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Oligosaccharide-derivatized dendrimers: defined multivalent inhibitors of the adherence of the cholera toxin B subunit and the heat labile enterotoxin of *E. coli* to GM1

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Poly(propylene imine) dendrimers having four or eight primary amino groups and a Starburst™ (PAMAM) dendrimer having eight primary amino groups were used as core molecules, to which phenylisothiocyanate derivatized (PITC) galβ1-3galNAcβ1-4[sialic acidα2-3]-galβ1-4glc (oligo-GM1) residues were covalently attached to yield multivalent oligosaccharides. The synthesis of the oligo-GM1-PITC derivatized dendrimers was monitored using high performance thin layer chromatography, infrared spectroscopy, sialic acid content, and mass spectroscopy. The ability of multivalent oligo-GM1-PITC dendrimers to inhibit the binding of ^{125}I-labeled cholera toxin B subunit and the heat labile enterotoxin of *E. coli* to GM1-coated microtiter wells was determined. IC_{50}s obtained for the oligo-GM1-PITC dendrimers, GM1, and the oligosaccharide moiety of GM1 indicated that the derivatized dendrimers inhibited binding of the choleragenoid and the heat labile enterotoxin to GM1-coated wells at a molar concentration five- to 15-fold lower than native GM1 and more than 1,000-fold lower than that of the free oligosaccharide.

Keywords*: dendrimer, oligosaccharide, ganglioside, cholera toxin, heat labile enterotoxin of *E. coli

Introduction

Cholera toxin, a protein secreted by the bacterium *Vibrio cholerae*, consists of an A subunit and a homopentameric B, binding, subunit [1]. The cell surface ligands recognized by the B subunit are GM1 [2] and fucosylated GM1 [3]. Once bound, the A subunit enters the cell, the disulfide bond linking the two peptides comprising the A subunit (A1 and A2) is reduced, and the A1 subunit is able to induce the irreversible activation of adenylate cyclase [4]. The clinical result is an overwhelming and continuous efflux of digestive fluids into the lumen of the intestine which results in diarrhea, massive dehydration, and, if untreated, death of the affected individual. The heat labile enterotoxin of *E. coli*, a structurally similar GM1 binding toxin [1], also affects the cells of the gut and is a causative agent of traveller's diarrhea [5]. Inhibition of the adherence of the B subunits of these toxins to cell surface GM1 should prevent the clinical problems associated with infection by the bacteria that produce them.

Previous studies in this lab indicated that the adherence of cholera toxin and the heat-labile enterotoxin of *E. coli* to GM1-coated plastic wells, was inhibited by GM1 and derivatives thereof [6]. The most effective inhibitor was a mixture of poly-L-lysine molecules (molecular weight was less than 5000) to which an average of eight oligosaccharide residues from GM1 (oligo-GM1) were linked to primary amino groups on the poly-L-lysine by reductive amination. These observations, coupled with the ability of gangliosides to form micelles in an aqueous environment, and to cluster in patches on the cell surface [7], support the hypothesis that a spherical molecule coated with oligo-GM1 residues should be an effective ligand for both cholera toxin and the heat labile enterotoxin of *E. coli*.

Dendrimers, hyperbranched polymers that have a defined structure and are synthesized in a stepwise approach by repetitive reaction sequences [8], were used as core molecules to which oligo-GM1 moieties were attached. This approach allowed for the synthesis of defined, multivalent oligo-GM1 compounds. Molecular modeling predicted that the structure of the dendrimer cores would allow for a radial distribution of the added oligosaccharide residues, effectively clustering them into an 'artificial' micelle.

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Materials and methods

Materials

V. cholera toxin B subunit horseradish peroxidase conjugate (CT-B-HRP), *V. cholera* toxin B subunit (CT-B or choleragenoid) and goat anti-choleragenoid antibody were obtained from List Biological Laboratories (Campbell, CA), heat labile enterotoxin of *E. coli* from ICN Pharmaceuticals, Inc. (Costa Mesa, CA), $Na^{125}I$ from New England Nuclear (Boston, MA), generation 1 Starburst (PAMAM) dendrimer, acrylonitrile, 1,4-diaminobutane, boranemethyl sulfide complex (BH_3 concentration of 10.0–10.2 M), anhydrous methanol, anhydrous tetrahydrofuran, sodium cyanoborohydride, thiophosgene, and 2-(4-aminophenyl)-ethylamine from Aldrich (Milwaukee, WI), ganglioside standards from Matreya Inc. (Pleasant Gap, PA), Supersignal CL-HRP Substrate System from Pierce (Rockford, IL), Bio-Gel P-2 [fine 45–90 mm (wet)] from Bio-Rad (Hercules, CA), centricon-10 and centricon-50 filters from Amicon Corp. (Lexington, MA), PD-10 (sephadex G-25M) columns, Sephadex G-25, and DEAE-Sephadex A-25 from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden), HPTLC (Silica Gel-60) plates from VWR (Bridgeport, NJ) Immunolon 1 Removawell strips from Dynatech Labs Inc. (Chantilly, VA), Alchemy™ III (3D molecular modeling software) from Tripos Associates, Inc. (St Louis, MO), a CarboPac PA1 (4 × 250 mm) HPLC column and HPLC system from Dionex (Sunnyvale, CA), and X-OMAT AR film from Eastman Kodak Co. (Rochester, NY).

