Liposomes Targeted to Deliver Antisecretory Agents to Jejunal Mucosa

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ABSTRACT

The B subunit of cholera toxin has been covalently attached to the surface of liposomes made from a mixture of phosphatidylethanolamine, phosphatidylcholine and cholesterol. Adenylate cyclase inhibitors and chloride conductance inhibitors were encapsulated within the liposomes. These "targeted" liposomes were used to study the combined effects of this novel delivery system, and a limited number of possible antisecretory agents, on net fluid flux into the pig jejunum.

A state of net secretory fluid flux was induced in isolated jejunal loops in weanling pigs by adding theophylline or cholera toxin to the lumen of the isolated loops. There was no reduction in net fluid secretion when liposome suspensions without encapsulated secretory inhibitors were added to fluid in the lumen of loops treated with theophylline. There was also no reduction in net fluid secretion when miconazole, α-phenylcinnamate or 5 nitro-2-(3-phenethylamino)benzoate were encapsulated within targeted liposomes added to isolated jejunal loops. The net fluid flux induced by exposure of jejunal loops to theophylline was significantly reduced by adding targeted liposomes containing 2′-deoxy-3′-AMP. The reduction involved a reversal of net secretory fluid flux to an absorptive value.

The net fluid secretory response to treatment of loops with cholera toxin was also inhibited by treating loops with targeted liposomes containing 2′-deoxy-3′-AMP. However, the reversal of secretion was less complete for secretion induced by cholera toxin than for secretion induced by theophylline. The reduced antisecretory efficacy versus cholera toxin was not improved by encapsulating higher concentrations of 2′-deoxy-3′-AMP. A larger dose of the inhibitor delivered with increased numbers of liposomes caused a significant reduction in net fluid secretion. Occupancy of mucosal receptors by native cholera toxin may be a factor limiting access of liposomes with surface B subunit, and may reduce the utility of the B subunit of cholera toxin as a targeting agent for delivering antisecretory agents to the intestinal mucosa.

RÉSUMÉ

La sous-unité B de la toxine du choléra a été fixée à l'aide de liens covalents à la surface de liposomes fabriqués à partir d'un mélange de phosphatidyléthanolamine, phosphatidylcholine et de cholestérol. Des inhibiteurs de l'adénylate cyclase et de la conduction du chlore ont été encapsulés à l'intérieur des liposomes. Ces liposomes ont été employés pour l'étude de ce nouvel agent de libération ainsi que de différents agents antisécrétoires sur des intestins de porc.

Une sécrétion intestinale positive à partir d'une anse jéjunale de porcelets sevrés a été induite à l'aide de théophylline ou de la toxine du choléra. La suspension de liposomes non-chargés ainsi que des suspensions de liposomes contenant du miconazole, du 2-phénylcinnamate ou du 5-nitro-2-benzoate n'ont pas modifié l'activité sécrétoire de la théophylline sur l'intestin. Par contre l'activité sécrétoire de la théophylline et de la toxine du choléra a été renversée en un processus d'absorption lorsque des liposomes contenant du 2′-désoxy-3′-AMP ont été utilisés. Cependant, le renversement fut moins marqué pour la toxine du choléra.

Une augmentation des concentrations de 2′-désoxy-3′-AMP à l'intérieur des liposomes n'a pas augmenté l'effet anti-sécrétoire de la toxine du choléra. Par contre une augmentation de la dose d'inhibiteur couplée à une augmentation du nombre de liposomes a produit une diminution significative de sécrétion. L'occupation du récepteur par la toxine naturelle du choléra semblerait être un facteur limitant l'accès de la sous-unité B de la toxine liée aux liposomes ce qui indirectement en réduit l'activité des agents qui y sont attachés. (*Traduit par Dr Pascal Dubreuil*)

INTRODUCTION

Secretory diarrhea arises from the activation of regulatory processes that normally limit movement of electrolytes and water into the intestinal lumen. The main elements of the secretory mechanism include the intracellular second messenger, adenosine-3′,5′-cyclic monophosphate (cAMP) and the regulated chloride conductance protein located in the apical membrane of intestinal epithelial cells. Conductive transport of Cl^- into the intestinal lumen drives paracellular Na^+ and water movement into the lumen (1).

Antisecretory strategies have targeted a variety of processes including Ca^{2+}-binding proteins (2,3) and endorphin receptors (4,5) with varying degrees of success. Even with partial successes of these indirect approaches, the adenylate cyclase that produces cAMP and the chloride conductance protein which releases Cl^- into the intestinal lumen remain as the major targets for antisecretory therapy.

