Cholera Toxin B-Mediated Targeting of Lipid Vesicles Containing Ganglioside GM1 to Mucosal Epithelial Cells

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Purpose. To determine whether the non-toxic pentameric B subunit of Cholera toxin (CTB) binding to ganglioside GM1 on both the lipid vesicles and epithelial cells may provide a means to target lipid vesicles to mucosal cells expressing surface GM1.

Methods. Sonicated lipid vesicles containing ganglioside GM1 were prepared. Inter-vesicle cross-linking due to pentameric CTB binding to these GM1 vesicles was determined with a sub-micron particle analyzer. Association of CTB to GM1 vesicles was analyzed with continuous sucrose gradient centrifugation. CTB-mediated binding of GM1 vesicles to human mucosal epithelial cells (Caco-2 and HT-29), mucous membranes of mouse trachea, and nasal tissues were detected with fluorescent labeled vesicles.

Results. An increase in lipid particle size due to binding of CTB to lipid vesicles and inter-vesicles cross-linking was detected. At a 30-to-1 mole ratio of membrane-bound GM1-to-CTB, optimum increase in GM1 vesicle aggregation, was detected. Under such conditions, all the added CTB molecules were associated with GM1 vesicles. Time course analysis showed that inter-vesicles cross linking by CTB was detectable within 10 min. and reached a maximum value at 60 min. CTB associated GM1-vesicles bind to mucosal epithelial cells HT-29 and Caco-2 with similar affinity [$K_d = 7.8 \times 10^{-4}$ M lipid (Caco-2) and 7.6×10^{-4} M lipid (HT-29)]. GM1 mediated binding specificity was demonstrated by blocking with anti-GM1 antibody and the insignificant degree of CTB-associated GM1 vesicle binding to GM1 deficient C6 cells.

Conclusions. The CTB-mediated GM1 binding to multiple membrane surfaces provides selective localization of GM1 vesicles to GM1 expressing mucosal cells and tissues. The strategy may be useful in localizing drugs and proteins to gut and respiratory tract mucosa.

KEY WORDS: Cholera toxin B; ganglioside GM1; mucosa; liposome; drug targeting; drug delivery.

INTRODUCTION

A number of enterotoxins bind to the epithelial cells lining the mucosal surfaces through membrane-bound ligands. One such enterotoxin from Cholera (*vibrio cholerae*), cholera toxin, has been well studied for its molecular interactions with ligands expressed on epithelial cells of mucosa. Each cholera toxin molecule contains five B subunits (CTB) and one A subunit (CTA). The pentamer B subunits are responsible for selective binding to the epithelial cells through cell surface monosialated glycolipid or ganglioside GM1 (1). Upon binding to five GM1 on the cell surface, the CTB pentamer is hypothesized to mediate insertion of the cytotoxic subunit A into cytoplasm. Once it is inside, the A subunit of cholera toxin catalyzes the ADP-ribosylation of the signal transduction protein Gs-α of the adenylate cyclase system. ADP-ribosylation of Gs-α blocks its capacity to hydrolyze bound GTP to GDP; as a result, GTP activates adenylate cyclase, leading to intracellular accumulation of cAMP (2). An elevated cAMP concentration in the small intestine results in the secretion of fluid to the intestinal tract in mediating cholera-dependent pathogenicity.

A number of methods have been developed to separate and purify the functional B and A subunits of cholera toxin to homogeneity. As a result, the possibility of using CTB without its cytotoxic component CTA to target drugs and proteins becomes feasible. Recently, a number of cytokine-CTB and drug or protein-CTB conjugates have been constructed using molecular cloning or chemical conjugation techniques to target drugs or proteins (3–5). While these approaches provide binding specificity of CTB to cells expressing GM1, they often require extensive time investment in synthesis or cloning to obtain functional hybrid molecules. Moreover, the capacity of the final hybrid molecule is usually a one-to-five CTB holo protein per drug or protein of interest.

CTB-mediated targeting of drug and proteins to mucosal surfaces can be greatly improved if one can develop a generalized carrier expressing surface CTB. This can be achieved using lipid vesicles expressing ganglioside GM1 and CTB. Addition of CTB to these vesicles will noncovalently link CTB to vesicles. If additional sites on CTB are available for binding to GM1 expressed on cell surfaces, this strategy will permit targeting of drugs associated with or encapsulated in lipid vesicles to epithelial cells expressing surface GM1. We have developed such a carrier, characterized its molecular interactions in optimizing the CTB-expressed vesicles binding to epithelial cells in culture, and demonstrated the targeting effects of CTB-expressed lipid vesicles to mucosal tissues.

MATERIALS AND METHODS

Materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) were purchased from Sygena, Inc. (Cambridge, MA). N-(7-nitro-2,1,3-benzoxadiazol-4yl)-PE(NBD-PE) was obtained from Avanti Polar Lipids (Alabaster, AL). Ganglioside GM1 and rabbit antiserum against ganglioside GM1 were purchased from Matreya, Inc. (Pleasant Gap, PA) and CTB from Sigma (St. Louis, MO).