Ganglioside Isolation

Intact GM1 was purified from bovine brain as described previously [6, 9]. It was also obtained by the partial acid hydrolysis of disialo-, trisialo-, or mixed gangliosides using the hydrolysis procedure described by Warren [10] with a shortened reaction time. Briefly, 200 mg of gangliosides were dissolved by sonication in 1 ml of 0.1 N H_2SO_4 and incubated at 80 °C for 45 min. After neutralizing the hydrolysate with 10 N NaOH, the sample was diluted to 5 ml with methanol:chloroform:water (2:1:0.26, by vol) and centrifuged to precipitate insoluble material. GM1 was isolated from the supernatant by chromatography on a DEAE-Sephadex A-25 column (100 ml bed vol). The column was eluted with a step gradient of methanol:chloroform:water (60:30:8, by vol) to methanol:chloroform:1.0 M sodium acetate (60:30:8, by vol. [11]). GM1 was recovered in the fraction eluted with methanol:chloroform:0.3 M sodium acetate (60:30:8, by vol). After the GM1 fraction was eluted, polysialyated lipids were recovered using methanol:chloroform:1 M sodium acetate (60:30:8, by vol). Ganglioside containing fractions were rotoevaporated to remove organic solvents and dialyzed against water to remove salts prior to being dried under vacuum from the frozen state (lyophilized). Purity of each fraction was determined using HPTLC with chloroform:methanol:0.3% $CaCl_2$ (60:35:8, by vol) or chloroform:isopropanol:50 mM KCl (2:13.4:4.6, by vol). Sialic acid containing glycosphingolipids were visualized with resorcinol spray [12]. GM1 was identified by its comobility with standard GM1, plus its ability to function as a ligand for the binding subunit of cholera toxin. Recovered polysialylated gangliosides were rehydrolyzed and the procedure to isolate GM1 repeated.

To determine whether the GM1 used for the isolation of oligo-GM1 contained both *N*-acetyl- and *N*-glycolyneuraminic acid, the GM1 was hydrolyzed [10] and the released carbohydrate was analyzed by anion exchange chromatography using pulsed amperometric detection (Dinoex, technical note 20, 1989).

Sythesis of GM1 derivatives

The oligosaccharide moiety of GM1 (oligo-GM1) was isolated chemically as described previously [13]. Synthesis of the phenylisothiocyanate derivative of oligo-GM1 was based on previously reported methods in which other carbohydrates were used [14, 15]. Briefly, 11 mM oligo-GM1 was reductively aminated at 37 °C for 90 h in 122 mM 2-(4-aminophenyl)ethylamine in 200 mM borate buffer, pH 8.0, containing 32 mM sodium cyanoborohydride. The aminophenyl derivative of oligo-GM1 was separated from unreacted oligo-GM1 by chromatography on a Biol-Gel P2 column. Twenty-five mM pyridine acetate buffer, pH 7.0, was used as the eluent and the aminophenyl derivative was eluted prior to oligo-GM1. Aliquots of each fraction were analyzed by HPTLC with chloroform:methanol:0.3% $CaCl_2$ (60:35:8, by vol) as the developing solvent and visualized using resorcinol. Fractions containing the aminophenyl derivative were combined and lyophilized. Synthesis of the phenylisothiocyanate derivative was accomplished by incubating 9.6 μmol of the aminophenyl derivative with 21.1 μmol of thiosphosgene in 1 ml of 75% ethanol at room temperature for 1 h. Volatile organic compounds were removed by evaporation under nitrogen, residual water by lyophilization. The remaining solid contained the phenylisothiocyanate derivative of oligo-GM1 (oligo-GM1-PITC) plus any unreacted aminophenyl derivative (see Figure 1). Purity of each derivative of oligo-GM1 was determined by HPTLC with butanol:acetic acid:water (2:1:1, by vol) or methanol:n-butanol:water (2:1:1, by vol) used to develop the chromatograph and resorcinol to visualize sialic acid containing compounds. Low resolution negative ion fast atom bombardment (-Fab) mass spectroscopy was used to determine the mass of the synthesized oligo-GM1-PITC.

Synthesis of poly(propylene imine) dendrimers

First and second generation dendrimers, with four or eight primary amino groups, respectively, were synthesized according to the method described by de Brabander-van den Berg and Meijer [8] except borane methylsulfide in

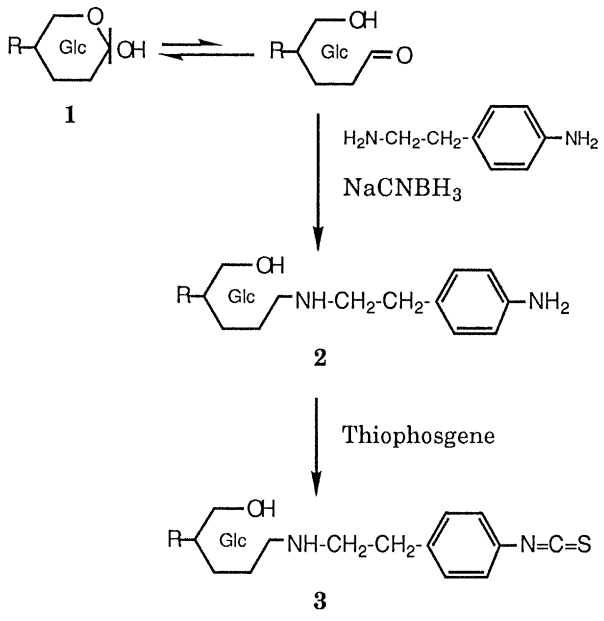


Figure 1. Flow chart for the synthesis of the phenylisothiocyanate derivative of the oligosaccharide galβ1-3galNAcβ1-4[sialic acid α2-3]galβ1-4glc (**1**, oligo-GM1). Compound **1** was converted to **2**, the aminophenyl derivative, by reductive amination using 2-(4-amino phenyl)ethylamine and sodium cyanoborohydride in borate buffer, pH 8.0. Compound **3**, the phenylisothiocyanate derivative of oligo-GM1 was produced by reacting compound **2** with thiophosgene in 75% ethanol. The **R** indicates the galβ1-3galNAcβ1-4[sialic acid α2-3]galβ1-4 portion of the oligosaccharide.

