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Inhibition of the Adherence of Cholera Toxin and the Heat-labile Enterotoxin of *Escherichia coli* to Cell-Surface GM1 by Oligosaccharide-derivatized Dendrimers

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ABSTRACT. The adherence of either cholera toxin or the heat-labile enterotoxin of *Escherichia coli* to monosialoganglioside gal(β1–3)galNAc(β1–4)[sialic acid (α2–3)]gal(β1–4)glcβ1–ceramide (GM1) present on the surface of epithelial cells lining the intestine is the first step of a series that results in the induction of a watery diarrhea. While cholera is more severe, both can lead to death as a result of dehydration. To determine the potential of defined multivalent oligosaccharides, synthesized by the covalent attachment of multiple phenylisothiocyanate (PITC) derivatives of gal(β1–3)galNAc(β1–4)[sialic acid(α2–3)]gal(β1–4)glc (oligo-GM1) to the arms of a poly(propylene imine) dendrimer, as therapeutic agents for these diseases, their ability to inhibit adherence of the toxins to cell surface-associated GM1 was determined. They not only inhibited choleragenoid (binding subunit of cholera toxin) binding to GM1-treated NCTC-2071 cells (chemically transformed murine fibroblasts) at 5°, but also inhibited adherence of the choleragenoid, cholera toxin, and heat-labile enterotoxin of *E. coli* to GM1-treated NCTC-2071 cells at 37°. Inhibition was observed whether the toxin was preincubated with the oligo-GM1-PITC-derivatized dendrimer prior to addition to cells or given just after the addition of the derivatized dendrimer to cells. The derivatized dendrimer had no effect on cell viability, as monitored by trypan blue exclusion. Blue-shifts in tryptophan fluorescence emission spectra maxima induced by adherence of either choleragenoid, cholera holotoxin, or the heat-labile enterotoxin of *E. coli* to oligo-GM1-PITC-derivatized dendrimers were similar to those induced by adherence to GM1 or oligo-GM1. Comparable shifts were not observed when the toxins were incubated with gangliosides that fail to function as receptors. BIOCHEM PHARMACOL **56**;5:591–597, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cholera toxin; heat-labile enterotoxin of *E. coli*; ganglioside GM1; dendrimer

Cholera toxin, produced by *Vibrio cholerae*, and the heat-labile enterotoxin of *Escherichia coli* are enterotoxins that can adhere to ganglioside GM1§ present on epithelial cells lining the intestine. As a result, cyclic AMP production is enhanced, which leads to cellular changes that result in a watery diarrhea [1]. Evidence indicates that the eighth cholera pandemic has begun [2], and it is well known that, when untreated, death can result from dehydration. Although the "traveler's" diarrhea caused by the heat-labile enterotoxin of *E. coli* is less severe than cholera, it, too, can result in death due to dehydration. Both diseases can be treated using oral rehydration. It should also be possible to reduce the severity of the disease and possibly prevent its expression by inhibiting the adherence of these toxins to their cell surface receptors.

In previous work, we observed that while the oligosaccharide portion of ganglioside GM1 (oligo-GM1) was a poor inhibitor of the binding of cholera toxin or the heat-labile enterotoxin of *E. coli* to immobilized GM1, multivalent oligosaccharide preparations made by linking multiple oligo-GM1 residues to poly-L-lysine were as effective or better than GM1 at inhibiting toxin adherence [3]. These observations can be explained by the crystal structures obtained for each toxin, which indicate that there are five binding sites in each pentameric binding subunit [4, 5]. The presence of a clustered ligand provides multiple sites to which each of the five binding sites can adhere. Based upon the crystal structures, more defined multivalent-oligo-GM1 preparations were synthesized by linking PITC-derivatized oligo-GM1 residues to poly(propylene imine) dendrimers, hyper-branched polymers that radiate from a central core [6]. Covalent attachment of multiple PITC derivatives of oligo-GM1 to first or second generation poly(propylene imine) dendrimers resulted in molecules that were able to inhibit adherence of the binding subunit of cholera toxin (choleragenoid) and the heat-labile enterotoxin of *E. coli* to immobilized GM1 as well as or better than did GM1,

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§ *Abbreviations:* GM1, gal(β1–3)galNAc(β1–4)[sialic acid(α2–3)]gal(β1–4)glcβ1–ceramide; oligo-GM1, gal(β1–3)galNAc(β1–4)[sialic acid (α2–3)]gal(β1–4)glc; and PITC, phenylisothiocyanate.

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their natural ligand [7]. A possible reason for the efficacy of the oligosaccharide-derivatized dendrimers is that the dendrimer core promotes the radial distribution of the added oligosaccharide moieties, effectively clustering them into an "artificial" micelle.