Animal and Cell Lines

Human intestinal cell lines HT-29 and Caco-2, and surface GM1 deficient, rat glial tumor cell line C6 were obtained from the American Type Culture Collection. HT-29 and Caco-2 were grown in RPMI-1640 and DMEM, respectively, with 10% fetal calf serum. C6 cells were grown in Ham's F-10 with 15% horse

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ABBREVIATIONS: CTB, cholera toxin B subunit; GM1, ganglioside GM1; NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4yl)-PE; BSA, bovine serum albumin; DMPC, 1,2-Dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol; PBS, phosphate buffered saline; GI, gastrointestinal tract.

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serum and 2.5% fetal bovine serum. For vesicle-cell binding experiments, 1×10^6 cells were seeded in six-well plates overnight, to form monolayers.

C57BL mice were obtained from the University of Washington, Seattle, WA. To isolate nasal tissues for the GM1 lipid vesicle binding experiment, the mice were euthanized by cervical dislocation. After the cheek muscle was removed, the nasal mucosal tissues were dissected from the nasal septum and the weight of the tissue was recorded for the binding experiment. To isolate the trachea, the thoracic cavity was first exposed, then the lung and trachea tissue were separated. Subsequently, the trachea was dissected and used for binding experiments and for histochemistry.

All animal studies were performed according to the approved protocols of the Animal Care Committee at the University of Washington.

Lipid Vesicle Preparation

Lipid vesicles containing DMPC, DMPG, cholesterol and GM1 (molar ratio of 6:1:3:0.001) were prepared by sonication to obtain small unilamellar vesicles as described previously (6–8). Lipid vesicles without GM1 were made with same ratio of lipids and cholesterol. Fluorescent labeled vesicles used in the tissue binding experiment were prepared by the addition of 1 mole % NBD-PE to both test and control vesicles. Briefly, the mixtures containing 50 or 100 μmol lipid were dissolved in 3 ml of chloroform:methanol (2:1, v/v). The organic solvent was removed under a gentle stream of N_2 and vacuum desiccated. Phosphate buffered saline (PBS) was added to hydrate the lipids, and the mixture was subsequently sonicated in a bath-type sonicator (Hicksville, NY) until a translucent unilamellar vesicle suspension was obtained. Under these conditions, the suspensions mainly contained small unilamellar vesicles as determined by negative-stained electron microscopy. The number of lipid and GM1 molecules in each vesicle was estimated according to Enoch & Strittmatter, 1979 (21).

Characterization of Lipid Vesicle Size

To determine CTB-dependent aggregation of GM1 vesicles, varying amounts of CTB (e.g., 0–2 μg) were incubated with 1 μmol lipids in 30 μl of PBS at 37°C for 30 min. Subsequently, 2 ml of PBS was added to stop the reaction; and the mixture was transferred into a cuvette and analyzed with a Sub-Micron Particle Analyzer (Coulter, N4). To analyze the time-dependent aggregation of vesicles, CTB was incubated with GM1-vesicles in a fixed mole ratio (12:1) of GM1-to-CTB and the reaction was stopped as described above at indicated time (0–120 min) prior to determination of the lipid particle size. Control studies using vesicles without GM1 incubated with CTB, and bovine serum albumin (BSA) substituted for CTB, were performed in the same experiment. All the samples were tested in quadruplicate, and the data were presented as mean ± S.E.

CTB Binding to GM1 Vesicles

To determine binding of CTB to lipid vesicles, we first radiolabeled CTB with [^{125}I] using chloramine T, according to Hunter and Greenwood (22), to a final specific activity of 7100 cpm/μg. [^{3}H]-labeled lipid vesicles, either with or without GM1, prepared with a trace amount of hexadecyl[^{3}H]cholestanyl ether (CE) (25) [final specificity 3.5×10^9 cpm/mol of total lipid] were first mixed with total lipids inorganic solvent and the rest of liposome preparation procedure remained the same as above. Then, 6 μmol [^{3}H]-labeled lipid vesicles in 85 μl PBS, pH 7.4 were incubated at 37°C with a varying amount of [^{125}I]-CTB to a final GM1-to-CTB mole ratio of 40, 24 and 12 or PBS in a final volume of 200 μl. [^{3}H]-labeled lipid vesicles without GM1 were incubated with [^{125}I]-CTB as a control. After a 30 min. incubation, the CTB-vesicle mixture was loaded on 5 ml of a linear 5–20% sucrose gradient with 0.5 ml of a 65% of sucrose cushion. The duplicate samples were centrifuged in a 50 Ti rotor (Beckman) at 226,000x*g* for 5 h at 4°C (6). GM1 vesicles or [^{125}I]-CTB were also loaded separately on the sucrose gradients as controls. Subsequently, the gradients were fractionated from the bottom using a peristaltic pump. Each fraction was counted for [^{125}I] radioactivity to detect CTB content, 50 μl of each fraction was counted for [^{3}H] cholesterol radioactivity to detect lipid content. Data were expressed as the average of duplicates which were within 5% of the average value.