anhydrous tetrahydrofuran (THF), instead of catalytic hydrogenation, was used to reduce nitriles on the dendrimer intermediates to the corresponding primary amines [16]. The reduction was carried out as follows. Under a nitrogen atmosphere, 16.6 mmol dendrimer – $(CN)_4$ was dissolved in 100 ml anhydrous THF and heated to 50 °C, prior to the gradual addition of 73.2 mmol of borane methylsulfide. After heating at 50 °C for 1 h, 100 ml of anhydrous methanol was slowly added, and excess borane was converted to methyl borate by bubbling anhydrous HCl through the mixture. The reaction mixture was then refluxed for 1 h at 65 °C to form the dendrimer generation 1-amine hydrochloride salt. The contents of the flask were rotoevaporated to dryness. Methyl borate was removed by resuspending the residue in 50 ml of methanol and drying it by rotoevaporation three times. Unreduced dendrimer was removed from the first generation product by dissolving the residue in 100 ml of water and extracting it three times with 100 ml of chloroform. The extracted aqueous phase was dried, yielding the dendrimer-$(NH_2 \cdot HCl)_4$ (generation 1.0). A second generation dendrimer was obtained by converting the dendrimer-$(NH_2 \cdot HCl)_4$ to dendrimer-$(CN)_8$ and then reducing it to obtain dendrimer-$(NH_2 \cdot HCl)_8$ (generation 2.0) using the procedures described above except that the molar ratio of borane methylsulfide to dendrimer-$(CN)_8$ was 8.5 : 1 (see Figure 2).

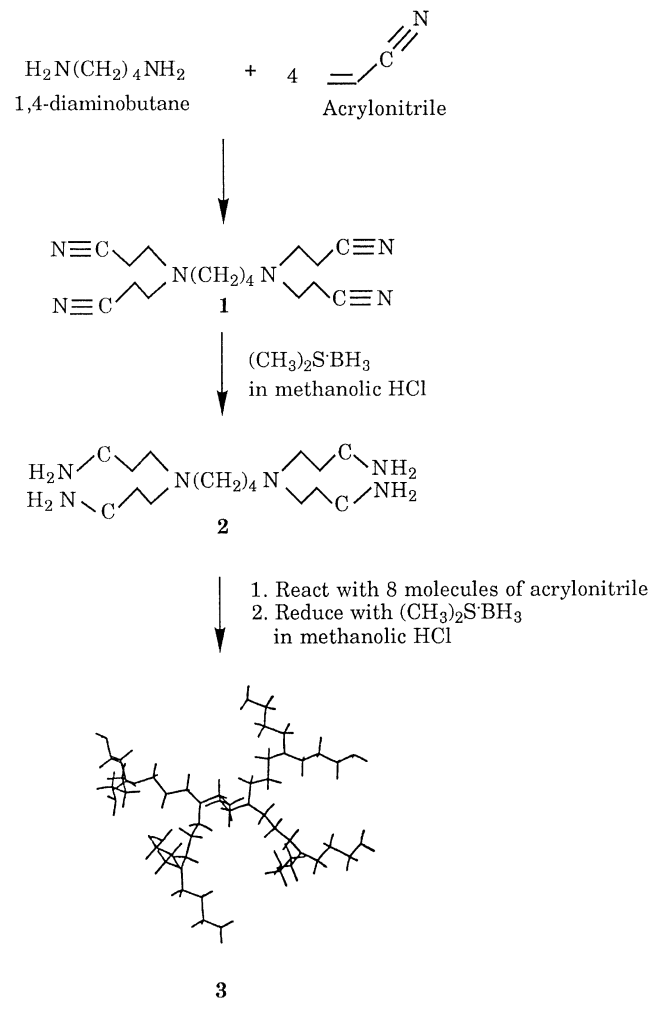


Figure 2. Flow chart for the synthesis of tetra- and octa(propylene imine) dendrimers. Synthesis of **1**, an 0.5 generation poly(propylene imine) dendrimer was accomplished by allowing 1,4 diaminobutane to react with acrylonitrile. Borane methyl sulfide was used to reduce **1** to produce the tetra(propylene imine) dendrimer with four terminal primary amino groups, **2**. Repetition of the addition and reduction reactions yielded **3**, the octa(propylene imine) dendrimer, with eight terminal primary amino groups. Compound **3** is drawn in its computer-modeled, lowest energy conformation.

Synthesis of oligo-GM1 dendrimers

Coupling of the oligo-GM1-PITC to each of the dendrimer cores was accomplished by combining oligo-GM1-PITC with the dendrimer in 0.2 M borate buffer, pH 9.0. A 5:1 molar ratio of oligosaccharide-PITC to dendrimer was used for dendrimers with four primary amino groups, and a 9:1 molar ratio for dendrimers having eight primary amino groups. In each case, the mixture was stirred for 15 h at 37 °C. When the Starburst™ dendrimer was used the reaction time was increased to 30 h and a 20:1 molar ratio of oligo-GM1-PITC to dendrimer was used. Oligo-GM1 dendrimers were isolated from lower molecular weight

components by size exclusion centrifugation, using the appropriate Centricon™ filter. Retentates were rinsed three times by addition of water followed by reconcentration prior to drying under vacuum from the frozen state. Recovery of oligo-GM1 dendrimers was confirmed using HPTLC with either n-butanol:glacial acetic acid:H_2O (2:1:1, by vol) or chloroform:methanol:0.3% calcium chloride (60:35:8, by vol) as the developing solvent. Plates were visualized with both ninhydrin (to detect primary amines) and resorcinol (to detect sialic acid). Total sialic acid content of each oligo-GM1 dendrimer was determined using the thiobarbituric acid procedure [10]. Samples containing known concentrations of GM1 were treated in the same way in order to account for any loss of sialic acid due to incomplete hydrolysis or destruction of released sialic acid.