To determine whether oligo-GM1-PITC-derivatized dendrimers might be of therapeutic value, their ability to inhibit adherence of choleragenoid, cholera holotoxin, and the heat-labile enterotoxin of *E. coli* to cell surface GM1 was determined.

MATERIALS AND METHODS

Materials

NCTC-2071 cells were from the American Type Culture Collection. *V. cholerae* toxin B subunit (CT-B or choleragenoid) and goat anti-choleragenoid antibody were obtained from List Biological Laboratories. Heat-labile enterotoxin of *E. coli* was purchased from ICN Pharmaceuticals, Inc. *V. cholerae* holotoxin, rabbit anti-goat Cy3 conjugate, trypsin, bacitracin, and NCTC-135 tissue culture medium were from the Sigma Chemical Co. $Na^{125}I$ was obtained from New England Nuclear and acrylamide from Boehringer Mannheim; ganglioside standards were from Matreya Inc. VANEX® 0.2 μm microspin centrifuge inserts were bought from Vangard International Inc. Falcon tissue culture flasks and centrifuge tubes were from Becton Dickinson Labware, and BSA, fraction V (RIA grade), was obtained from the United States Biochemical Corp.

Synthesis of Oligo-GM1-derivatized Dendrimers

Oligo-GM1-PITC-derivatized tetra- and octa(propylene imine) dendrimers, containing four and an average of seven oligo-GM1 moieties, respectively, were prepared as previously described [7].

^{125}I Labeling of the Toxins

Proteins were labeled with ^{125}I using the chloramine T procedure described by Williams *et al.* [8]. Purity of the labeled toxin was determined by looking for the presence of contaminating labeled proteins after SDS–PAGE of the labeled toxin on a 5% stacking, 10% running gel under reducing and nonreducing conditions [9]. Gels were dried and labeled proteins visualized by autoradiography.

Cell Culture

Chemically transformed, GM1-deficient, mouse fibroblast NCTC-2071 cells were cultured, aseptically, in defined NCTC-135 medium with L-glutamine and 0.22% sodium bicarbonate. Cultures were grown at 37° in 95% air/5% CO_2 and 90% humidity. Confluent cells were harvested by first exposing them for 1 min at 37° to 0.05% trypsin/versene containing 0.1% glucose and then rapping the flask sharply to dislodge the cells. Trypsin activity was inhibited by the addition of an approximately equal volume of NCTC-135 medium supplemented with 3% FBS, and the cells were pelleted by centrifugation at 200 *g* for 5 min. The supernatant was discarded, and the cells were resuspended in fresh, unsupplemented, NCTC-135 medium. The number of viable cells was determined by counting trypan blue negative cells in a hemocytometer. Then aliquots containing $\sim 5 \times 10^6$ cells were seeded into 75 cm^2 flasks in a total volume of 10 mL of fresh NCTC-135 medium.

Cell-Binding Assay

Because NCTC-2071 cells contain little cell surface GM1, it was necessary to determine how much GM1 had to be added to the cells to provide binding sites for the toxins, and how much labeled toxin would be used in the assays. Therefore, NCTC-2071 cells were grown for 18 hr prior to harvest in medium supplemented with increasing amounts of GM1 (0–500 nM). After harvesting cells by scraping them into PBS, they were recovered by low speed centrifugation and washed three times with PBS. After the third rinse, cells were resuspended in a small volume of NCTC-135 medium, and the number of viable cells was determined by counting trypan blue negative cells in a hemocytometer. Then aliquots containing 3×10^5 viable cells in 100 μL were added to an equivalent volume of medium containing 12 nM labeled choleragenoid. After 1 hr at 16° samples were transferred to 0.2 μm filter microcentrifuge tube inserts. Nonspecific binding sites on the filters were blocked prior to use by filtering 500 μL of PBS containing 0.1% BSA through them. Samples were centrifuged at 1000 *g* for 5 min. Cells retained by the filters were rinsed three times by repetitive addition of 200 μL of PBS followed by centrifugation. Bound label was determined by counting that associated with cells retained on the filter inserts in a gamma counter. Binding to added GM1 was obtained by subtracting counts associated with cells grown in medium alone from those associated with cells grown in medium containing GM1. Results indicated that incubation of the cells with as little as 50 nM GM1 resulted in adherence of a significant amount of labeled choleragenoid and that the amount of choleragenoid that adhered to the cells was linearly related to the amount of GM1 added to the medium (Fig. 1A). When cells that had been incubated with 50 nM GM1 were incubated subsequently for 1 hr at 16° with increasing amounts of labeled choleragenoid, binding was found to be saturable (Fig. 1B). To avoid saturation of binding sites on GM1-treated cells, subsequent experiments were carried out using cells grown for 18 hr in medium containing 200 nM GM1 and a final concentration of 2 nM labeled protein to monitor binding.