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Bulk addition of inhibitors of cAMP production or conductive Cl^- transport to the intestinal lumen has not been an effective antisecretory strategy (6,7). The poor efficacy of inhibitors supplied by this route has been attributed to problems in effective and specific delivery of therapeutic agents to sub-populations of intestinal cells which are specialized for a role in fluid secretion. Liposomes might be useful as a delivery vehicle that could reduce the required dose of inhibitors and minimize the side effects of antidiarrheal therapy. Liposomal delivery systems may be improved by attachment of surface ligands that will direct liposomes to bind to, and deliver contents into the intestinal cells containing surface receptors for the binding of bacterial enterotoxins. Coupling the B subunit of cholera toxin to liposome surfaces significantly increases the apparent binding affinity of the B subunit for the jejunal brush border surface (8).

Potential antisecretory agents were encapsulated within liposomes, and liposomes were coupled to a modified form of the B subunit of cholera toxin. The targeted liposomes produced by this procedure were tested for inhibitory effects on intestinal secretion by an *in situ* intestinal loop preparation.

MATERIALS AND METHODS

LIPOSOME PREPARATION AND CHARACTERIZATION

Egg yolk phosphatidylcholine and cholesterol in an 18:1 molar ratio (140 μmol of total lipid) was dissolved in chloroform and dried under vacuum in a rotary evaporator to deposit the lipid as a thin film on the wall of a flask. ^3H cholesterol (0.1 μCi) was added to the lipid mixture to measure lipid content of liposome suspensions. The dried lipid film on the side wall of the flask was dispersed by vigorous mixing with an aqueous buffer system containing 10 mM mannitol, 136 mM NaCl, 10 mM K_2HPO_4 (pH 7.4), as well as any agent intended for encapsulation within the liposomes. The aqueous resuspension system also contained 0.28 μCi 1-[^{14}C]mannitol to serve as a marker for liposome volume measurements and calculations of trapping efficiency. The lipid suspension was treated with five freeze thaw cycles to increase interlamellar spacings and trapped volumes (9). Liposome suspensions produced by vortex mixing were size-sorted by serial passage through two polycarbonate filters (0.4 μm pore size) using a high pressure filtration system (Extruder, Lipex Biomembranes Inc., Vancouver, British Columbia).

Unincorporated solute was removed by gel filtration of liposomes suspensions with Sephadex G-50. Fractions collected from the column were sampled to measure the content of ^3H and ^{14}C by dual label scintillation counting. The specific activity of the 1-[^{14}C]mannitol was used to convert dpm to nmol of mannitol. The equivalence of 10 nmol mannitol per μL was used to calculate the aqueous volume trapped within a sample of vesicles. This volume, or trapped space, was expressed as nL per nmole of lipid as determined from the amount of ^3H cholesterol in the sample.

Dipalmitoylphosphatidylethanolamine (DPPE) was used as a source of a functional group for coupling the B-subunit of cholera toxin to liposome surface. The DPPE was incorporated by addition into the lipid solution in chloroform. Vesicle space was measured as a function of the percentage of DPPE in the lipid mixture. Mayer *et al* (9) have reported that repeated passage through the extruder apparatus favors the formation of unilamellar liposomes. Vesicle suspensions were filtered up to four times to determine the effect of this treatment on trapped space within the vesicles.

Trapping efficiency was expressed as the percent of the total aqueous solute that could be retained within the vesicle space during resuspension of a lipid film. Calculations of entrapped space and efficiency of solute trapping were both based on vesicular content of 1-[^{14}C]mannitol. Trapping efficiency was measured as a function of varying proportions of DPPE, and total concentrations of lipid in the resuspension buffer.

CHOLERA TOXIN B SUBUNIT DERIVATIZATION

The B subunit of cholera toxin was treated with N-succinimidyl S-thioacetate (SATA) to introduce reactive sulfhydryl groups onto the surface of the protein. The reaction between SATA and cholera toxin B subunit was carried out for 30 min at 20°C in a 400 μL reaction volume containing 1.0 μmol of SATA and 10 nmol of B subunit in 50 mM Na_2CO_3 (pH 9.5). This ratio of reactants gave a tenfold molar excess of SATA over total lysine residues in the B subunit. The reaction mixture was separated from unreacted SATA by gel filtration in 10 mM MES buffer (pH 5.5). Modified B subunit was collected and deacetylated for two hours in a solution containing 50 mM hydroxylamine-HCl, 5.0 mM $NaHPO_4$ and 2.5 mM EDTA (pH 7.5). The sulfhydryl content of the deacetylated product was determined by reaction with Ellman's reagent (10).