^{125}I labeling of the cholera toxin B subunit and the heat labile enterotoxin of *E. coli*

This was accomplished using the chloromine T procedure described by Williams *et al.* [17]. Purity of the labeled toxin was determined using SDS-polyacrylamide gel electrophoresis under reducing and nonreducing conditions [18]. Labeled proteins were visualized by autoradiography.

HPTLC overlay experiments [19]

These were done to determine whether ^{125}I-labeled cholera toxin B subunit could bind to the oligo-GM1 dendrimers and whether that adherence involved the GM1 binding sites on the toxin. Briefly, 0.65 nmol of GM1, asialo-GM1, and oligo-GM1-PITC dendrimers were chromatographed using HPTLC (methanol:glacial acetic acid:H_2O, 15:7.5:0.25, by vol). The plates were air dried, and dipped in an 0.075% solution of polyisobutyl methacrylate [20]. Nonspecific binding sites were blocked by incubating the plate in 0.1% BSA in phosphate buffered saline for 1 h at room temperature. The plate was then incubated in 0.1% BSA in phosphate buffered saline containing CT-B-HRP conjugate (1:50 000 dilution) for 1 h at room temperature. Following incubation, the plate was rinsed seven times in phosphate buffered saline, pH 7.2. Adherence of the B subunit was detected using SuperSignal™ CL-HRP, an enhanced chemiluminescent substrate for HRP. When experiments to determine whether GM1 could block the interaction between the toxin and oligo-GM1-PITC dendrimers were carried out the CT-B-HRP was incubated with 6.5 μM GM1 for 1 h at 37 °C prior to its incubation with samples on the HPTLC plate.

Well-binding assays

These were done to determine the effectiveness of the oligo-GM1 dendrimers at inhibiting the adherence of ^{125}I-labeled cholera toxin B subunit and heat labile enterotoxin to GM1-coated wells [6]. Briefly, GM1 in methanol was added to plastic microtiter wells and allowed to dry. Potential nonspecific binding sites were blocked by incubating the wells with phosphate buffered saline containing 0.1% BSA for 1 h at 37 °C. After 1 h, the buffer was removed and the wells used for the binding assay. Wells lacking GM1, but blocked in the same way, were used to determine nonspecific binding. Cholera toxin B subunit or the heat labile enterotoxin (~6 nM) was preincubated for 1 h at 37 °C in the presence or absence of inhibitor in phosphate buffered saline (pH 7.2) containing 0.1% BSA and then added to GM1-coated or control wells. After incubating for 1 h at 37 °C, the toxin was removed, the wells washed seven times with PBS, and bound ^{125}I-labeled toxin determined by counting in a gamma counter.

Results

Characterization of the oligo-GM1 derivatives

A thin layer chromatograph of GM1 and the oligo-GM1 derivatives is shown in Figure 3. The plate was developed in methanol:n-butanol:water (2:1:1, by vol) and the sialic acid containing compounds visualized using resorcinol spray. Rf values were 0.84, 0.76, 0.48, and 0.80, for GM1, oligo-GM1, oligo-GM1 phenylamine, and oligo-GM1-PITC, respectively. Average percentage yields were 31% for oligo-GM1, and 80% for the aminophenyl derivative. Purity of the derivatives as determined by densitometric scanning of bands on a thin layer chromatograph (Stratagene Eagle Eye scanner and NIH Image software) was essentially 100% for oligo-GM1 and 90% for the oligo-GM1 aminophenyl derivative. Densitometric scanning indicated that the phenylisothiocyanate derivative, which was not isolated from unreacted oligo-GM1 aminophenyl derivative, was

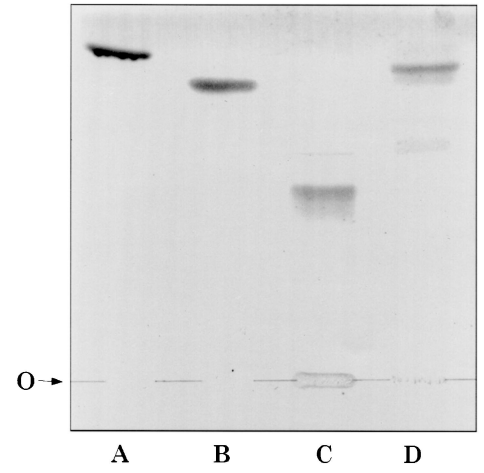


Figure 3. Thin layer chromatogram showing resorcinol positive bands obtained for oligo-GM1 derivatives. Compounds shown in each lane are: (1) GM1; (B) oligo-GM1; (C) the aminophenyl derivative of oligo-GM1; and (D) the phenylisothiocyanate derivative of oligo-GM1. The plate was developed in methanol: n-butanol: water (2:1:1, by vol) and the origin is indicated by the O.

about 80% pure. It was not necessary to isolate the oligo-GM1-PITC derivative because it was the only product that would covalently bind to the amino groups of the dendrimer under the conditions used. The oligosaccharides contained *N*-acetyl and *N*-glycolyl derivatives of sialic acid since each was present in the GM1 used (94% and 6%, respectively). Low resolution -Fab mass spectroscopic analysis of the oligo-GM1-PITC, produced two major peaks with molecular masses of 1185 and 1201 ($\pm$0.5 mass units).