The effectiveness of $(\text{oligo-GM1-PITC})_7$ octa(propylene imine) at inhibiting the adherence of labeled choleragenoid or toxin to GM1-treated cells was determined as follows. Two nanomolar labeled protein was preincubated for 1 hr at 37° with different concentrations of $(\text{oligo-GM1-PITC})_7$ octa(propylene imine) prior to its addition to GM1-treated

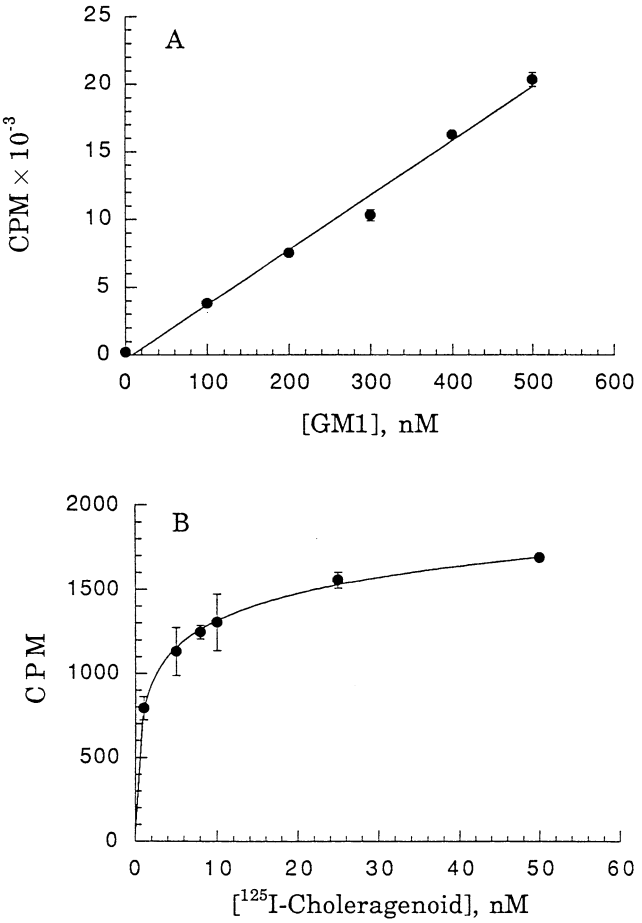

FIG. 1. Adherence of ^{125}I-labeled choleragenoid to NCTC-2071 cells. (A) Cells were grown in the presence of media containing different amounts of GM1 prior to harvest 18 hr later and incubation with 6 nM ^{125}I-labeled choleragenoid for 1 hr at 16°. (B) Cells were grown in media containing 50 nM GM1 for 18 hr prior to a 1-hr incubation at 16° with the indicated concentrations of labeled choleragenoid. Cell-associated labeled choleragenoid was determined by counting in a γ counter. Values shown are the average counts obtained for quadruplicate samples and replicate experiments gave similar results. Error bars indicate the standard deviation in counts obtained for four samples.

or control (grown in medium alone) cells. The concentration of ligand used was based on the amount found previously to inhibit adherence of labeled choleragenoid or holotoxin to GM1-coated plastic wells by 50% (IC_{50} values). For 6 nM choleragenoid and heat-labile enterotoxin, those values were 3 and 6 nM, respectively, for (oligo-GM1-PITC)$_7$ octa(propylene imine) [7], whereas for 6 nM cholera toxin it was 7 nM (unpublished results). To minimize endocytosis of labeled choleragenoid or toxin, initial incubations of the cells with toxin plus dendrimer or toxin alone were done at 16° [10]. Subsequently, analogous experiments were done at 37°. After 1 hr, samples were transferred to filter microcentrifuge tube inserts and treated as described above. Specific binding was defined as the amount of label bound to GM1-treated cells minus that bound to the same number of control cells. Values for each experimental point are the average of quadruplicate samples, and each experiment was usually done twice. Experiments were also carried out in which the preincubation of toxin with dendrimer was omitted. In these experiments, inhibitor was added to the cells just prior to the addition of labeled toxin; the rest of the procedure was unchanged. In some of the assays done at 37°, 100 μg of bacitracin was added/mL of incubation medium to reduce the possibility of receptor-mediated endocytosis [11] during the incubation period.

To determine the effect of (oligo-GM1-PITC)$_7$ octa (propylene imine) on NCTC-2071 cell viability, confluent cells were grown in medium containing it at a concentration of 500 nM. This concentration was selected because it was ten times the highest concentration of derivatized dendrimer used in most studies. After 18 hr, medium was removed, the cells were harvested into PBS by scraping, and the number of trypan blue negative and positive cells was counted using a hemocytometer.