CHOLERA TOXIN B SUBUNIT ATTACHMENT TO THE SURFACE OF LIPOSOMES

The DPPE (40 mg) was dissolved in dry chloroform, methanol and triethylamine (16 mL:2 mL:20 mg) and mixed with 20 mg m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) under anhydrous conditions for 24 h (11). The R_f of the product was measured by thin layer chromatography on silica gel in a solvent of chloroform, methanol and glacial acetic acid (65:25:13) (11). Maleimide-conjugated DPPE was added to the chloroform solution of phosphatidylcholine and cholesterol as 0.25% by weight of the total lipid (140 μmol) used for a single liposome preparation.

The modified B subunit with added SH groups was added immediately after the final passage of the aqueous liposome resuspension system containing maleimide-substituted DPPE through the pressure filtration system. The reaction mixture was maintained at 4°C for 16 h. The liposome suspension was separated from unconjugated B subunit by passage over a Sephadex G-50 desalting column equilibrated with 10 mM MES buffer (pH 5.5). The efficiency of the coupling reaction was monitored with [^{125}I]B subunit.

MEASUREMENT OF INTESTINAL SECRETION

Weanling pigs 17 to 20 kg were anesthetized with halothane and 10 cm jejunal loops were prepared, starting 30 cm distal to the ligament of Trietz, by transecting the gut and inserting

lucite cannulae. Net fluid flux in the loops was determined by the two compartment kinetic model of Berger and Steele (12), using [^{3}H]-polyethyleneglycol 4000 (1 μCi per L) as a dilution marker. Net and unidirectional chloride flux measurements were based on net fluid flux, content of Cl^- in the flux solution and the changes in concentration of ^{36}Cl marker added to the flux solutions (13). All measurements were carried out in an isotonic electrolyte mixture formulated to match the normal composition of pig jejunal loop fluid (13).

A state of net secretion was induced by adding 10 mM theophylline to jejunal loop fluid. Theophylline and targeted liposomes were combined in jejunal loop fluid when attempting to inhibit the net secretory effect of the theophylline.

Cholera toxin (crude lyophilized culture filtrate of *Vibrio cholerae*, Wyeth Labs, Philadelphia, Pennsylvania) was dissolved in 0.15 M NaCl and incubated in jejunal loops for 90 min prior to flux measurements. Loops were emptied and flux measurements were made in jejunal loop fluid without additional cholera toxin.

POTENTIAL SECRETORY INHIBITORS

Miconazole (1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)-methoxy] ethyl]-1 H-imidazole) (14) and adenosine- 2′-deoxy-3′-monophosphate(2′-d-3′-AMP) were used as adenylate cyclase inhibitors (15,16). α-Phenylcinnamate (αPC) and 5-nitro-2-(3-phenyl-ethylamino)-benzoate (NPEB) were chosen as potential chloride channel blockers (17,18). A 10 mM solution of each inhibitor was encapsulated within liposomes by including the inhibitor in 1.0 mL of solution used to disperse the dried lipid film. Trapping efficiencies were calculated from the 1-[^{14}C]mannitol content of the vesicle suspension. The suspension was divided into six or eight equal fractions for addition to jejunal loop fluid used in flux measurements.

STATISTICAL PROCEDURES

An unpaired Student's *t*-test was used to assess the statistical significance of treatment effects.