Cell and Tissue Binding

Vesicles Binding to Cells

For CTB-mediated vesicle binding to HT-29 and Caco-2 cells, CTB-bound vesicles were prepared by first incubating 2.5 μmol NBD-labeled vesicles with 0–1 μg CTB in 50 μl for 30 min. at 37°C. Then, the CTB-vesicle suspensions were diluted to 10-fold the original volume with PBS and added to 1×10^6 cells in six-well plates. After they were incubated at 37°C for 30 min., the unbound vesicles were washed three times with 5 ml PBS. Subsequently, 2 ml of PBS containing 5 mM EDTA and 1% Triton X-100 was added to solubilize cell-associated vesicles; fluorescent labeled vesicles were quantitated with a spectrofluorometer (Hitachi F4500). NBD-PE fluorescence was measured at λ_{ex} = 460 nm and λ_{em} = 530 nm. Fluorescent labeled vesicles without GM1 were also used as a control. Specificity of CTB-bound GM1 vesicles binding to cell surface GM1 was assayed by incubating HT-29 cells with rabbit anti-GM1 serum in PBS at 37°C for 30 min. before exposing to CTB-bound GM1 vesicles.

Vesicles Binding to Tissues

CTB-bound vesicles were first prepared by incubating 5 μmol vesicles with 1.2 μg CTB (GM1-to-CTB mole ratio = 24) in 50 μl for 30 min. at 37°C to arm GM1 vesicles with CTB. Under this incubation condition, we found that all CTB added was associated with GM1 vesicles (Figure 3E). These vesicle-CTB suspensions (control or test preparations) were diluted 10-fold of the original volume with PBS, and subsequently incubated with tracheal or nasal tissues, dissected from the same mice. Thirty minutes later, these samples were washed three times with 5 ml of PBS, and solubilized with 2 ml of PBS containing 5 mM EDTA and 1% Triton. Tissue-associated fluorescence, due to vesicle binding to cells, was determined with a fluorometer as described above.

All tests were done in quadruplicate, and data expressed were mean ± S.E of % total dose.

Scatchard Analysis

To determine the binding affinity of CTB-associated GM1 liposomes, GM1-to-CTB ratio was fixed at 24 and varying concentrations of total lipid were added to the cells. Preparation and incubation condition for CTB-GM1 liposome mixture was the same as described above. The concentration range 0–2.6 mM of total lipid was incubated with cells and determination of cell associated lipid vesicles was the same as described above. Data from the cell binding were further analyzed by Scatchard analysis (26). The data of NBD-vesicles binding to HT 29 and Caco 2 cells were transformed and plotted according to the following equation

$$\frac{[CTB - vesicles]_b}{[CTB - vesicles]_f} = -\frac{[CTB - vesicles]_b}{K_d} + \frac{n[E]_t}{K_d}$$

where $[CTB\text{-}vesicles]_b$ and $[CTB\text{-}vesicles]_f$ represent the concentration of cell associated or free CTB-vesicles, respectively. K_d is the dissociation constant, and $n[E]_t$ is the product of total concentration of CTB-vesicle binding site (n) and total cell numbers $[E]_t$. The data for each set of CTB-lipid vesicles binding experiments were fitted by linear regression method. K_d was determined from the slope of the fitted curve.

RESULTS

Binding of CTB to Vesicles Containing Ganglioside GM1

To characterize the interactions of vesicles containing GM1 and the pentamer of CTB, we first incubated them together and determined the time- and concentration-dependent effects by measuring vesicle aggregation using a sub-micron particle sizer. As shown in Figure 1, an apparent increase in vesicle size can be detected with increasing amounts of CTB added into the GM1 vesicle suspension. A minimum of 0.2 μg of CTB (equivalent to 30-to-1 GM1-to-CTB mole ratio) was needed

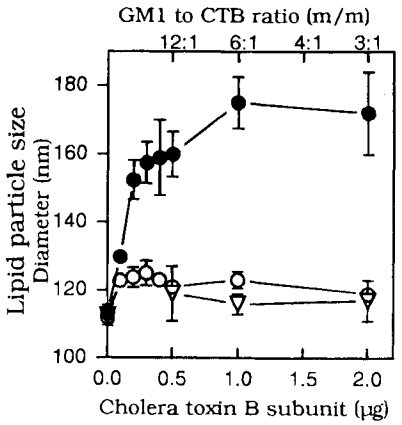

Fig. 1. Concentration dependent cholera toxin B binding to the lipid vesicles. One μmol of GM1-vesicles (●) or normal control vesicles without GM1 (○) were incubated with a varying amount of CTB in a final volume of 30 μl PBS at 37°C for 30 min. GM1-vesicles were also incubated with an equivalent amount of BSA as a control (▽). The mixtures were diluted to 2 ml for size estimation as described in the Materials and Methods. The top X axis shows mole ratio of GM1-to-CTB. All samples were tested in quadruplicate and data were presented as mean ± S.E.