Immunofluorescence

The ability of (oligo-GM1-PITC)$_7$ octa(propylene imine) to inhibit adherence of 6 nM choleragenoid to GM1-treated and control cells was also monitored using immunofluorescence. Cells (3×10^5) were seeded and grown in each of eight separate chambers on a microscope slide. Cells in half of the chambers were treated with GM1 as above, the others with medium alone. Choleragenoid (6 nM) was preincubated at 37° for 1 hr in the presence or absence of 30 nM (oligo-GM1-PITC)$_7$ octa(propylene imine) or 30 nM GM1, in PBS containing 0.1% BSA and then added to cells that had been rinsed three times with 500 μL PBS containing 0.1% BSA. After a 1-hr incubation at 5°, cells were rinsed three times with PBS to remove unbound choleragenoid and then fixed at room temperature in 200 μL of 4% paraformaldehyde. Fixative was removed after 10 min, and cells were rinsed three times with PBS prior to incubation at room temperature for 1 hr with goat anti-choleragenoid IgG in PBS containing 0.1% BSA. Cells were washed to remove excess primary antibody and bound antibody fluorescently labeled by incubating the cells with rabbit anti-goat IgG Cy3 conjugate in PBS containing 0.1% BSA. Then cells were washed with PBS, and immunofluorescence was observed using a fluorescence microscope equipped with a rhodamine filter. Nonspecific fluorescence was accounted for by monitoring a cell sample not exposed to choleragenoid but fixed and stained in the same way.

Tryptophan Fluorescence

The effect of potential ligands on the intrinsic tryptophan fluorescence of choleragenoid and the holotoxins was monitored using an Aminco-Bowman® series 2 luminescence spectrometer and associated software from SLM-Aminco®. Choleragenoid or toxin (0.5 μM) in PBS, pH 7.2, was incubated at 37° for 1 hr and then allowed to come

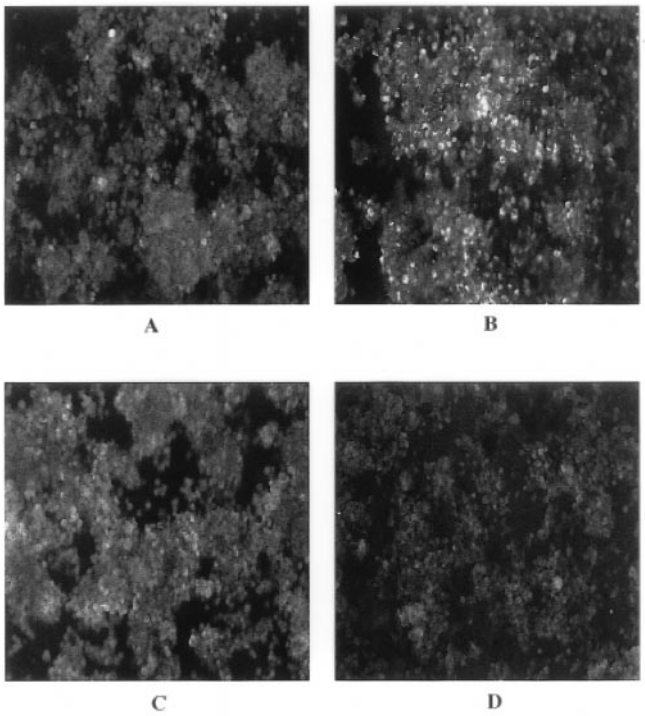

FIG. 2. Indirect immunofluorescence analysis of choleragenoid adherence to NCTC-2071 cells. Photomicrographs show: (A) choleragenoid adherence to control cells; (B) choleragenoid adherence to NCTC-2071 cells that had been grown for 18 h in media containing 200 nM GM1; (C) adherence of choleragenoid pre-incubated with 30 nM GM1 for 1 hr at 37° prior to overlay of cells treated with GM1 as in (B); and (D) adherence of choleragenoid preincubated with 30 nM (oligo-GM1-PITC)$_7$ octa(propylene imine) for 1 hr at 37° prior to overlay of cells treated with GM1 as in (B). Photomicrographs are shown at low magnification to maximize the number of cells seen. Data shown are from a single experiment. Two experiments in which rhodamine conjugated rabbit anti-goat IgG was used as the secondary antibody gave similar results but the fluorescent signal was weaker.

to room temperature prior to monitoring its tryptophan fluorescence emission spectra. Samples were excited at 282 nm, and the resulting fluorescence was monitored over the wavelength range of 290–430 nm. Samples containing choleragenoid or toxin plus the ligand to be studied in a 1:5 molar ratio (toxin:ligand) were incubated at 37° for 1 hr prior to recording their emission spectra. A five-fold concentration of ligand was used because five molecules of oligo-GM1 were needed to occupy each of the binding sites in one molecule of toxin and, to be consistent, the same ratio was used in each experiment.