Dendrimer cores

IR was routinely used to monitor the presence of nitriles and primary amines during the stepwise synthesis of the tetra- and octa(propylene imine) dendrimers. Generation 0.5 and 1.5 nitrile containing dendrimers, had a characteristic peak at $\sim 2260\ cm^{-1}$. This peak was absent in generation 1.0 and 2.0 dendrimers which had four and eight primary amino groups, respectively, and absorbed in the frequency range indicative of primary amines, 3350–3500 cm^{-1}. Analysis of generation 1.0 and 2.0 poly(propylene imine) dendrimers by HPTLC, using the solvents described above, showed that they remained at the origin. The compounds were strongly positive when visualized using ninhydrin spray. Generation 0.5 and 1.5, which lacked primary amines and moved from the origin upon HPTLC, were ninhydrin negative. The mass of the first generation dendrimer as determined by low resolution -Fab mass spectroscopy was 317 ($\pm$0.5 mass units).

Isolation and characterization of oligo-GM1 derivatized dendrimers

Centrifugation in centricons with appropriate molecular weight cutoff filters proved effective for separating oligo-GM1-PITC dendrimers from underivatized dendrimers and oligo-GM1-PITC. Both the oligo-GM1-PITC and underivatized dendrimer core could be detected in the filtrate when analyzed by HPTLC (data not shown). Oligo-GM1-PITC dendrimers, recovered in the retentate, remained at the origin when analyzed by HPTLC using the previously described mobile phases. Oligo-GM1-PITC poly(propylene imine) dendrimers were resorcinol positive and ninhydrin negative. In contrast, the oligo-GM1-PITC Starburst™ dendrimer was both resorcinol and ninhydrin positive, even though more rigorous conditions were used (see methods) for its synthesis compared to those used for the linkage of oligo-GM1-PITC to the corresponding poly(propylene imine) dendrimer.

Analysis of the sialic acid content of each of the dendrimers indicated that generation 1.0 poly(propylene imine) oligo-GM1-PITC dendrimers had four sialic acid residues, generation 2.0 poly(propylene imine) oligo-GM1-PITC dendrimers had an average of seven sialosyl residues, while Starburst™ oligo-GM1-PITC dendrimers had an average of six sialosyl residues. Since each oligo-GM1:PITC moiety has only one sialosyl residue, the results indicate that under the conditions used, an average of seven of the eight primary amines present on the second generation poly(propylene imine) dendrimer and an average of six of the eight primary amines on the Starburst™ dendrimer were derivatized with oligo-GM1-PITC. The generation 1.0 poly(propylene imine) dendrimer was fully derivatized.

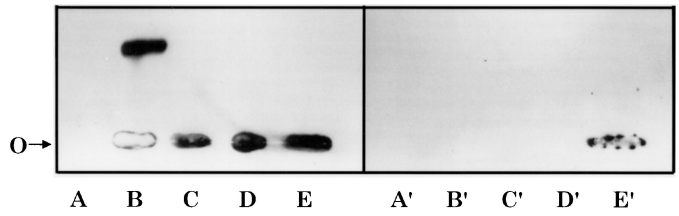


Figure 4. Adherence of horseradish peroxidase conjugated cholera toxin (HRP-CT) to GM1 and oligo-GM1-PITC derivatized dendrimers. Enhanced chemiluminescent detection was used to monitor adherence of the toxin to: (A) asialo GM1; (B) GM1; (C) tetra(propylene imine)(oligo-GM1-PITC)$_4$ (D) octa(propylene imine) (oligo-GM1-PITC)$_7$; and (E) polyclonal anti cholera toxin binding subunit antibody. Lanes A′–E′ show the same compounds as in A–E overlaid with HRP-CT that was preincubated with GM1 (for complete experimental details see the Experimental procedures section of the text). Plates were developed in methanol: glacial acetic acid:water (15:7.5:0.25, by vol). The origin is indicated by the O.

HPTLC overlay experiments

Using a chemiluminescent substrate of horseradish peroxidase to detect CT-B-HRP, it was determined that CT-B-HRP adhered to GM1, to first and second generation poly(propylene imine)oligo-GM1-PITC dendrimers, and to a polyclonal goat anti-choleragenoid antibody, adsorbed to the HPTLC plate after the plate was run (Figure 4). Oligo-GM1-PITC Starburst™ dendrimer was not tested under these conditions. CT-B-HRP did not adhere to asialo-GM1. When the HPTLC overlay experiment was repeated using CT-B-HRP preincubated with 6.5 μM GM1, the toxin no longer bound to either GM1 or the poly(propylene imine)oligo-GM1-PITC dendrimers, but did adhere to the anti-choleragenoid antibody.

Well-binding assays

All oligo-GM1 dendrimers were more effective at inhibiting the adherence of ^{125}I-labeled cholera toxin B subunit and the heat labile enterotoxin to GM1-coated wells than was GM1 (Figure 5). The concentrations of octa(propylene imine) (oligo-GM1-PITC)$_7$ dendrimer needed to inhibit adherence of the cholera toxin B subunit and heat labile enterotoxin to GM1 by 50% (IC_{50}) were 3 nM and 6 nM, respectively, compared to 45 nM and 30 nM for GM1. The IC_{50} for inhibition of adherence of the cholera toxin B subunit was 7–8 nM for both the tetra(propylene imine) (oligo-GM1-PITC)$_4$ and Starburst™ (oligo-GM1-PITC)$_6$ dendrimers. In contrast, the IC_{50} for free oligo-GM1