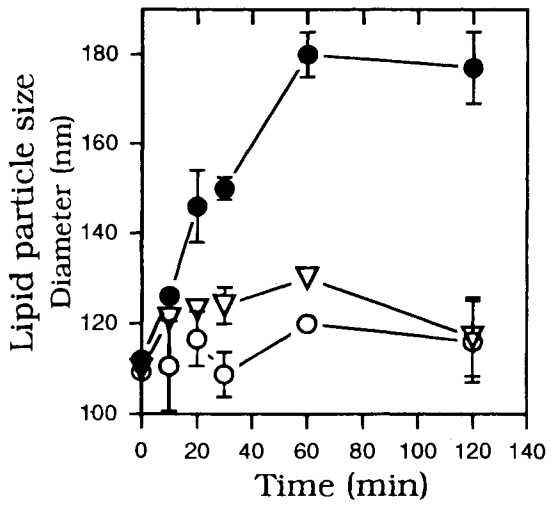

Fig. 2. Time-course of cholera toxin B mediated vesicle aggregation. One μmol of the GM1-vesicles (●) or control vesicles without GM1 (○) were incubated with CTB (GM1-to-CTB = 12:1, m/m) in 30 μl PBS, and the reaction was stopped at indicated time by dilution as described in the Materials and Methods. Lipid particle (vesicle) size was determined as described in Figure 1 in 2 ml. As a protein control for CTB, an equivalent amount BSA was incubated with GM1 vesicles (▽). All samples were tested in quadruplicate and data were presented as mean ± S.E.

to induce detectable increases in vesicle size. The CTB-GM1 interaction was specific in that control vesicles without GM1 or GM1-vesicles incubated with bovine serum albumin, failed to induce a similar degree of vesicle aggregation (Figure 1). Furthermore, addition of CTB beyond 2 μg in the suspension did not produce any additional increase in lipid particle size (Figure 1). Under these experimental conditions, no self-aggregation of CTB can be detected using size-exclusion chromatography, photon correlation spectroscopy and native polyacryamide gel electrophoresis (Data not shown).

Next, the kinetics of the CTB- and GM1-dependent vesicle size increase were characterized with a fixed GM1-to-CTB mole ratio of 12:1. CTB-induced GM1 vesicle aggregation can be detected within 10 min., and it reaches a maximum by 60 min. (Figure 2). No additional increase in the aggregation size of vesicles was detectable beyond 60 min. Control experiments substituting bovine serum albumin for CTB or removing GM1 from the vesicles failed to induce a similar degree of vesicle aggregation, indicating the specificity of CTB binding to GM1 on the vesicle surface.

The association of CTB to GM1 vesicles was further analyzed by sucrose gradient centrifugation to determine the extent of CTB association to GM1 vesicles. We tested GM1-to-CTB ratios of 12, 24, and 40 to determine whether all the CTB in the mixtures was associated with GM1 vesicles. As shown in Figure 3 (D–F), when the CTB-GM1 vesicle mixture was separated in a sucrose gradient, all the CTB added was associated with GM1 vesicles which banded at the top of the gradient (Figure 3B); whereas the free CTB alone distributed into the bottom half of the gradient (Figure 3A). CTB binding to GM1 vesicles was specific in that the mixture containing control vesicles (without GM1) and CTB did not co-distribute at the top of gradient despite a shift in the distribution of CTB toward