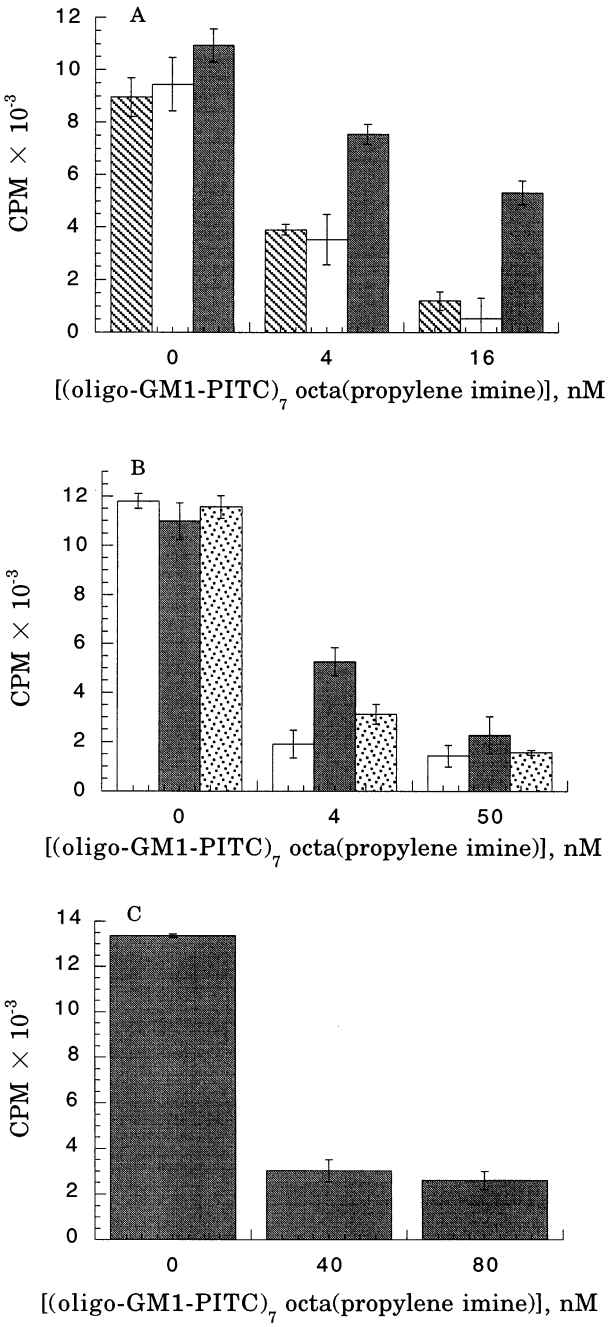

FIG. 3. Adherence of ^{125}I-labeled (A) choleragenoid, (B) cholera toxin, and (C) the heat labile enterotoxin of *E. coli* to GM1-treated NCTC-2071 cells in the absence or presence of (oligo-GM1-PITC)$_7$ octa(propylene imine). Average cell-associated counts are indicated by (▨) for experiments in which labeled choleragenoid was preincubated with (oligo-GM1-PITC)$_7$ octa(propylene imine) for 1 hr at 37° prior to incubation with cells for 1 hr at 16°, by (□) for experiments in which labeled choleragenoid or toxin was preincubated with derivatized dendrimer for 1 hr at 37° prior to incubation with cells for 1 hr at 37°, by (■) for experiments in which dendrimer and choleragenoid or toxin were added directly to the cells which were then incubated for 1 hr at 37°, and by (▤) for experiments in which toxin and dendrimer were added directly to cells which were then incubated for 1 hr at 37° in media containing 100 μg/mL of bacitracin. Cell-associated label was determined by counting in a gamma counter. The concentration of choleragenoid or toxin (2 nM) and number of cells (3 × 10^5) grown in the presence of 200 nM GM1 was the same for each assay. Each value shown is the average of at least three separate measurements. Error bars indicate the standard deviation.