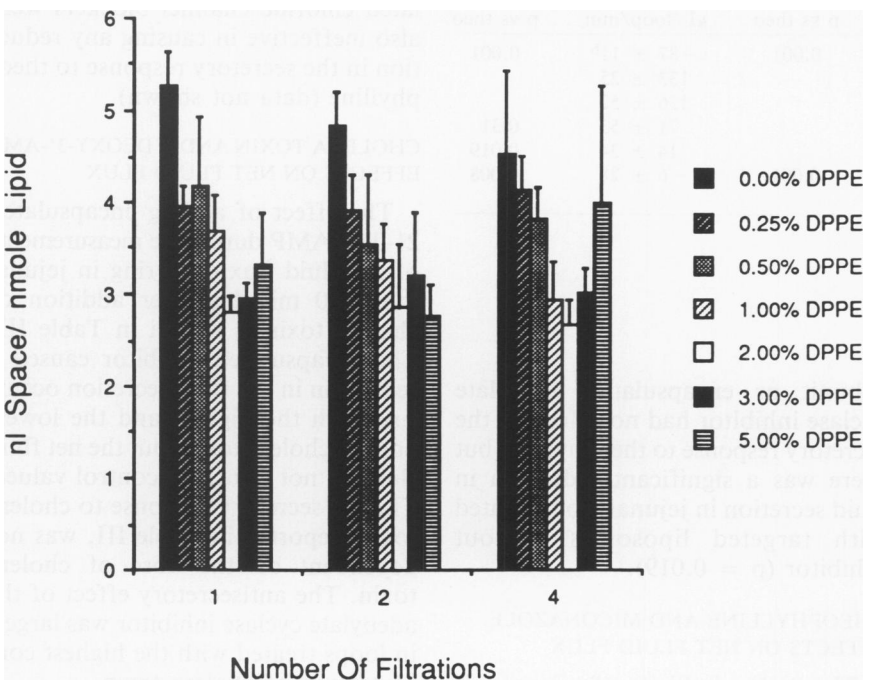

Fig. 1. The effect of dipalmitoylphosphatidylethanolamine on the aqueous volume trapped within liposomes. Liposome suspensions were size sorted by serial ultrafiltration through 0.4 μm pore size polycarbonate filters. Enclosed space was determined by measuring the amount of 1-[^{14}C]mannitol associated with vesicle lipid in the void volume elution peak from a Sephadex G-50 column. Bars are averages ± standard deviations from nine separate measurements.

RESULTS

LIPOSOME COMPOSITION, PREPARATION AND PROPERTIES

The effect of vesicle properties of DPPE addition to the lipid mixture used for liposome preparation was measured. An optimal level of DPPE would provide enough sites for B subunit attachment without inducing significant changes in the properties of the vesicles. Increasing the amount of DPPE in the lipid mixture reduced the effective space within liposomes (Fig. 1).

Maximizing trapping efficiency is important when liposomes are used as a delivery vehicle for expensive agents. Trapping efficiency was expressed as the percentage of the 1-[^{14}C]mannitol aqueous marker that was located within the liposomes after the normal preparation steps of dispersion, freeze-thaw cycling and high pressure ultrafiltration. Increasing amounts of DPPE in the lipid mixture decreased trapping efficiencies (Fig. 2). Lipid concentrations above 100 mg per mL are reported to give higher trapping efficiencies (9), but ultrafiltration was difficult with more concentrated lipid. As a compromise, the lipid mixture was made with 0.25% DPPE, and resuspended for liposome formation (100 mg of lipid per mL of aqueous medium).

ENCAPSULATION OF INHIBITORS AND DOSE CALCULATIONS

Standard liposome preparations involved resuspension of 140 μmol of phospholipid and cholesterol in an aqueous volume of 1.0 mL, containing 1-[^{14}C]mannitol and secretory inhibitor. The internal volume was determined from the 1-[^{14}C]mannitol content of a sample of the final liposome suspension. Actual doses of inhibitor delivered in 100 μL samples of liposome suspension varied with trapping efficiency between batches of vesicles, with a normal range of 5 to 20 nmol of inhibitor per 10 cm loop contained within 1 to 4 μmol of liposome lipid (data not shown).

THEOPHYLLINE AND 2′DEOXY-3′-AMP EFFECTS ON NET FLUID FLUX

Net fluid flux is reported as μL of fluid per loop per minute, with negative values representing net absorption.

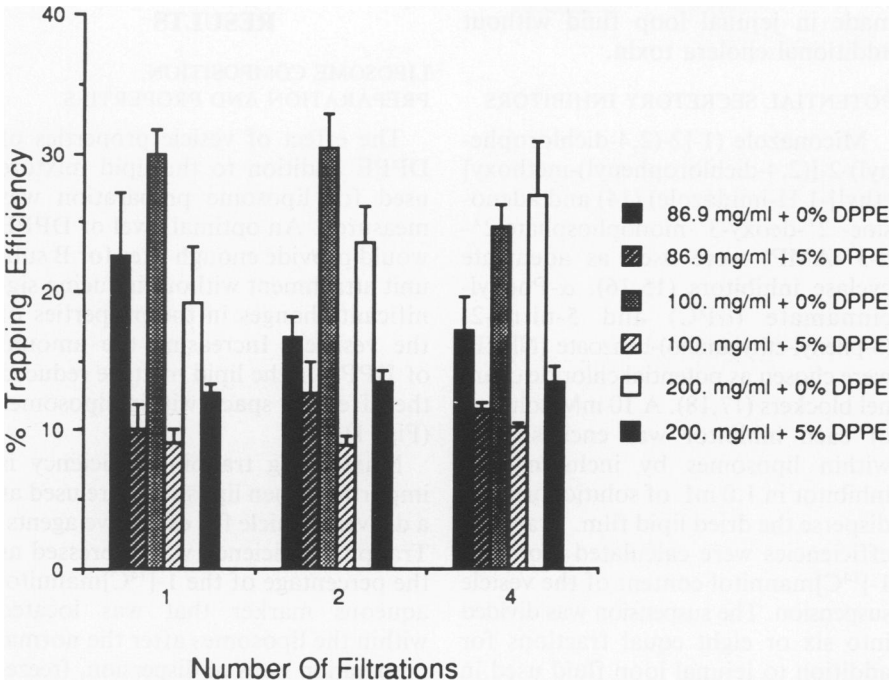

Fig. 2. The effect of dipalmitoylphosphatidylethanolamine on the efficiency of trapping mannitol within liposomes. Liposome suspensions were size sorted by serial ultrafiltration through 0.4 μm pore size polycarbonate filters. Trapping efficiency refers to the percent of the 1-[^{14}C]mannitol present in the resuspension buffer that was associated with vesicle lipid in the void volume elution peak from a Sephadex G-50 column. Bars are averages ± standard deviations of nine measurements.

