),3 cholesterol, carboxyfluorescein, dioleoyl-L-a-phosphatidylcholine (DOPC), cholesterol ox- dase, filipin, nystatin, a protease inhibitor mixture, verapamil, 4-aminopyridine, tetraethylammonium, and quinine were obtained from Sigma. Polyethylene glycol 200, 300, 400, 600, and 1000 were purchased from Nacalai Tesque (Kyoto, Japan). o-Conotoxin, charybdotoxin, and margatoxin were purchased from Peptide Institute Inc. (Osaka, Japan). Mouse anti-caveolin-1 and Lyn antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-labeled sheep anti-mouse IgG were purchased from Amersham Biosciences. Dulbecco’s Eagle’s medium and fetal bovine serum were purchased from Invitrogen. The fluorescent probes, sodium green and N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE), were obtained from Molecular Probes (Eugene, OR). All other chemicals were of the highest grade available from commercial sources.

**Beta Toxin and Derivative**—The expression and purification of recombinant beta toxin was performed as described previously (15). To prepare beta toxin derivative (BTD) for labeling of the toxin, plasmid pTB-1 containing the entire beta toxin gene (13) was used as a template for PCR to add BamHI site and EcoRI site for subcloning into the vector using a forward primer 5’-GGGATCCAGTAGATATAGATAGAAAC-3’ (BamHI site is underlined) and a reverse primer 5’-GGGTTACG- GATATCGAACTAC-3’ (EcoRI site is underlined). The PCR prod- ucts digested with BamHI and EcoRI were inserted into pGEX-2TK (Amersham Biosciences). The resultant plasmid, named pGE282XT, enabled the expression of glutathione S-transferase (GST)-beta toxin derivative (GST-BTD) which contained a pentapeptide recognized by the cAMP-dependent protein kinase. The accuracy of the final DNA construction was confirmed by DNA sequencing.

**Cells**—Cell lines (Vero cells, CHO cells, CHO-K1 cells, COS-7 cells, intestine 407 cells, HeLa cells, MDCK cells, PC12 cells, mouse masto- cytoma P-815 cells, and HL 60 cells) were obtained from Riken Cell Bank (Tsukuba, Japan). Vero cells, CHO cells, CHO-K1 cells, COS-7 cells, intestine 407 cells, HeLa cells, and MDCK cells were cultured in Dulbecco’s Eagle’s medium supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. PC12 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum, 5% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. P-815 cells and HL 60 cells were cultured in RPMI 1640 medium containing 2% heat-inactivated fetal bovine serum, 100 unit/ml peni- cillin, 100 μg/ml streptomycin, and 2 mM glutamine. All incubation steps were carried out at 37 °C in a 5% CO2 atmosphere.

**Mouse Lethality Test and Cytotoxicity Assay**—The lethality of beta toxin and its derivative toward mice was determined as described previously (15). A group of 6 male ddY mice weighing about 25 g each was used. Each mouse was injected intravenously with 0.1 ml of the toxin solution, and deaths occurring within 24 h were recorded.

For cytotoxicity assays, cells were seeded in 48-well culture plates (1 × 105 cells in 0.2 ml/well) and cultured for 24 h at 37 °C. Beta toxin was added to each well and additionally incubated for a further 12 h. After the incubation, the cells in each well were washed with phosphate-buffered saline containing Mg2+ and Ca2+. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy- phenyl)-2(4-sulfophenyl)-2H-tetrazolium inner salt conversion assay (Promega, Madison, WI). The absorbance was read at 490 nm using an enzyme-linked immunosorbent assay plate reader. Percentage of cell viability was calculated as follows: the mean absorbance value of a toxin group/that of a control. Release of lactate dehydrogenase (LDH) from HL 60 cells was determined with a microplate assay according to the manufacturer’s instructions (Roche Applied Science).