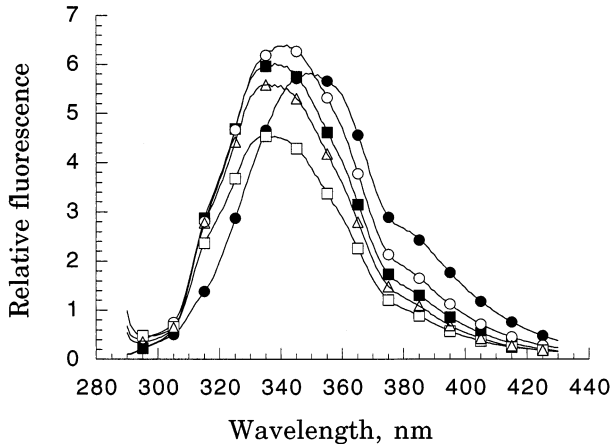

FIG. 4. Tryptophan fluorescence emission spectra for choleragenoid plus oligo-GM1-containing compounds. Choleragenoid was incubated at 37° for 1 hr in the absence (●——●), or presence of a five-fold molar excess of GM1 (○——○), oligo-GM1 (■——■), (oligo-GM1-PITC)$_7$ octa(propylene imine) (□——□), or (oligo-GM1-PITC)$_4$ tetra(propylene imine) (△——△). The excitation wavelength was 282 nm. Note that incubation of the choleragenoid with any of the oligo-GM1-containing ligands resulted in a similar blue-shift in the fluorescence emission maximum. Data shown are from a single experiment; however, each experiment was done twice and comparable results were obtained.

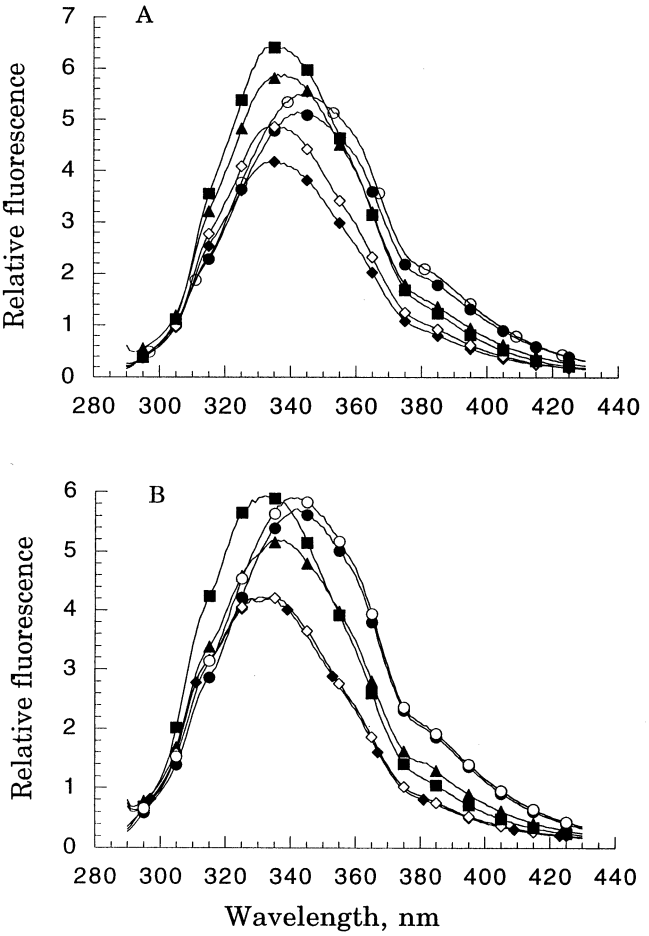

FIG. 5. Tryptophan fluorescence emission spectra for (A) cholera toxin and (B) the heat labile enterotoxin of *E. coli* plus oligo-GM1-containing compounds. Cholera toxin or the heat labile enterotoxin of *E. coli* was incubated at 37° for 1 hr in the absence (●——●) or presence of a five-fold molar excess of GM1 (▲——▲), (oligo-GM1-PITC)$_7$ octa(propylene imine) (◆——◆), (oligo-GM1-PITC)$_4$ tetra(propylene imine) (◇——◇), oligo-GM1 (■——■), or asialo-GM1 (○——○). The excitation wavelength was 282 nm. Note that a similar blue-shift in emission maximum was obtained when the toxin was incubated with any of the oligo-GM1-containing compounds. Incubation with asialo-GM1 did not induce a change in the emission maximum. Data shown are from a single experiment; however, each experiment was done twice and comparable results were obtained.

RESULTS

Adherence of Choleragenoid and the Intact Toxins to GM1-containing NCTC-2071 Cells

Immunofluorescent analysis of choleragenoid binding to GM1-treated and control cells indicated that more choleragenoid adhered to cells grown in medium containing 200 nM GM1 than to cells grown in medium alone (Fig. 2, A and B). Experiments to determine the effectiveness of GM1 and (oligo-GM1-PITC)$_7$ octa(propylene imine) at inhibiting adherence of choleragenoid to GM1-treated cells indicated that preincubation of choleragenoid with (oligo-GM1-PITC)$_7$ octa(propylene imine) markedly reduced the amount of choleragenoid associated with cells compared with that seen when the derivatized dendrimer was replaced with an equivalent concentration of GM1 (Fig. 2, C and D). Cells prepared in the same manner, but not exposed to choleragenoid, showed little fluorescence, indicating that there was minimal nonspecific association of the antibodies used.