TABLE I. Net fluid flux in jejunal loops exposed to theophylline

Condition	Experiment 1 μL/loop/min	p vs theo	Experiment 2 μL/loop/min	p vs theo
Control	−78 ± 11[a]	0.001	−87 ± 11[b]	0.001
Theophylline	75 ± 13		133 ± 25	
Theo + Lipo	nd[c]		156 ± 52	
Theo + Lipo + 2′-d-3′ AMP	nd		71 ± 53	0.31
Theo + Lipo + B subunit	nd		14 ± 34	0.019
Theo + Lipo + B subunit + 2′-d-3′ AMP	−45 ± 10	0.001	−6 ± 28	0.008

[a]Mean ± SEM for flux in 12 loops
[b]Mean ± SEM for flux in 20 loops
[c]nd = not determined

Positive values represent appearance of fluid in the loops (net fluid secretion). The averages of two control loops in six pigs, −78 ± 11 μL per loop per minute (Table I), was typical of the expected normal fluid flux in this region of the pig gut (6,7,12). Net fluid secretion (75 ± 13 μL per loop per minute) was induced in jejunal loops by the addition of 10 mM theophylline to the jejunal loop fluid.

The encapsulated adenylate cyclase inhibitor was partially effective as an antagonist of this secretory response (Table I). Liposomes without surface B subunit or encapsulated adenylate cyclase inhibitor had no effect on the secretory response to theophylline, but there was a significant reduction in fluid secretion in jejunal loops treated with targeted liposomes without inhibitor (p = 0.019).

THEOPHYLLINE AND MICONAZOLE EFFECTS ON NET FLUID FLUX

The hydrophobicity of miconazole created problems of reduced amounts of enclosed liposome space and reduced trapping efficiencies. Amounts of miconazole delivered in a standard liposome dose per loop (1 to 3 nmol) were substantially lower than the dose of 2′-d-3′-AMP. Addition of encapsulated miconazole to flux solutions did not reduce the secretory response to theophylline. The net fluid flux in control loops was −68 ± 7 μL/loop/min, in loops treated with theophylline 37 ± 23 μL/ loop/min, and in loops with theophylline plus liposomes containing miconazole 51 ± 23 μL/loop/min (data not shown).

CHOLERA TOXIN AND CHLORIDE CONDUCTANCE INHIBITOR EFFECTS ON NET FLUID FLUX

Loop contents were drained following 90 min of exposure to cholera toxin (50 mg crude filtrate per loop) and replaced with liposome suspensions of isotonic saline solutions. After 10 min of exposure to the liposomes containing either αPC or NPEB the loops were drained and refilled with jejunal loop fluid to start the fluid flux measurements. Neither αPC nor NPEB had any effect of fluid flux induced by previous exposure to cholera toxin (Table II). The net flux of Cl^- into the loops tended to increase in the presence of the agents that are known to inhibit conductive transport of Cl^- in patch clamp experiments. Encapsulated chloride channel blockers were also ineffective in causing any reduction in the secretory response to theophylline (data not shown).

CHOLERA TOXIN AND 2′DEOXY-3′-AMP EFFECTS ON NET FLUID FLUX

The effect of adding encapsulated 2′-d-3′-AMP during the measurements of net fluid flux occurring in jejunal loops 90 minutes after addition of cholera toxin is shown in Table III. The encapsulated inhibitor caused a reduction in the fluid secretion occurring with the highest and the lowest dose of cholera toxin, but the net fluid flux did not return to control values. The net secretory response to cholera toxin, reported in Table III, was not dependent on the dose of cholera toxin. The antisecretory effect of the adenylate cyclase inhibitor was largest in loops treated with the highest concentration of cholera toxin.