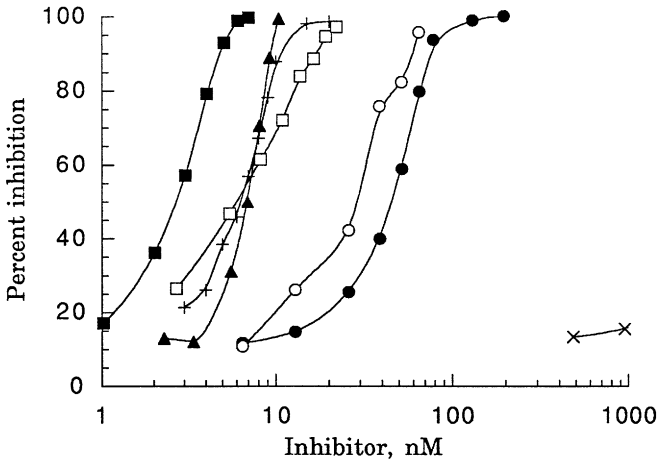


Figure 5. Inhibition of adherence of ^{125}I labeled choleragenoid and heat labile enterotoxin of *E. coli* to GM1-coated plastic wells by oligo-GM1-containing ligands. ^{125}I-labeled binding subunit or toxin was preincubated with inhibitor and then added to GM1-coated plastic wells. After incubating for 1 h at 37 °C, binding subunit or toxin was removed, the wells washed, and adherent choleragenoid or toxin detected using a gamma counter. Each point is the average of quadruplicate samples. Replicate experiments gave similar results. Open circles and squares indicate the inhibition of heat labile enterotoxin by GM1 and the octa(propylene imine) (oligo-GM1-PITC)$_7$ dendrimer, respectively. Inhibition of choleragenoid adherence to GM1 is indicated by closed circles, to octa(propylene imine) (oligo-GM1-PITC)$_7$ by closed squares, to tetra(propylene imine) (oligo-GM1-PITC)$_4$ by closed triangles, to Starburst™ (oligo-GM1-PITC)$_6$ by $+-+$, and to oligo-GM1 by $\times-\times$. The first two points are included for oligo-GM1 to indicate the relatively high (μM) concentration needed to inhibit adherence of the cholera toxin B subunit.

was $\sim$ 10 μM for the choleragenoid. This observation agrees with previous work that indicated the IC_{50}s for oligo-GM1 were in the μM range for both the cholera toxin binding subunit and the heat labile enterotoxin [6]. If a comparison is made between the average number of oligosaccharide residues present on the dendrimers and the number of free oligosaccharide residues needed for 50% inhibition there is still a greater than 250-fold difference in concentration. Concentrations of underivatized dendrimer equivalent to the amount of derivatized dendrimer used did not inhibit adherence of either the heat labile enterotoxin or the choleragenoid to GM1-coated wells.

Discussion

The fact that GM1 is the cell-surface receptor for cholera toxin and the heat labile enterotoxin of *E. coli* has been well documented (*eg* 5, 21–23). Previous studies also indicated that the spatial arrangement of GM1 oligosaccharides is important for the efficient adherence of the toxins [6, 24]. The results of this study indicate that oligo-GM1-PITC-derivatized dendrimers were synthesized and support the hypothesis that the oligosaccharide moieties of GM1, when presented in a clustered arrangement, are as effective or better ligands than GM1 for the adherence of the binding subunit of cholera toxin and the heat labile enterotoxin. Inhibition of toxin adherence to imobilized GM1 by the derivatized dendrimer is seen even though all of the binding sites on the toxin may not be occupied by the oligosaccharide residues of the dendrimer [*eg* the tetra(propylene imine)(oligo-GM1-PITC)$_4$]. In addition to the affinity of the toxin for the clustered oligosaccharide, adherence of the toxin to the dendrimer may sterically inhibit binding by the vacant binding site(s) to immobilized GM1.

The results indicate that the phenylisothiocyanate derivative of oligo-GM1 was synthesized. That both steps in its synthesis yielded a different derivative of the isolated oligosaccharide was supported by changes in mobility upon HPTLC. However, it was the experimentally determined mass of the product that confirmed the identification of the derivative. The two major masses obtained were those predicted for the sodium salt of a phenylisothiocyanate derivative of oligo-GM1 containing either *N*-acetyl- or *N*-glycolylneuraminic acid (calculated masses = 1184 and 1200, respectively, observed 1185 ± 0.5 and 1201 ± 0.5). The presence of both NANA and NGNA is in agreement with previous reports that both derivatives are in gangliosides isolated from bovine tissue [25, 26]. Finally, the effectiveness of the oligosaccharide dendrimers as ligands for the binding subunit of cholera toxin indicates that the oligo-GM1 portion of the product was not modified during its conversion to oligo-GM1-PITC. The reasons for using the phenylisothiocyanate derivative of oligo-GM1 instead of reductive amination to directly couple the oligosaccharide to the dendrimer were that: (1) the phenylisothiocyanate provided an additional 8.7 Å spacer which computer modeling indicated would provide a better 'fit' between ligand and toxin; and (2) its covalent linkage to the dendrimer was efficient. As others have noted (27), reductive amination reactions in aqueous medium are usually slow when molar amounts of aldehyde or amine are insufficient to drive the reaction. Synthesis of the phenylamine derivative by reductive amination was feasible because the molar amount of amine far exceeded the amount of oligo-GM1.

The results also indicate that first and second generation poly(propylene imine) dendrimers were prepared. Their synthesis was facilitated by the solubilities of the different intermediates. The presence of terminal nitrile groups on the 0.5 and 1.5 generation dendrimers resulted in their being readily soluble in most organic solvents. In contrast, the terminal amino groups on the first and second generation poly(propylene imine) dendrimers made them soluble in water. The different solubilities permitted the use of solvent extraction as a purification tool.