**Ion Assays**—HL 60 cells (1 × 105 cells/ml) were inoculated in 6-well plates in Hanks’ balanced solution (HBSS). For the K+ efflux assay, the cells were incubated with the toxin at 37 °C for various times. K+ concentrations in the supernatants were determined at the given times with an atomic absorption spectrophotometer (Hitachi Z-8200, Tokyo, Japan). The Na+ activity was measured by Na+ K+ ATPase, and the Ca2+ concentration was measured in a microplate fluorometer MTP-32 (Corona Electric Co., Katsuda, Japan) with the following filters: excitation 485 nm and emission 538 nm for sodium green and excitation 355 nm and emission 475 nm for MQAE. The data are expressed as percents of fluorescence intensity from the toxin-untreated cells (control) (18).

**Estimation of the Functional Diameter of the Membrane Pore**—Toxin-induced lysis of HL 60 cells was assayed at 37 °C in HBSS containing polyethylene glycols of different sizes at a concentration equivalent to 40 mOsm. The total osmotic pressure of the extracellular space was adjusted to 295 mOsm by changing the concentrations of NaCl. The values for the hydrodynamic diameters of polyethylene glycols were taken from the reports of Stelzer and Gerstel (19) and Saburo et al. (20), where the hydrodynamic diameters were calculated on the basis of the viscosity of the polyethylene glycol solutions.

**Preparation of Radiolabeled Toxin Derivatives**—Beta toxin was radiola- beled with 125I by the following two methods. The purified toxin (15 μg) was incubated with 38.2 GBq of Na125I (643.8 GBq/mg; PerkinElmer Life Sciences) and IOAD-BEADS iodination reagent (Pierce) in 60 μl of phosphate-buffered saline for 15 min at room temperature. Beta toxin (15 μg) was incubated with 250 μCi of 125I-labeled Bolton-Hunter reagent (74 TBq/mmol; Amersham Biosciences) as described previously (17). Radiolabeled proteins were separated from free iodine by gel filtration on a Sephadex G-25 column (0.75 × 100 cm). To determine the yield of the reaction, the radioactivity in the supernatant was counted with a scintillation counter (Aloka Co., Tokyo, Japan).

**Phosphorylation of BTD with 32P** was performed as follows. Approximately 200 μg of GST-BTD was loaded onto a glutathione–Sepharose 4B column (bed volume, 200 μl; Amersham Biosciences), and then phosphorylated using the catalytic subunit of bovine heart protein kinase (Sigma) and [γ-32P]ATP (167 TBq/mmol; ICN Biochemicals, Costa Mesa, CA) according to the protocol recommended by the manu- facturer. The specific activity of 32P-labeled GST-BTD was about 32000 cpm/μg protein. 32P-BTD was prepared from 32P-labeled GST-BTD by gel filtration on a Sephacryl S-200 column (0.5 × 20 cm) and then dialyzed in 0.5M sodium phosphate buffer at pH 7.4 containing 150 mM NaCl. The specific activity of Na32P-labeled and 125I-labeled Bolton-Hunter reagent-labeled beta toxin was 440 and 560 kcpm/μg protein, respectively.

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**Flotation Centrifugation on a Sucrose Gradient**—HL 60 cells (1 × 106 cells/ml) were incubated with 1% Triton X-100. Each fluorescent probe was added to the lysed cells to a final concentration of 10 μM for sodium green (Na+ assay) and MQAE (Ca2+ assay). The cell suspensions were centrifuged at 17,100 rpm for 30 min at 4 °C. The supernatants were subjected to SDS-PAGE and autoradiographed.

**Immunoblot Analysis of Lipid Raft Marker Proteins**—Aliquots of the supernatants were subjected to SDS-PAGE and autoradiographed.
SDS-PAGE gel, followed by transfer to a polyvinylidene difluoride membrane. The membrane was blocked with 2% Tween 20 and 5% skim milk and incubated first with the primary antibody in TBS containing 1% skim milk, then with a horseradish peroxidase-conjugated secondary antibody, and finally with an enhanced chemiluminescence analysis kit (Amersham Biosciences).