Experiments in which the amount of labeled choleragenoid or holotoxin associated with the cells was determined provided a more quantitative assessment of the efficacy of (oligo-GM1-PITC)$_7$ octa(propylene imine) as an inhibitor. A significant reduction in adherence of ^{125}I-labeled choleragenoid, cholera holotoxin, or heat-labile enterotoxin of *E. coli* to GM1-treated cells was seen when (oligo-GM1-PITC)$_7$ octa(propylene imine) was present (Fig. 3, A–C). Inhibition of choleragenoid binding to GM1-treated cells by (oligo-GM1-PITC)$_7$ octa(propylene imine) depended upon the concentration of derivatized dendrimer used. While significant inhibition ($P = 0.0002$, two-tailed unpaired *t*-test) of choleragenoid adherence to GM1-treated cells was obtained when the choleragenoid and (oligo-GM1-PITC)$_7$ octa(propylene imine) were added directly to GM1-treated cells, it was less than that obtained when the choleragenoid was preincubated with the oligo-GM1-PITC-derivatized dendrimer prior to addition to the cells (51 and 94%, respectively). Bacitracin reduced the amount of cell-associated cholera holotoxin (Fig. 3B). Counts per minute shown in the graphs were obtained by subtracting label associated with control cells incubated with choleragenoid or holotoxin alone. This was done because the addition of 4 or 16 nM (oligo-GM1-PITC)$_7$ octa(propylene imine) plus labeled choleragenoid or holo-

toxin directly to control cells resulted in the adherence of as much label to the control cells as was found in association with GM1-treated cells. Interestingly, this binding was always significantly less than the amount of label associated with GM1-treated cells incubated with choleragenoid or toxin alone. At a concentration of derivatized dendrimer equal to or greater than ~50 nM, binding to control cells went down to about 2–3 times that seen with control cells minus derivatized dendrimer (usually 2000–3000 compared with <1000 cpm for control cells incubated with choleragenoid or holotoxin alone). This effect was not as pronounced when labeled choleragenoid was preincubated with the derivatized dendrimer prior to addition to control cells. The cause of the enhanced binding to control cells was not determined.

Effect of (Oligo-GM1-PITC)$_7$ octa(propylene imine) on NCTC-2071 Cell Viability

Exposure of the cells to 500 nM (oligo-GM1-PITC)$_7$ octa(propylene imine) for 18 hr at 37° had no significant effect on cell viability, as determined by trypan blue exclusion. Cell counts obtained for quadruplicate samples indicated that the average percent of trypan-blue negative (viable) cells was 77 ± 2.6% for cells grown in medium containing (oligo-GM1-PITC)$_7$ octa(propylene imine) compared with 78 ± 4.9% for cells grown in medium alone.

Tryptophan Fluorescence Analyses

Choleragenoid, excited by ultraviolet light with a wavelength of 282 nm, had a tryptophan emission spectra with a maximum at approximately 350 nm. Similar emission spectra were observed when the choleragenoid was incubated with a five-fold molar excess of either of three gangliosides, GD1a, GT1b, or asialo-GM1, that fail to function as ligands for the binding subunit. In contrast, incubation of choleragenoid with a 5-fold molar excess of GM1, oligo-GM1, (oligo-GM1-PITC)$_7$ octa(propylene imine), or (oligo-GM1-PITC)$_4$ tetra(propylene imine) resulted in almost identical ~12 nm blue-shifts in the wavelength at which maximum fluorescence emission was observed (Fig. 4). Similar changes in fluorescent emission maxima were observed when either cholera holotoxin or the heat-labile enterotoxin of *E. coli* was incubated with the same oligo-GM1-containing compounds (Fig. 5). No blue-shift was observed when the holotoxins were incubated with asialo-GM1. The reduction in maximum fluorescent intensity seen with the derivatized dendrimers may be due to quenching by the PITC residues.

DISCUSSION

In previous work [3, 7], we observed that nanomolar concentrations of GM1 or multivalent, nonlipid, oligo-GM1 derivatives were able to inhibit adherence of labeled choleragenoid and the heat-labile enterotoxin of *E. coli* to GM1-coated plastic wells by 50%, whereas comparable concentrations of the oligosaccharide portion of GM1 were ineffective. The effectiveness of GM1 as a ligand appears to reflect its ability to form micelles when present in an aqueous environment at concentrations above 10^{-9} M [12, 13]. To determine whether a "multivalent" oligosaccharide might be of physiological interest, the ability of (oligo-GM1-PITC)$_7$ octa(propylene imine) to inhibit adherence of choleragenoid and the holotoxins to cells maintained *in vitro* was determined. NCTC-2071 murine fibroblasts were used for these studies because: 1) the cells are naturally deficient in GM1 [14], which permitted the amount of cell surface GM1 to be controlled experimentally; 2) the cells could be grown in serum-free medium which eliminated the possibility of the cells incorporating gangliosides present in association with serum components; and 3) cells not supplemented with exogenous GM1 could serve as negative controls in the cell-binding experiments. The finding by Moss *et al.* [14] that added GM1 taken up by NCTC-2071 cells made the cells responsive to the cellular effects of cholera toxin indicates that results obtained with this model should be relevant to cells that normally express GM1 on their cell surface.