Oligo-GM1-PITC derivatives were prepared using both generation one and two poly(propylene imine) dendrimers as well as the Starburst™ dendrimer. The molar ratio of

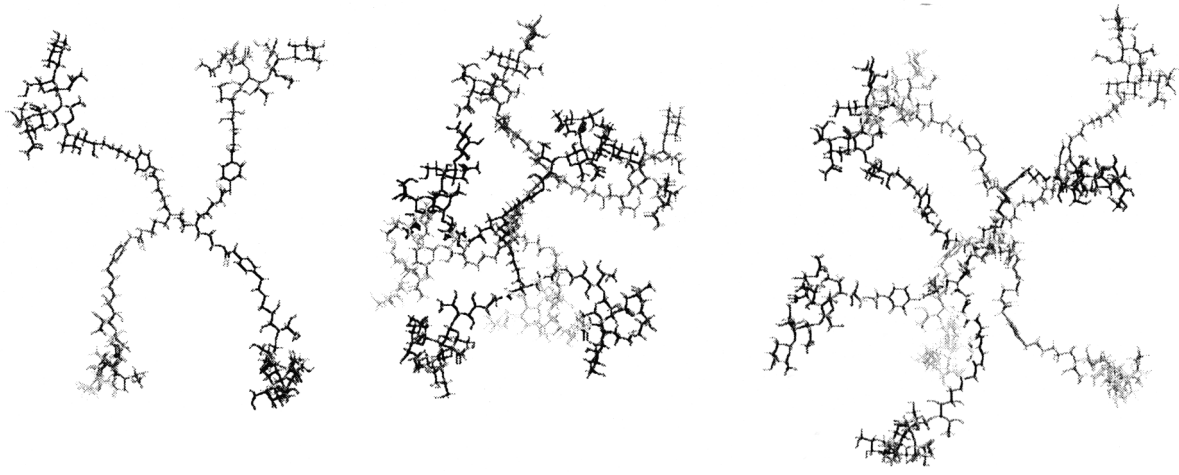


Figure 6. Predicted lowest-energy conformation of the fully derivatized oligo-GM1-PITC poly(propylene imine) and Starburst™ dendrimers. Energy conformations were predicted using the program Alchemy III. The tetra(propylene imine) oligo-GM1-PITC dendrimer is on the left, the octa(propylene imine) oligo-GM1-PITC dendrimer is in the center, and the Starburst™ oligo-GM1-PITC dendrimer is on the right. An oligo-GM1-PITC moiety can be seen at the end of each dendrimer arm. Structures shown are uniformly scaled to show their relative sizes.

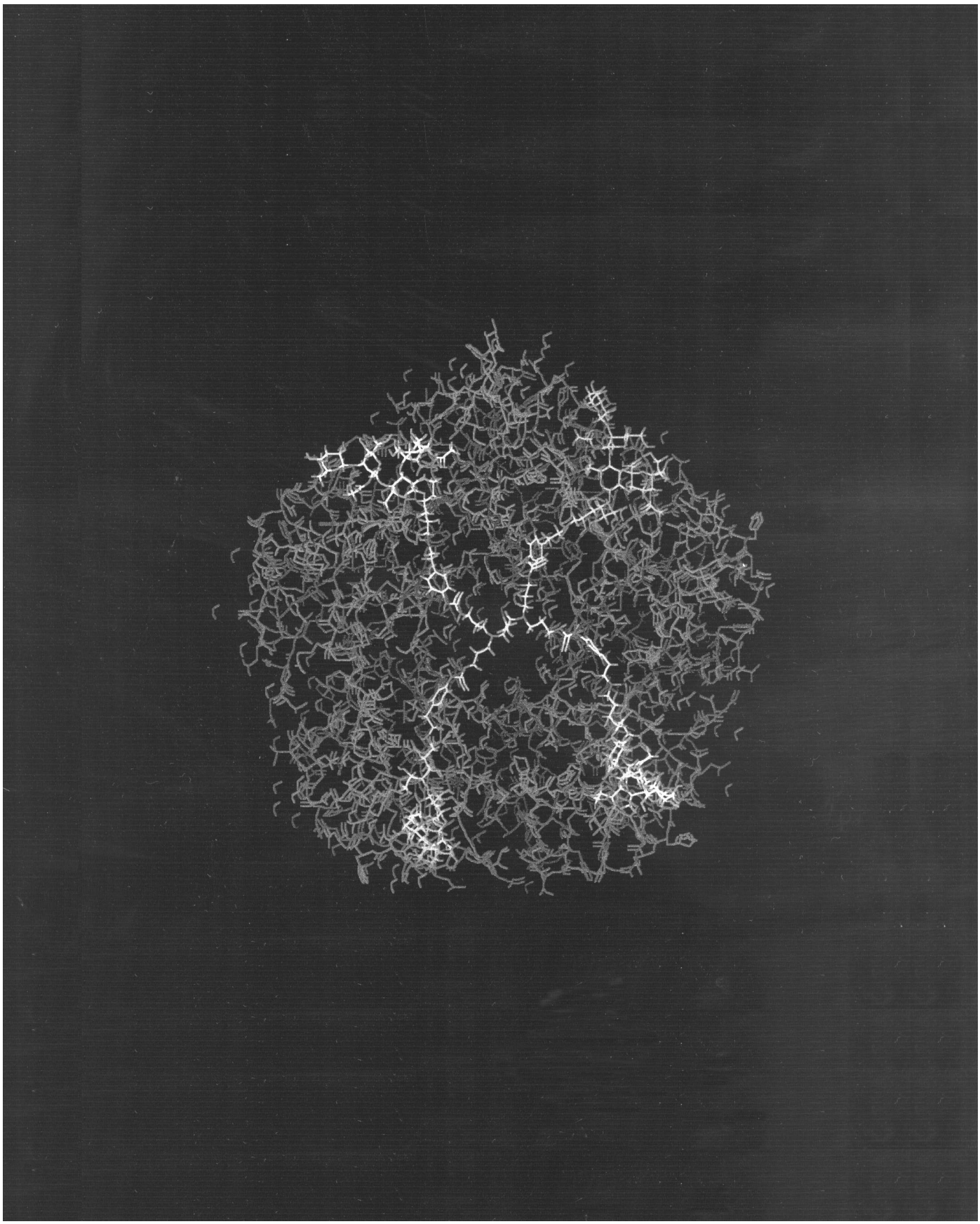
sialic acid per dendrimer molecule indicates that derivatization of the second generation dendrimers was not as efficient as that of the first. The second generation Starburst™ PAMAM dendrimer was least efficiently modified, with an average of only six oligo-GM1-PITC residues added, even though both the reaction time and concentration of oligo-GM1-PITC used were increased two-fold. This observation indicates that the molecular modeling (see below) prediction of increased steric hindrance around the Starburst™ dendrimer core relative to that of the poly(propylene imine) dendrimer may be correct.