**Cholesterol Depletion and Repletion**—To remove cholesterol, HL 60 cells (2–4 × 10⁶ cells/ml) were incubated for 1 h at 37 °C in the presence or absence of 10 mM MβCD in HBSS and then washed with HBSS. For the cholesterol repletion experiment (22), 40 mg of cholesterol was coated on the walls of a glass tube, and 5 ml of 50 mM MβCD in HBSS was added and incubated after sonication for 15 h at 37 °C. The resulting solution (50 mM MβCD/cholesterol) was filtered and added back to cholesterol-depleted cells (2–10⁶ cells/ml) by incubation for 2 h at 37 °C with the indicated concentrations of MβCD/cholesterol. Cholesterol contents were assayed spectrophotometrically using a diagnostic kit (Cholesterol C-Test, Wako Pure Chemical, Osaka, Japan).

**Liposomes**—DOPC-cholesterol (1:1) liposomes containing carboxyfluorescein (CF) were prepared, and CF release was monitored by a procedure described previously (23). The binding of ³²P-BTD to liposomes was performed as described previously (23).

**RESULTS**

**Morphological Alterations of Cells Induced by Beta Toxin**—HL 60 cells were small and round, and displayed distinct dark outlines, as shown in Fig. 1A, when the cells were observed by phase-contrast microscopy. However, when HL 60 cells were incubated with 2.5 µg/ml of beta toxin at 37 °C, the cells began to swell within 1 h, and a large number of cells became swollen and translucent within 2 h, but the blebs did not, as shown in Fig. 1B. At this time, swollen cells with a granular-like nucleus not seen in control cells were observed. However, DNA from cells treated with the toxin did not exhibit laddering classically associated with apoptosis (data not shown), suggesting that changes in the nuclei of cells treated with the toxin do not occur under these conditions. The cells were completely lysed after 6 h of incubation (data not shown).

**Fig. 1.** Morphological changes in HL 60 cells following toxin exposure. HL 60 cells were incubated without toxin (A) or with 2.5 µg/ml of beta toxin (B) in HBSS at 37 °C for 120 min.

When the cells were incubated with the toxin at concentrations from 0.5 to 10.0 µg/ml at 37 °C for 1 h, swollen cell counts increased in a dose-dependent manner under the conditions (data not shown). The effects of beta toxin on various cell lines (Vero cells, CHO cells, MDCK cells, CHO-K1 cells, COS-7 cells, P-815 cells, PC12 cells, HeLa cells, and intestine 407 cells) were investigated. The toxin had no effect on these cell lines, even when incubated with 50 µg/ml of the toxin at 37 °C for 12 h (data not shown). Our data show that, of the 10 cell lines tested, only the HL 60 cell line was susceptible to beta toxin.

**Fig. 2.** Time courses of efflux of K⁺ and LDH and influx of Ca²⁺, Na⁺, and Cl⁻ in HL 60 cells treated with beta toxin. A, HL 60 cells were incubated with various concentrations of beta toxin for various times at 37 °C. Extracellular concentrations of K⁺ (closed symbols) and LDH (open symbols) were determined as described under “Experimental Procedures.” Data are given as a means ± S.E. (n = 3). ■ or □, beta toxin at 0.5 µg/ml; ● or ○, beta toxin at 1.0 µg/ml; ● or ○, beta toxin at 5.0 µg/ml. B, HL 60 cells were incubated with beta toxin (5.0 µg/ml) for various times at 37 °C. Extracellular concentrations of Na⁺ (▲), Ca²⁺ (□), and Ca²⁺ (○) were determined as described under “Experimental Procedures.” Data are given as a means ± S.E. (n = 3).
Efflux of K⁺ and Lactate Dehydrogenase (LDH) and Influx of Ca²⁺, Na⁺, and Cl⁻ in the Cells Treated with Beta Toxin—To test whether beta toxin affects the membrane permeability of the cells, we measured the efflux of K⁺ and LDH from the cells and the influx of Ca²⁺, Na⁺, and Cl⁻ into the cells. HL 60 cells were incubated with various concentrations of beta toxin in HBSS at 37 °C. As shown in Fig. 2A, beta toxin at concentrations from 0.5 to 5.0 μg/ml induced efflux of K⁺ from the cells in a dose- and time-dependent manner. Incubation of the cells in the absence of beta toxin at 37 °C or in the presence of beta toxin at 4 °C did not induce efflux of K⁺ (data not shown). Furthermore, when the cells were incubated with 2.5 μg/ml of beta toxin at 4 °C for 2 h, washed, and incubated at 37 °C for 1 h, efflux of K⁺ was observed (data not shown). Fig. 2B shows that the toxin (5.0 μg/ml) induced influx of Ca²⁺, Na⁺, and Cl⁻ and that these events reached a maximum after 1 h of incubation at 37 °C. Beta toxin at concentrations below 5.0 μg/ml induced LDH leakage from HL 60 cells in a dose- and time-dependent manner, as shown in Fig. 2A. LDH release induced by 5.0 μg/ml of beta toxin began after 1 h and reached a maximum after 6 h, showing that lysis of the cells began after 1 h of incubation of the cells with the toxin and was complete after 6 h. It is therefore likely that release of K⁺ and uptake of Ca²⁺, Na⁺, and Cl⁻ induced by the toxin increased until lysis of the cells began.