Cell-binding experiments done at 16° indicated that the adherence of ^{125}I-labeled choleragenoid to GM1-treated cells was saturable, supporting the receptor-mediated nature of the adherence. The fact that ^{125}I-labeled choleragenoid binding to cells at 16° was linearly related to the amount of GM1 added to the medium in which the cells were grown prior to harvest and assay further supported identification of the added GM1 as the receptor molecule.

The more visible reduction in immunofluorescence seen when choleragenoid was preincubated with (oligo-GM1-PITC)$_7$ octa(propylene imine) prior to incubation with GM1-treated cells at 5° than when it was preincubated with GM1 agrees with previous observations that indicated that the derivatized dendrimer was a more effective inhibitor of choleragenoid adherence to GM1-immobilized on plastic than was GM1 [7]. The lack of adherence of choleragenoid to control cells and its marked adherence to GM1-treated cells agree with the observations of Moss *et al.* [14]. The results also provide a visual illustration of why intact GM1 should not be considered as a possible drug to inhibit cell surface adherence of a GM1-binding toxin: it can associate with the cell membrane, making it more susceptible to the toxin.

Inhibition of adherence of choleragenoid or the holotoxins by (oligo-GM1-PITC)$_7$ octa(propylene imine) to GM1-treated cells was also seen when the temperature was increased to either 16° or 37°. When labeled choleragenoid or toxin was preincubated with derivatized dendrimer prior to addition and incubation with GM1-treated cells, the percent inhibition of adherence was greater than when they were just added to and then incubated with the cells for 1 hr at 37°. These results indicate that the degree of inhibition depends upon adherence of the toxin to derivatized dendrimer prior to its adherence to cell surface GM1, and

that once the binding subunit of the toxin associates with the (oligo-GM1-PITC)$_7$ octa(propylene imine) its ability to associate with cells is reduced significantly. While adherence of the labeled toxins was reduced significantly when higher concentrations of (oligo-GM1-PITC)$_7$ octa (propylene imine) were used, some of the toxin did associate with cells, and that association was greater at 37° than at 16°. The fact that addition of bacitracin reduced the amount of cholera holotoxin associated with the cells indicates that some of the toxin may have been endocytosed.

The comparable blue-shifts in the tryptophan fluorescence emission maxima induced by the adherence of the choleragenoid and holotoxins to GM1, oligo-GM1, (oligo-GM1-PITC)$_4$ tetra(propylene imine), or (oligo-GM1-PITC)$_7$ octa (propylene imine) indicate that each ligand had a similar effect on the micro-environment of the single tryptophan residue contained in each carbohydrate binding site of the binding subunit. The lack of change in emission maxima seen when the toxins were incubated with asialo-GM1, GD1a, or GT1b, lipids that are not ligands for the toxins, indicates that the blue-shift in emission spectra resulted from the binding of the toxins to the oligo-GM1-containing ligands. These data support the hypothesis that the oligo-GM1-PITC-derivatized dendrimers mimic GM1 and oligo-GM1 in their ability to function as ligands for choleragenoid, cholera holotoxin, and the heat-labile enterotoxin of *E. coli*.

The results of this study support the hypothesis that defined, polyvalent oligosaccharides that can serve as effective ligands for pathogens that adhere to the oligosaccharide portion of cell surface glycoconjugates can be synthesized, using dendrimers as carrier molecules. The oligo-GM1-PITC-derivatized dendrimers used in this study were tested using a clearly defined model. The availability of x-ray crystallographic structures of the toxins [4, 5], plus the fact that their oligosaccharide receptors were well-defined [15–17], enabled us to synthesize a polyvalent oligosaccharide ligand that, according to computer modeling, was large enough for adherence by more than one of the five binding sites present in the pentameric binding subunit [7] and may be of therapeutic value.

Although the derivatized dendrimers used as ligands in this study are not as simple as those that might be designed using computer modeling [e.g. Ref. 18], the approach used does have some advantages. It can be applied to the development of polyvalent oligosaccharide ligands for use in the study of other pathogen–oligosaccharide interactions for which the carbohydrate ligand is known, even though the structure of the protein is not defined, as well as in studies to ascertain the effect of clustered oligosaccharides on cell development and/or cell–cell interactions.

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