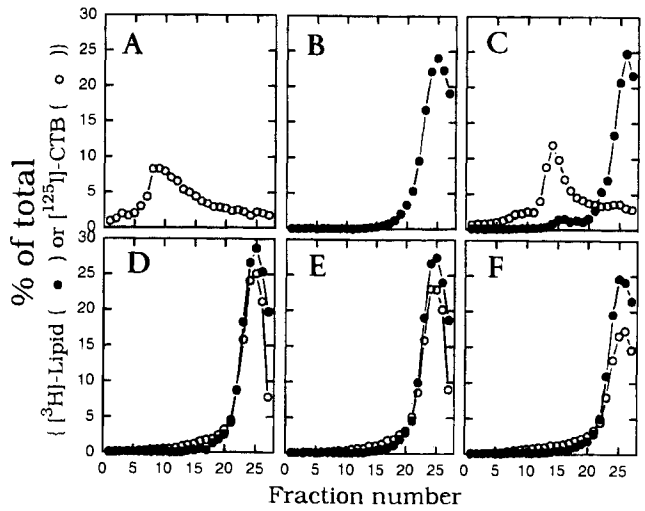

Fig. 3. Sucrose gradient centrifugation analysis of CTB binding to lipid vesicles. Two micrograms [^{125}I]-CTB (panel A) or 6 μmol [^{3}H]-labeled GM1 vesicle (panel B) was incubated separately in PBS; GM1 vesicles were incubated with 0.89 μg [CTB:GM1 = 40] (panel D), 1.5 μg [CTB:GM1 = 24] (panel E) and 3 μg of [CTB:GM1 = 12] CTB (panel F). Lipid vesicles without GM1 (6 μmole) were incubated with 3 μg CTB as a control (panel C). The resulting mixtures, separated on sucrose gradients by ultracentrifugation, were fractionated from the bottom of the tube. Each fraction was analyzed for [^{125}I]-CTB (○) and [^{3}H]-lipid (●); mean of duplicate samples were expressed as % of total loaded onto each gradient.

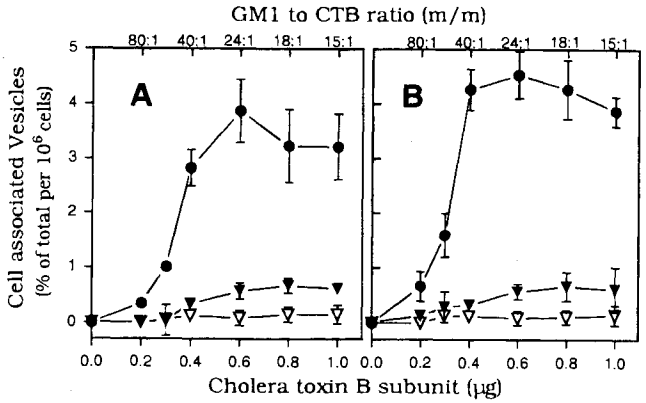

Fig. 4. Binding of GM1 vesicles to HT-29 (panel A), and Caco-2 (panel B) colonic epithelial cells. Fluorescent (NBD-PE) labeled GM1-vesicles (2.5 μmol) were first incubated with CTB at indicated GM1-to-CTB mole ratio. The mixture of CTB + GM1-vesicles (●), CTB + control vesicles (▽) or BSA + GM1 vesicle (▼) was added to 1 × 10^6 monolayer cells at 37°C for 30 min. After removing the unbound vesicles, the cell-associated vesicles were lysed with 1% Triton and EDTA. Cell-associated NBD-PE fluorescence was expressed as % of total added to the cells. The same sets of symbols were used for both panels A and B. Data expressed were mean ± S.E.

the top of the gradient. Together, these data indicate that CTB-mediated GM1 vesicle aggregation paralleled a tight and selective binding interaction between CTB and GM1 on lipid vesicles.

Characterization of CTB-Dependent GM1-Vesicles Binding to Epithelial Cells

To determine whether CTB-bound GM1-vesicles will provide enhanced binding to epithelial cells expressing surface GM1, we incubated CTB-GM1 vesicle with two epithelial cell lines, Caco-2 and HT-29. Cholera toxin was shown previously to bind to these cells (9,10). In our preliminary immunochemical staining experiment with anti-GM1 antibody, we confirmed that both of these cells expressed a significant amount of surface GM1 while glial cell C6 did not. Therefore, using NBD-PE as a membrane-bound fluorescent marker, CTB-mediated binding of vesicles expressing GM1 was detected as cell-associated NBD fluorescence, as described in the Materials and Methods. As shown in Figure 4, binding of vesicles to both Caco-2 and HT-29 cells was dependent on the amount of CTB added into the fixed concentration of vesicles expressing GM1, and hence the mole ratio of GM1 to CTB. Specific binding mediated by CTB was detectable at 0.2 to 0.3 μg of CTB, which is equivalent to a GM1-to-CTB mole ratio of 80-to-60. A maximum vesicle binding to cells was detected at a GM1-to-CTB mole ratio of 24. At this mole ratio, all the CTB added was associated with GM1 vesicles (Figure 3E). No additional vesicle-cell association was detectable beyond this ratio; in fact, a slightly reduced vesicle binding was detected at the high dose of CTB for both cell lines (Figures 4A and B). We also observed a higher degree of CTB-mediated GM1-vesicle binding to Caco-2 cells than to HT-29 cells under identical conditions (Figures 4A and B). However, both cell lines demonstrated minimal binding of GM1 vesicles when CTB was substituted with BSA. In addition, normal vesicles without GM1, either in the absence or presence of CTB, did not bind significantly to HT-29 cells.

To further characterize the specificity of CTB-dependent GM1 vesicle binding to epithelial cells, we used anti-GM1 antibody to inhibit CTB-GM1 vesicle binding to HT-29 cells. As shown in Figure 5A, anti-GM1 antiserum, but not control serum, significantly reduced CTB-dependent binding of GM1-vesicles to HT-29 cells ($p < 0.02$). In addition, CTB-associated GM1 vesicles did not bind significantly to C6 glial cells, deficient in surface GM1 (24) (Figure 5B).