The Effect of Polyethylene Glycols on the Events Induced by the Toxin—To test whether the toxin forms functional pores in HL 60 cells, the cells were incubated with beta toxin in the presence of various sizes of polyethylene glycols at 37 °C for 60 min, and the effects of the polyethylene glycols on the toxin-induced K⁺ efflux, Ca²⁺ influx, and swelling of cells were investigated (Table I). The toxin induced no swelling of the cells in the presence of polyethylene glycols 600 and 1000 (molecular size of 1.6 and 1.8 nm, respectively), and the toxin induced Ca²⁺ influx by about 25% of the control under these conditions.

### Table I

**Effect of polyethylene glycols with different hydrodynamic diameters on beta toxin-induced actions in HL 60 cells**

| Polyethylene glycols | K⁺ efflux | Ca²⁺ influx | Cell swelling | Hydrodynamic diameter of PEG |
|----------------------|-----------|-------------|---------------|-----------------------------|
| None                 | 101.0 ± 6.4 | 100.5 ± 9.2 | +++ | - |
| PEG200               | 95.4 ± 5.3  | 72.9 ± 13.2 | +++ | - |
| PEG300               | 96.8 ± 4.1  | 50.5 ± 8.8  | +++ | - |
| PEG400               | 95.1 ± 3.8  | 31.4 ± 4.9  | ++ | 1.12 |
| PEG600               | 94.2 ± 4.9  | 25.8 ± 5.2  | ++ | 1.16 |
| PEG1000              | 95.0 ± 5.5  | 25.4 ± 4.7  | ++ | 1.36 |

a K⁺ efflux and Ca²⁺ influx are expressed as a percentage of the data in the absence of polyethylene glycol.

b p < 0.05.

Betax [23] was purified and labeled Bolton-Hunter reagent, to modify amino group. However, these labeled toxins drastically lost the activity. To resolve this problem, we constructed a GST-beta toxin fusion protein containing a protein kinase recognition site (RRXSV) at the N terminus of the toxin (Fig. 3). The fusion protein was purified, phosphorylated, and cleaved with thrombin. The phosphorylated beta toxin derivative (32p-BTD) showed no loss of activity, compared with that of the wild-type toxin. To investigate the possible interaction of beta toxin with lipid rafts of HL 60 cells, 32p-BTD was incubated with HL 60 cells in RPMI 1640 medium containing 20% heat-inactivated fetal bovine serum at 37 °C for 15 and 30 min, and the cells were treated with 1% Triton X-100 at 4 °C. The Triton X-100-insoluble components were fractionated by sucrose density gradient centrifugation, as described under “Experimental Procedures.” The fractions were subjected to SDS-PAGE analysis and autoradiography. Low density fractions (fractions 3–5) showed three of the labeled bands of about 35 kDa, which is the expected size of monomeric toxin, and of about 191 and 228 kDa, which are the expected sizes of hexameric and heptameric toxins, respectively, as shown in Fig. 4A. These results suggest that these oligomers of beta toxin are associated with the detergent-insoluble fraction. When the cells were incubated with the toxin at 37 °C for 60 min, the detergent-insoluble fractions showed two faint labeled bands at 35 and 191 kDa (Fig. 4A), indicating that the oligomer at 228 kDa disappeared within 1 h. When rafts markers in the fractions obtained by sucrose density gradient centrifugation were analyzed using anti-caveolin-1 and anti-Lyn, caveolin-1 and Lyn were only detected in the low density fractions (fractions 3–5) (Fig. 4, C and D). Next, when cholesterol was measured in these fractions, more than 85% of cholesterol was detected in the fractions (fractions 3–5) (Fig. 4B). Therefore, it appears that the fractions (fractions 3–5) are lipid rafts, suggesting that monomers and oligomers of beta toxin are specifically located within lipid rafts in HL 60 cells. No oligomer of the toxin was detected in the absence of polyethylene glycol.
when the toxin was incubated with HL 60 cells at 4 °C, or when the toxin was incubated with MDCK cells, an insensitive cell, under these conditions (data not shown).