A separate trial was carried out with the timing protocol described above for the conductance inhibitors. After 90 min of exposure to 50 mg *V. cholerae*

TABLE II. Net fluid flux in jejunal loops exposed to cholera toxin followed by conductance inhibitors

Condition	HOH flux μL/min	Net Cl^- flux μeq/min	HOH flux μL/min	Net Cl^- flux μeq/min
Control	−17 ± 2[a]	−2.0 ± 2	−34 ± 8[b]	−3.8 ± 1
Cholera toxin	31 ± 4	4.7 ± 2	76 ± 12	8.0 ± 1
CT + αPC	35 ± 5	6.1 ± 2		
CT + NPEB			76 ± 11	11.1 ± 1

[a]Mean ± SEM n = 12
[b]Mean ± SEM n = 12

TABLE III. Net fluid flux in jejunal loops exposed to cholera toxin

Condition	200 mg CT/loop μL/loop/min	100 mg CT/loop μL/loop/min	50 mg CT/loop μL/loop/min
Control	−21 ± 12[a]	−28 ± 12[b]	−99 ± 12[c]
Cholera toxin	87 ± 20	81 ± 28	17 ± 4
Cholera toxin, 2′-d-3′-AMP	23 ± 17[d]	77 ± 29	−1 ± 12

[a]Mean ± SEM n = 12
[b]Mean ± SEM n = 6
[c]Mean ± SEM n = 15
[d]Significantly different from cholera toxin flux p = 0.006

filtrate per loop the loop contents were drained, and liposome suspensions or isotonic saline solutions added. The loops were drained after 10 min and refilled with jejunal loop fluid for initiating the fluid flux measurements. There was no substantial improvement in inhibitor efficacy with this procedure for delivery of adenylate cyclase inhibitor immediately before measuring fluid flux. Fluid flux in control loops was −1 ± 16, in loops treated with cholera toxin was 46 ± 10, and with cholera toxin followed by 10 min with liposomes containing 2′-d-3′-AMP was 27 ± 7 μL/loop/min (p = 0.18 for a reduced secretory response to cholera toxin).

The timing of treatment with the normal dose of encapsulated 2′-d-3′-AMP was changed to expose the loops to the inhibitor prior to addition of cholera toxin. For this experiment targeted liposomes containing inhibitor were added to ligated jejunal loops and incubated in the loops for 15 min prior to emptying the loops and adding cholera toxin. Loops were drained after 90 min and net fluid flux was measured in a standard 20 minute assay. Loops exposed to targeted liposomes before cholera toxin showed a full secretory response to the toxin (124 ± 11 μL/loop/min) compared to secretion in loops treated only with cholera toxin (114 ± 17 μL/loop/min) (data not shown).

EFFECTS OF CHANGING 2′DEOXY-3′-AMP CONCENTRATION AND LIPOSOME AMOUNTS

The delivery protocol for the inhibitor was modified to test if amounts of 2′-d-3′-AMP in the liposomes were sufficient to inhibit the adenylate cyclase activity resulting from prior exposure to cholera toxin. The first modification involved increasing the concentration of inhibitor during liposome formation from 10 to 50 mM. The liposome suspensions containing 50 mM 2′-d-3′-AMP were less effective than those with lower, 10 mM concentration. The corresponding net fluid fluxes for this experiment were: control, −4 ± 21; cholera toxin, 78 ± 10; and cholera toxin plus encapsulated 50 mM 2′-d-3′-AMP, 66 ± 15 μL/loop/min, with 12 observations for each treatment. The p value for significance between the two treatments receiving cholera toxin was 0.65 (data not shown).

Larger amounts of inhibitor were also delivered by increasing the dose of liposomes containing 10 mM 2′-d-3′-AMP. The dose per loop was increased fourfold, from 2.5 μmol to 10 μmol of liposome lipid. Jejunal loops were exposed to cholera toxin for 90 min, drained, and filled with 145 mM NaCl or with liposomes suspended in 145 mM NaCl for 10 min prior to measuring net fluid flux. This approach was more successful in reducing the secretory effect of cholera toxin. Fluid flux values with four times the normal dose of targeted liposomes were: control, −121 ± 14; cholera toxin, 74 ± 28; and cholera toxin plus 10 mM 2′-d-3′-AMP encapsulated in 10 μmol of liposomal lipid, 12 ± 10 μL/loop/min, with 12 observations for each treatment. The p value for a significant antisecretory effect of the inhibitor was 0.033.

COMPETITION FOR MUCOSAL RECEPTORS BETWEEN B SUBUNIT AND INTACT CHOLERA TOXIN

The secretory dose response to cholera toxin reported in Table III indicated that even the lowest dose of cholera toxin used in these experiments may be saturating surface receptors on secretory enterocytes. This possibility was investigated via an indirect competition experiment. Purified B subunit was added to jejunal loops and allowed to equilibrate for 10 min prior to draining the loops and adding 50 mg of crude *V. cholerae* culture filtrate to the loops. Preincubation of loops with amounts of isolated B subunit as low as 10 μg per 10 cm intestinal loop reduced the net fluid flux response to cholera toxin (Table IV). Previous estimates of the potency of the crude culture filtrate of *V. cholerae* indicated fairly similar amounts of B subunit protein in 50 mg of crude culture filtrate and in 10 μg of isolated B subunit (6). Without a clear response to increased amounts of B subunit this is incomplete evidence of competition for surface receptors between cholera toxin used to induce secretion into intestinal loops and immobilized B subunit on the surface of the liposomes.