Taken together, these data indicate that CTB-associated GM1-vesicles provide a CTB-dependent targeting of these vesicles to epithelial cells, probably through GM1, expressed on cell surface.

CTB-Mediated Association of GM1 Vesicles to Respiratory Epithelial Cells

We next determined whether CTB-bound GM1-vesicles could enhance localization of vesicles to epithelial cells in the respiratory tract. We used intra-nasal and trachea tissue from the mouse to determine CTB-mediated binding of GM1 vesicles, labeled with NBD-PE. Histochemical analysis on these tissues has revealed a high level expression of ganglioside GM1 on the mucosal membrane (data not shown). Therefore, we incubated freshly isolated mouse respiratory tract tissues with the GM1 vesicle-CTB suspension and tissue-associated vesicles were quantitated by measuring NBD fluorescence bound to the tissue. As shown in Table 1, a significantly higher accumulation of GM1 vesicles in tissue was detected only in the presence of CTB; about 50% lower tissue association of GM1 vesicle was observed when BSA or buffer (PBS) was substituted for CTB. A similar reduction in vesicle association was observed for

Table 1. Binding of CTB-Bound GM1-Vesicles to Tissues in Mouse Respiratory Tract

Tissue	Tissue associated vesicle fluorescence [arbitrary unit (mean ± S.E.) per 10 mg tissue]				
	PBS	G − L + CTB[a]	G − L[b]	G − L + BSA[c]	L + CTB[d]
Nasal	0.14 ± 0.02 (n[e] = 12)	62.76 ± 6.5[f] (n = 12)	30.0 ± 9.3 (n = 4)	30.5 ± 2.5 (n = 4)	23.65 ± 8.4 (n = 4)
Trachea	0.14 ± 0.02 (n = 12)	19.20 ± 3.7[g] (n = 12)	5.5 ± 0.5 (n = 4)	9.75 ± 0.8 (n = 4)	6.53 ± 2.6 (n = 4)

[a] G − L + CTB: Indicated tissues were incubated together with CTB-fluorescent labeled GM1-vesicle (GM1-to-CTB = 24:1, m/m) suspension. Unbound vesicles were washed with PBS. Finally, 2 ml PBS containing detergent was added to solubilize the tissue. Vesicle binding to tissue was quantitated with a fluorometer at λ_{ex} = 460 nm, and λ_{em} = 530 nm.
[b] G − L: Tissues were incubated with GM1-vesicles without CTB addition.
[c] G − L + BSA: Tissues were incubated with GM1-vesicles preincubated with BSA instead of CTB.
[d] L + CTB: Equivalent control vesicles without GM1 were preincubated with CTB prior to their addition to tissue.
[e] *n* indicates numbers of mice tested.
[f] G − L + CTB vs. G − L $p < 0.02$, vs. G − L + BSA $p < 0.05$, vs. L + CTB $p < 0.02$.
[g] G − L + CTB vs. G − L $p < 0.02$, vs. G − L + BSA $p < 0.05$, vs. L + CTB $p < 0.05$.

both intra-nasal mucosal and trachea tissues treated with control vesicles without GM1. Taken together, these data indicate that CTB can enhance GM1 vesicle binding to epithelial cells of the respiratory tract.