The Effect of Methyl-β-cyclodextrin, Cholesterol Oxidase, and Nystatin on the Cytotoxicity of Beta Toxin—It has been reported (29) that MβCD selectively encapsulates membrane cholesterol and does not deplete lipids other than cholesterol at concentrations of 5–10 mM. The effect of MβCD on the toxin-induced efflux of K⁺ and swelling was investigated. As shown in Table II, when HL 60 cells were incubated with 5 and 10 mM MβCD at 37 °C for 60 min, the cholesterol contents in those cells were decreased to about 55 and 30% of those in untreated control cells, respectively. Incubation of the toxin with HL 60 cells pretreated with 5 and 10 mM MβCD at 37 °C for 60 min pro-
HL 60 cells were incubated in the presence of MβCD, CO, or nystatin at 37 °C for 1 h. Next, various amounts of beta toxin were added to the cell suspensions. To restore cholesterol levels in MβCD-treated cells, cholesterol-depleted cells were incubated with MβCD-cholesterol complex (MβCD/Chol) at 37 °C for 2 h. Cell swelling was recorded after 2 h. Data are given as means ± S.E. (n = 4). Cell swelling was scored as follows: ++ +, 100% swelling; ++, 50–80% swelling; +, 20–40% swelling; −, no swelling. Cholesterol in cells was determined as described under "Experimental Procedures."

| Drugs          | Cholesterol back-addition | Beta Toxin µg/ml | Cholesterol in cells % control |
|----------------|---------------------------|------------------|--------------------------------|
| None           | −                         | +                | +                              | 100.0                          |
| 5 mM MβCD      | −                         | −                | +                              | 55.2 ± 8.6                     |
| 10 mM MβCD     | −                         | −                | +                              | 28.4 ± 4.3                     |
| 10 mM MβCD     | 1 mM MβCD/Chol            | −                | +                              | 61.3 ± 5.7                     |
| 10 mM MβCD     | 3 mM MβCD/Chol            | −                | +                              | 113.5 ± 9.0                    |
| CO (5 units/ml)| −                         | −                | +                              |                                |
| Nystatin (40 µg/ml) | −                   | −                | +                              |                                |