DISCUSSION

Enclosed liposome space and trapping efficiency were both reduced by adding DPPE to the lipid mixture. These results could have been effects of the palmitoyl side chains on assembly or on fluidity of the phospholipid bilayer of the liposomes. There was a necessary trade-off between the advantages of a phospholipid that was stable to oxidative attack during liposome preparation, and one which has acyl groups with lower melting points. It may be feasible to use low proportions

TABLE IV. Competition between intact cholera toxin and isolated B subunit

Condition	Net fluid secretion μL/loop/min	Net chloride secretion μeq/loop/min
Control	−45 ± 4[a]	−5.3 ± 0.7
Cholera toxin	86 ± 4	8.8 ± 0.9
10 μg B subunit before cholera toxin	46 ± 11[b]	4.1 ± 1.1
25 μg B subunit before cholera toxin	42 ± 8[b]	7.6 ± 1.6
50 μg B subunit before cholera toxin	42 ± 14[b]	7.1 ± 2.1

[a]Mean ± SEM n = 6
[b]Significantly less than flux with cholera toxin ($p < 0.05$)

of a stable phospholipid when it is only necessary to have a small number of functional groups available on the surface of a liposome. Huang and Mason reported an average surface area of 0.74 nm^2 per lipid head group in the outer plane of a liposome bilayer (19). A spherical liposome with a diameter of 100 nm would have a surface area of 3.14×10^4 nm^2. Assuming similar molecular weights for the phospholipids there would be 4.25×10^4 head groups per liposome surface. With 0.25% of the membrane lipids as DPPE there would be approximately 100 headgroups on the surface of one liposome. This should give sufficient sites for attachment of the B subunit of cholera toxin.

We have shown previously that 10 to 50 μmol of 2′-d-3′-AMP added into the lumen of isolated jejunal loops did not produce a measurable decrease in the net secretory fluid flux induced by theophylline or cholera toxin (6). In comparison to placing soluble agents in the intestinal lumen, the liposome system is severely limited in terms of the encapsulated volume available to hold aqueous solute. Delivery of secretory inhibitor by the targeted liposome system could only be effective if the liposomal vehicle were an effective delivery system, and if selective delivery could be achieved by attachment of a specific ligand to the liposome surface. The importance of selective inhibitor delivery to a specific cell population was supported by the effectiveness of much smaller doses of inhibitor (10–50 nmol) encapsulated within targeted liposomes in reducing the secretory response to theophylline.

Both theophylline and cholera toxin caused jejunal loops to change from an absorptive to a net secretory mode. Maximal net secretory capacity of 10 cm jejunal loops in this *in situ* model was approximately 150 μL/10 cm loop/min. In seven meters of small intestine this would be equivalent to 10 mL of net secretion per minute or 600 mL per hour. Assuming that cholera toxin reduces the normal absorptive capacity of the colon these rates may approximate maximal volumes of fecal fluid loss (10 to 15 L per day) measured in adult humans suffering from natural infections with *V. cholerae*.

The efficacy of antagonists of intestinal secretion must be assessed not just through preventing net fluid secretion, but in terms of restoring net fluid flux to normal levels. Net fluid flux is usually absorptive even in the jejunum, ranging from averages of −17 to −99 μL per 10 cm loop per min in the results summarized in the previous section. Simultaneous exposure of jejunal loops to levels of theophylline sufficient to produce a maximal secretory response, and to targeted liposomes containing 10 mM 2′-d-3′-AMP, reversed a net secretory response to a net absorptive response. The reduction in the absolute value of the net flux response to 10 mM theophylline was approximately 60% for both experiments. This reduction would likely be sufficient to prevent clinical dehydration and electrolyte losses arising from secretory diarrhea.

Chloride conductance inhibitors should hold the greatest potential for wide spectrum inhibitory effects toward secretory diarrhea. Specific inhibition of chloride conductance should prevent net fluid secretion into the gut lumen regardless of the intracellular second messenger compound which is responsible for the activation of chloride conductance activity. Bulk delivery of chloride conductance inhibitors to the fluid phase in the lumen did not reduce a net fluid secretory response to theophylline or to cholera toxin (7). The targeted liposome delivery system provided an excellent opportunity to assess the role of delivery or accessibility of the conductance inhibitors to the site of the conductance protein in secretory enterocytes. The lack of antisecretory effects seen with encapsulated αPC and NPEB in the *in situ* secretory model may raise questions about sidedness of inhibitor actions, or about the relationship of chloride channels assayed in *in vitro* preparations of membrane vesicles or cultured cells versus chloride channels involved in secretory ion flux *in situ*.