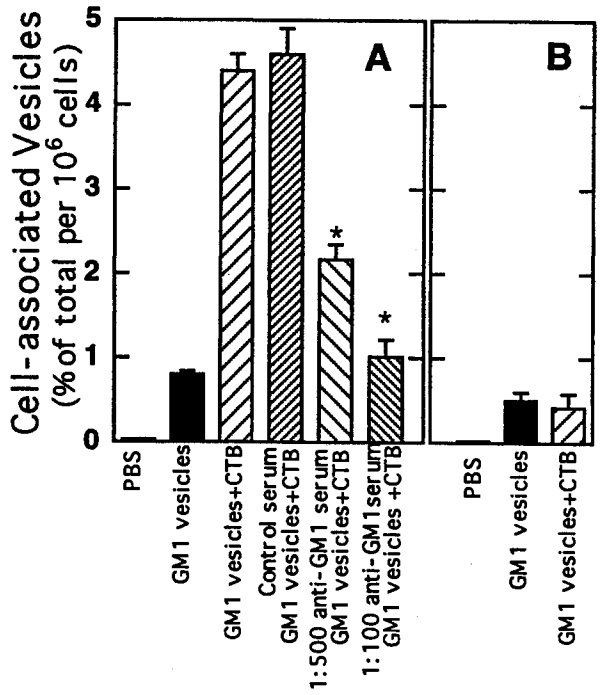

Fig. 5. Specificity of CTB-bound GM1-vesicles binding to cell surface GM1. Cell surface GM1 specific binding of the CTB-bound GM1-vesicles was determined with GM1 positive HT-29 cells (panel A) and GM1 deficient C6 cells (panel B). *Panel A*: HT 29 cells were blocked with 1:500 (▨) or 1:100 (▩) diluted rabbit anti-GM1 serum or control (▨) rabbit serum. These cells were incubated subsequently with CTB + GM1-vesicle (2.5 μmol) suspension for 30 min. Cells incubated with GM1 vesicles (■) or PBS (□) were also tested as controls. *Panel B*: C6 cells were incubated with PBS (□), GM1 vesicles (2.5 μmol) (■) or CTB + GM1 vesicle (2.5 μmol)(▨). GM1-to-CTB ratio was fixed at 24. Binding of GM1 vesicles to cells was determined as described in Figure 4. Data presented were mean ± S.E. of quadruplicate (* anti GM1 groups compare with control serum treatment, $p < 0.02$).

DISCUSSION

It is now well established that CTB recognizes and binds to ganglioside GM1 on the cell surface with high specificity and affinity (11,12). More recently, fine details of the physical interactions of GM1 binding to CTB have been elucidated by X-ray crystallography and surface plasmon resonance spectroscopy (13,14). Taking advantage of CTB-GM1 interactions, a number of drug and cytokine-CTB conjugates have been constructed to deliver drug and therapeutic proteins into cells expressing surface GM1 (15,16,23). In addition, CTB binding to vesicles expressing GM1 has been demonstrated to be similar to that of CTB binding to cell-surface GM1 ($K_d = 10^{-10}$ M) (17). To provide a more general carrier for the delivery of protein and drugs to GM1 expressing cells, a number of investigators have used CTB covalently conjugated to vesicle surfaces (4,5,18). While these approaches may provide target specificity for the vesicles, the efficiency of CTB conjugation and the multiple steps of exposure to cross-linking agents, essential for CTB expression, may potentially denature and inactivate drugs and proteins. To overcome these limitations, we have designed and characterized a novel strategy using a noncovalent approach in constructing CTB-expressed vesicles. Based on the ability of CTB to bind to both vesicles and cell surface GM1, we have successfully developed a more robust vesicle targeting scheme using CTB-associated GM1 vesicles as CTB-expressed vesicle carriers that are targeted to GM1-expressing cells (Figure 4–5). No covalent linking of CTB to the vesicle surface is necessary to produce CTB-mediated vesicle binding to epithelial cells and mucosal tissues (Figure 3–5 and Table 1).

Our GM1 vesicle-CTB interaction studies indicate that binding of CTB to GM1 vesicles and subsequent vesicle aggregation depend on the CTB concentrations for a given (lipid) vesicle concentration. As schematically presented in Figure 7, at low concentration of CTB, i.e., high GM1-to-CTB ratios, most of the CTB-bound GM1 vesicles are likely to be in the single vesicle suspension; as more CTB is added, additional GM1 binding sites on the CTB become available for binding

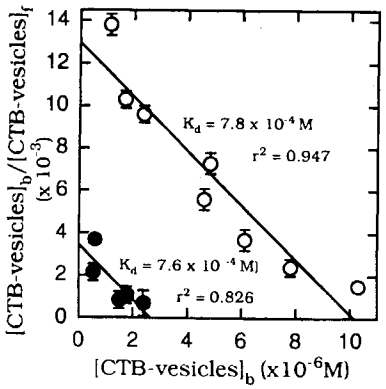

Fig. 6. Scatchard plot for CTB-bound GM1-lipid vesicles binding to Caco2 (○) or HT-29 cells (●). GM1 to CTB molar ratio was fixed at 24.

adjacent the vesicle as well as within the same vesicles. This hypothesis is contrary to the commonly accepted notion that five valency of CTB pentamers, each subunit with a single GM1 binding site, are fully occupied by cell-surface GM1 on a single vesicle or a single cell membrane. Our results of CTB concentration-dependent GM1 vesicle aggregations indicate that CTB induces inter-vesicle cross-linking suggesting that CTB binding across different cell and vesicle membranes is possible. These results suggest that additional GM1-binding sites are available for CTB that are bound to GM1 in vesicle. This hypothesis is consistent with observation that cholera toxin can agglutinate vesicles containing GM1, GM1-coated polystyrene spheres or erythrocytes (19,20), and lymphocytes and ganglioside-Sepharose (21). When most GM1 vesicles were armed with CTB and some degree of vesicle aggregation (i.e., 24:1 GM1-to-CTB) occurs, these CTB-expressed GM1-vesicles can bind to cell surfaces through the GM1 "receptor." Within sonicated vesicles measuring around 110 nm, and 0.01 mole percent of GM1 in the vesicle surface, we estimated that there are about 30 molecules of GM1 in each unilamellar vesicle with approximately 3×10^5 lipid molecules (22). At a 24:1 (m/m) GM1-to-CTB ratio, we estimated that there will be about 1 CTB molecule per vesicle. In other words, only one or two CTB molecules will be needed to produce specificity in CTB-mediated binding. In our experiments, any additional increase in CTB beyond this ratio did not lead to an increase in the number of vesicles bound to each cell (Figure 4), suggesting that only one or two CTB are required for each vesicle to achieve GM1-specific binding to the cells. Whether changes in the size and surface charge of the vesicles may require different GM1-to-CTB binding optima is yet to be experimentally determined.