Table II

Effect of various drugs on cell swelling induced by beta toxin

Reduced a decrease of about 50% of the control cells induced by the toxin and complete loss of swelling, respectively. Moreover, beta toxin-induced K⁺ efflux from HL 60 cells was diminished by prior treatment of the cells with 10 mM MβCD (Fig. 5). To analyze whether high cholesterol enrichment in lipid rafts is required for the binding of the toxin to cells, the effect of MβCD on the binding of the toxin to cells was investigated. After treatment of HL 60 cells with or without 10 mM MβCD at 37 °C for 60 min, the cells were incubated with 3²P-BTD at 37 °C for 30 min (Fig. 6). The low density fractions were subjected to SDS-PAGE analysis and autoradiography. The amount of oligomer of beta toxin in the fractions of MβCD-treated cells was markedly reduced, but not completely, compared with that in the fractions of untreated cells (Fig. 6B). Next, we investigated whether cholesterol in lipid rafts plays a role in direct binding of the toxin or integrity of binding site. MβCD-treated cells were incubated in the presence of cholesterol-saturated MβCD at 37 °C for 2 h. As shown in Table II, when the cells pretreated with 10 mM MβCD were incubated with 1 or 3 mM MβCD-cholesterol complex at 37 °C for 2 h, the cholesterol levels of cells treated with 10 mM MβCD were restored in a dose-dependent manner by the back-addition of cholesterol. Incubation of the 10 mM MβCD-treated cells with 1 and 3 mM MβCD-cholesterol complex recovered the sensitivity (about 50 and 100%, respectively) of the cells to the toxin (Table II). Furthermore, when 10 mM MβCD-treated cells were incubated with 3 mM MβCD-cholesterol complex at 37 °C for 2 h, and additionally incubated with 3²P-BTD for 30 min, 3²P-BTD was bound to lipid raft fractions in the cholesterol-depleted cells (Fig. 6C). These results show that the specificity of the cholesterol depletion was confirmed by the demonstration that cholesterol back-addition to depleted cells allowed transport to be restored. In addition, incubation of the toxin with the cells pretreated with 5 units/ml of CO, which oxidizes cholesterol in the membrane, caused complete loss of cell swelling (Table II) and K⁺ efflux (Fig. 5) induced by the toxin. No cells were stained by propidium iodinate, when the cells were treated with 10 mM MβCD or 5 units/ml of cholesterol oxidase under our experimental conditions (data not shown), indicating that the treated cells were alive under the conditions. To test the interaction of the toxin on cholesterol, the cells pretreated with 100 µg/ml nystatin, which specifically binds to cholesterol (27), were incubated with the toxin at 37 °C. Nystatin had no effect on swelling induced by the toxin, and in addition, cholesterol (100 µg/ml) had no effect on swelling induced by the toxin (data not shown). It therefore is apparent that the toxin does not directly bind to cholesterol in the membrane.

Effect of Beta Toxin on Phosphatidylcholine-Cholesterol Liposomes—To investigate whether the toxin forms a functional oligomer in artificial membranes, the toxin was incubated with phosphatidylcholine-cholesterol (1:1) liposomes containing CF at 4 and 37 °C for 3 h. Fig. 7A shows that beta toxin (5–50 µg/ml) dose-dependently caused CF-release from liposomes at 37 °C but not at 4 °C (data not shown). However, these results indicate that the sensitivity of the cells to the toxin is higher than that of liposomes to the toxin. To clarify the binding of beta toxin to the liposomes, 3²P-BTD was incubated with liposomes at 37 °C for 3 h. Next, washed and solubilized liposomes were subjected to SDS-PAGE analysis and autoradiography. As shown in Fig. 7B, 3²P-BTD bound to liposomes in a dose-dependent manner. The liposomes showed the presence of beta toxin migrating at 35 and 228 kDa, indicating that the toxin forms a heptamer in artificial membranes. It is interesting that a hexamer of 191 kDa was not observed in liposomes. Formation of the oligomer was elevated with an increase in doses of the toxin. Furthermore, bands of dimer, trimer, and tetramer were observed, compared with the band of 228 kDa. No oligomer was observed in liposomes, when incubated at 4 °C (data not shown).

**DISCUSSION**

The present study revealed that beta toxin induced morphological changes of HL 60 cells consisting of significant swelling without blebbing and cell lysis. Gibert et al. (30) reported that the toxin was weakly cytotoxic on intestinal 407 cells, but this result has not been confirmed by other laboratories. Steinthorsdottir et al. (15) reported that the toxin induced the release of...
arachidonic acid and leakage of inositol from human umbilical vein endothelial cells, a primary cell culture, and that the toxin formed a heat-stable oligomer in the cell membrane. However, it is not clear whether the oligomer formation responded directly to the formation of transmembrane channels related to the release of arachidonic acid and inositol from the cells, as pointed out by Tweten (31). Because of the absence of susceptible cell lines, it was difficult to obtain evidence of functional oligomer formation of the toxin and the mode of action of the toxin. The data presented here are the first to be published showing the HL 60 cell line as a cell line susceptible to beta toxin.