HPTLC overlay experiments indicated that the poly(propylene imine) oligo-GM1-PITC dendrimers were adhered to by the cholera toxin B subunit-HRP conjugate. The fact that binding of the toxin to the poly(propylene imine) oligo-GM1-PITC dendrimers was inhibited by preincubation of the binding subunit with GM1 indicated that the binding site for GM1 was either the same as, or overlapped with, that responsible for adherence to the oligo-GM1-PITC dendrimers.

Based on the molar concentration of derivatized dendrimer needed to inhibit the adherence of labeled cholera toxin B subunit and the heat labile enterotoxin to GM1-coated wells by 50% (IC_{50}), the octa(propylene imine) $(oligo\text{-}GM1\text{-}PITC)_7$ dendrimer appeared to be an effective ligand. It was a more efficient ligand for the cholera toxin B subunit than either the tetra(propylene imine) $(oligo\text{-}GM1\text{-}PITC)_4$ dendrimer or the Starburst™ $(oligo\text{-}GM1\text{-}PITC)_6$ dendrimer. The implication of this observation is that choleragenoid binding to the oligo-GM1-PITC dendrimers is not based solely on the number of oligo-GM1-PITC moieties, since the Starburst™ oligo-GM1-PITC dendrimer contained an average of six oligo-GM1-PITC residues while the tetra(propylene imine)oligo-GM1-PITC dendrimer had four.

To determine whether the differences might reflect the spatial arrangement of the oligo-GM1 residues, molecular modeling was used to predict the lowest energy conformation and size of each of the fully derivatized oligo-GM1-PITC dendrimers (Figure 6). The software used was Alchemy™ III. Each of the oligo-GM1-PITC dendrimers was predicted to be large enough to span the diameter of binding subunit of both cholera toxin and the heat labile enterotoxin. Using the predicted structures, average molecular distances between the oligo-GM1 moieties of the oligo-GM1-PITC dendrimers were compared to the geometry of the GM1 binding sites predicted by X-ray crystallographic structure determination of the toxin B subunit [28]. The octa(propylene imine)oligo-GM1-PITC dendrimer, which if completely derivatized was predicted to have its oligo-GM1-PITC moieties spaced approximately the same distance apart as the binding sites on the B subunit, was the best inhibitor. In contrast, the Starburst™ oligo-GM1-PITC dendrimer, which, if fully derivatized, was predicted to have its oligo-GM1-PITC moieties too far apart for optimal interaction with the binding sites on the B subunit, was a less effective inhibitor.

Molecular modeling also predicted that the poly(propylene imine) dendrimer would be more flexible than the Starburst™. The poly(propylene imine) dendrimer core is linear in nature, whereas the Starburst™ dendrimer core contains amide bonds. The presence of the amide bond restricts the freedom of rotation at that site thereby decreasing its molecular flexibility. The three dimensional structure of the core molecules predicted more 'intramolecular room'



around each of the arms of the poly(propylene imine) dendrimers compared to those of the Starburst™ dendrimer. Therefore, although the oligo-GM1-PITC moieties on the tetra(propylene imine) dendrimer may not exactly fit the binding sites on the B subunit, it is possible that the molecular flexibility of the dendrimer arms would allow the oligosaccharides to move into appropriate positions (Figure 7).

The comparable LC_{50}s obtained with the intact heat labile enterotoxin of *E. coli* and the binding subunit of cholera toxin indicate that the A subunit of the holotoxin does not markedly affect adherence to the dendrimers. This may be due to the fact that according to published X-ray crystallographic data the A subunit of both cholera toxin [29] and the heat labile enterotoxin [30] does not appear to significantly penetrate through the surface of the toxin containing the GM1-binding domains.

Although dendrimers are a relatively new class of macromolecules, there are a number of investigators studying their potential uses [31]. Data from this study indicate that dendrimers can be used as the basis for the synthesis of clustered oligosaccharides. In this case they facilitated the formation of a ligand for both the B subunit of cholera toxin and the heat labile enterotoxin of *E. coli* that is better than the natural ligand, GM1. The observation that an oligo-GM1-PITC derivatized dendrimer was a better inhibitor of adherence than was native GM1 is important because it may provide a model for developing compounds that can inhibit the adherence of pathogens or toxins that recognize glycosphingolipids as a ligand. The lipid *per se* can not be used because it has been shown that exogenous glycosphingolipids can become functional components of a cell's plasma membrane. As a result, a previously nonsusceptible cell can be made susceptible to the toxic agent [32].

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Figure 7. Overlay of the crystal structure of the choleragenoid with the Alchemy III predicted structure of the tetra(propylene imine)(oligo-GM1-PITC)$_4$ derivative. The relative size of the toxin, gray, and the tetra(propylene imine) oligo-GM1-PITC dendrimer, white are shown. The structures indicate that the potential exists for the choleragenoid (GM1-binding sites are located near the 'points' of the toxin pentamer) to adhere to more than one of the oligo-GM1-PITC moieties at the end of each arm of the dendrimer. In addition, it appears that adherence of the choleragenoid to three oligo-GM1-PITC moieties would result in blockage of the central pore.

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