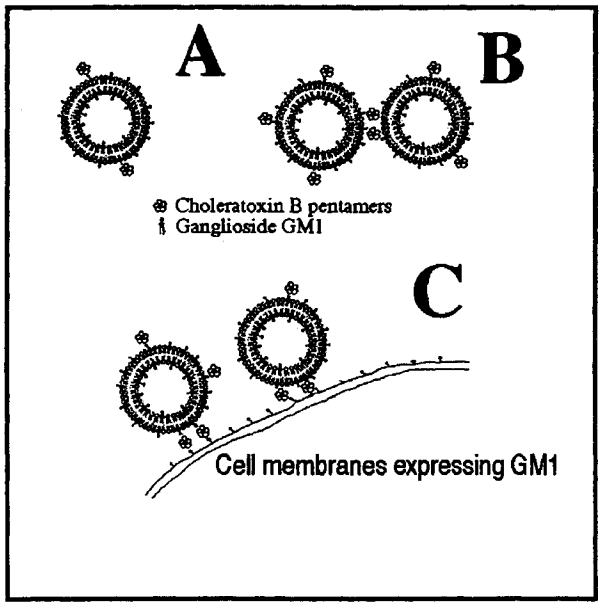

Fig. 7. Schematic representation of CTB-bound GM1-vesicles delivery system. *Panel A*: At low CTB-to-GM1 ratio, CTB molecules bind predominantly to a single vesicle. *Panel B*: At high CTB-to-GM1 ratio, more CTB molecules are available for binding to adjacent GM1-vesicle bilayers leading to inter-vesicle cross-linking. *Panel C*: Under optimal conditions, CTB-bound GM1-vesicles can bind selectively to cells that express GM1 on their surface.

Additional analysis of the CTB-bound GM1 vesicle binding to Caco-2 and HT-29 cells (Figure 6) indicates that these vesicles associate to cells with the dissociation constant K_d of 7.8×10^{-4} and 7.6×10^{-4} M total lipid in vesicle form, respectively. With 0.01% GM1 in the vesicle membrane and a GM1-to-CTB ratio of 24, the apparent dissociation constant of CTB to these cells was estimated to be similar to that of native CTB ($K_d = 10^{-10}$ M vs. 3.3×10^{-9} M). The similarity in dissociation constants between soluble CTB and vesicle-bound CTB suggests that there is little or no cooperative binding of multiple CTB molecules found on the surface of a single GM1 vesicle. Whether a higher density of GM1 in the lipid membrane (i.e., greater than 0.01% GM1 in lipid) may produce a cooperative binding effect is not known, and needs to be experimentally determined.

Regardless of the mechanisms of CTB-mediated binding of GM1-vesicles to cell surfaces, this strategy can be used to deliver both hydrophobic and hydrophilic drugs to mucosal epithelial cells that express a high degree of GM1. With intestinal epithelial cells, Caco-2 and HT-29, as well as mucosal tissue, we have shown that CTB-bound GM1 vesicles can selectively target these cells and tissues. Additionally, we found that this strategy is not limited to delivery of lipophilic drugs or marker (NBD-PE) but is also applicable for enhanced local delivery of a water soluble, hydrophilic enzyme, HRP to HT-29, a human epithelial cell (data not shown). Intracellular delivery may be further improved by choosing acid sensitive or other lipid membrane compositions which has been reviewed (27,28). Nevertheless, these results demonstrate that GM1-expressing vesicles can be constructed to deliver protein (such as cytokines and vaccine antigens) and DNA (such as CFTR, ADA, or alpha anti-trypsin genes) to epithelial cells in the respiratory and gastrointestinal (GI) tract. In the case of GI delivery, the increased localization of vesicles may provide extended absorption of the proteins in the upper GI tract and therefore potentially increase the bioavailability of the protein, as most of the food and drug absorption take place in the upper half of the ileum (provided some degree of absorption of protein is possible).

In summary, we have developed and characterized a novel CTB-associated vessicle generally applicable for target-selective localization to intestinal and mucosal epithelial cells. Non-

covalent attachment of CTB to vesicles is achieved by binding CTB to GM1 on vesicles; the resulting CTB-bound vesicles exhibit target selectivity with an affinity similar to that of the native CTB in solution. With a relatively robust vesicle construction scheme, this principle should be widely applicable for delivery of vaccines, proteins, genetic materials and other therapeutics with minimal modification.

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