In view of these results showing that treatment of the cells with the toxin induced simultaneous uptake of Ca$^{2+}$, Na$^+$, and Cl$^-$, positive and negative ions, into the cells, that the toxin-induced Ca$^{2+}$ uptake and swelling were markedly or completely blocked by polyethylene glycols 600 and 1000, and that the toxin-induced swelling of the cells was not inhibited by Ca$^{2+}$ channel blockers and K$^+$ channel blockers, it seems likely that the morphological change in the cells induced by the toxin is induced through pores formed by the toxin in the plasma membrane, indicating a marked influx of water into the cells. However, we cannot explain why the toxin-induced K$^+$ release is not inhibited by polyethylene glycols and K$^+$ channel blockers. Binding of the toxin to the membrane may activate K$^+$ channels insensitive to the K$^+$ channel blockers used.

Our results have revealed that the heptamer (212 kDa) and hexamer (191 kDa) of the toxin exist almost exclusively in lipid rafts of HL 60 cells by showing the distribution of $^{32}$P-BTD oligomers in low density fractions floating on sucrose density gradient, suggesting that oligomers of the toxin are formed in lipid rafts of the cells. MβCD reduced the binding of the toxin to lipid rafts and swelling induced by the toxin. On the other hand, incubation of the MβCD-treated cells with MβCD-cholesterol complex recovered the binding of the toxin to lipid rafts, formation of oligomers, and sensitivity of the treated cells to the toxin. However, treatment of the cell membranes with nystatin, which binds to cholesterol but does not deplete cellular cholesterol levels (27), had no effect on them, and addition of cholesterol did not interfere with swelling induced by the toxin, showing that the toxin does not directly interact with cholesterol. Several studies have reported that disruption or depletion of membrane-associated cholesterol results in major changes in the distribution, function, and integrity of raft-associated membrane components (29, 32, 33). It appears that the inhibitory effect seen with MβCD or CO could be due to changes in the properties of lipid rafts that occur when cholesterol is removed from lipid rafts by MβCD and CO. From these observations, we provide direct biochemical evidence indicating that beta toxin associates with receptors located mainly in lipid rafts of HL 60 cells, forms oligomers in lipid rafts, and induces cytotoxic effects. This finding is significant in that there has not been a previous description concerning a relationship between the biological activities and oligomer formation of the toxin.

The 191-kDa complex of beta toxin remained in the lipid rafts, and the 228-kDa complex disappeared before efflux of K$^+$ and influx of Ca$^{2+}$, Na$^+$, and Cl$^-$ reached a maximum. It therefore appears likely that formation of the 228-kDa complex is involved in efflux of K$^+$ and influx of Ca$^{2+}$, Na$^+$, and Cl$^-$.
addition, incubation of liposomes with the toxin caused formation of the 228-kDa complex of the toxin and release of CF from the liposomes. It is apparent that the 228-kDa complex is a functional oligomer, indicating that the toxin forms a heptameric oligomer as a functional pore in the biological membrane. Furthermore, it is likely that no membrane protein was included in the toxin complexes that were formed on the surface of the cells.

Shatursky et al. (16) reported that the toxin formed potential dependent and cation-selective channels in monolayer membranes composed of phosphatidylcholine and cholesterol and that the size of the pore was \(-12\) Å in diameter. The toxin-induced swelling of the cells was not significantly inhibited by polyethylene glycol 200 (with a hydrodynamic diameter of 1.12 nm), was significantly inhibited by polyethylene glycol 400 (a hydrodynamic diameter of 1.36 nm), and was completely inhibited by polyethylene glycol 600 (hydrodynamic diameter of 1.6 nm), was completely inhibited by polyethylene glycol 200 (with a hydrodynamic diameter of 1.12 nm), and was induced swelling of the cells.

Several bacterial pore-forming toxins have been reported to utilize lipid rafts to intoxicate cells. Aerolysin and Clostridium septicum alpha toxin bind to glycosylphosphatidylinositol-anchored proteins in lipid rafts (27, 34) and C. perfringens epsilon toxin and perfringolysin bind to cholesterol in lipid rafts (21, 24). It has been proposed that lipid rafts serve as concentrating platforms to promote pore formation of these toxins that form oligomers. The findings that beta toxin preferentially binds to lipid rafts and oligomerizes, and that the characteristics of beta toxin resemble those obtained by these pore-forming toxins strongly support that the toxin forms oligomers in membranes.

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