A good secretory dose response has been produced with *V. cholerae* culture filtrate in the range of 50 to 200 mg of lyophilized filtrate per 10 cm jejunal loop (6). The filtrate used in this study apparently saturated the secretory capacity of jejunal loops at the lowest dose used, the 50 mg per loop level. These high doses of cholera toxin could have produced a situation that was not favorable for reversal of the secretory response.

Reduced efficacy of 2′-d-3′-AMP against net fluid secretion induced by cholera toxin may be due to problems in delivery of the inhibitor to the fraction of the enterocyte population that has been targeted previously by cholera toxin. Saturation of secretory capacity (Table III) may imply saturation of the total population of ganglioside G_{M1} present on the mucosal brush border surface within the lumen of the jejunal loop. In this scenario there could be secretory enterocytes which have all surface ganglioside binding sites for the B subunit already occupied by holotoxin. This prior receptor occupancy could prevent the delivery of inhibitors that are encapsulated within liposomes made with surface B subunit. This effect could occur through occupancy of surface ganglioside G_{M1} site with cholera toxin, or through removal of these sites from the surface after binding to the B subunit of the holotoxin.

It may be significant that of the six different experiments reported above with cholera toxin as a secretory agent and 2′-d-3′-AMP as the secretory antagonist there was only one case (50 mg CT dose per loop in Table III) where the net fluid flux after exposure

to cholera toxin was actually returned to a negative or absorptive value. This effect could be related to different mechanisms of action of cholera toxin on two separate processes: stimulation of unidirectional Cl^- secretion versus inhibition of Na^+ and Cl^- absorption. Unidirectional Cl^- secretion may be stimulated primarily by cAMP, while NaCl absorption may be inhibited by increased cytosolic concentrations of Ca^{2+} ion (20,21). This situation could account for a failure of the adenylate cyclase inhibitor to restore the normal absorptive component of the net fluid flux.

To distinguish between the hypotheses presented in the previous paragraphs it may be necessary to measure the effects of secretory inhibitors encapsulated in targeted liposomes on net fluid flux produced by submaximal doses of cholera toxin. Inhibitor effectiveness could be determined where there is only partial occupancy of intestinal receptors with the native form of cholera toxin.

REFERENCES

1. **FIELD M.** Intestinal secretion: effect of cyclic AMP and its role in cholera. New Engl J Med 1971; 284: 1137–1144.
2. **DONOWITZ M, WICKS J, BATTISTI L.** Senokot inhibits Na and Cl transport in rat descending colon by a Ca^{2+} dependent mechanism. Gastroenterology 1984; 87: 503–512.
3. **DONOWITZ M, WICKS J, MADARA JL, SHARP GWG.** Studies on the role of calmodulin in Ca^{2+} regulation of rabbit ileal Na and Cl transport. Am J Physiol 1985; 248: G726–G740.
4. **DOBBINS J, RACUSEN L, BINDER HJ.** Effect of D-alanine methionine enkephalin on ion transport in rabbit ileum. J Clin Invest 1980; 66: 19–28.
5. **McKAY JS, LINAKER BD, TURNBERG LA.** Influence of opiates on ion transport across rabbit ileal mucosa. Gastroenterology 1981; 80: 279–284.
6. **FORSYTH GW, HAMILTON DL, SCOOT A, GOERTZ KE, KAPITANY RA.** Failure to reverse cholera toxin-induced intestinal secretion by agents which decrease mucosal cyclic AMP. Can J Physiol Pharmacol 1979; 57: 1004–1010.
7. **FORSYTH GW, GABRIEL SE.** Effects of chloride conductance inhibitors on fluid secretion into ligated ileal and jejunal loops in pigs. Am J Vet Res 1990; 51: 1551–1555.
8. **UWIERA RE, ROMANCYIA DA, WONG JP, FORSYTH GW.** Effect of covalent modification on the binding of cholera toxin B subunit to ileal brush border surfaces. Anal Biochem 1992; (In press).
9. **MAYER LD, HOPE MJ, CULLIS PR.** Vesicles of variable size produced by a rapid extrusion technique. Biochim Biophys Acta 1986; 858: 161–168.
10. **ELLMAN GL.** Detection of free sulfhydryl groups with dithio-nitrobenzoic acid. Arch Biochem Biophys 1959; 82: 70–77.
11. **HUTCHINSON FJ, JONES MN.** Lectin-mediated targeting of liposomes to a model surface: an ELISA method. FEBS Lett 1988; 234: 493–496.
12. **BERGER EY, STEELE JM.** The calculation of transfer rates in two compartment systems not in dynamic equilibrium. J Gen Physiol 1958; 41: 1135–1152.
13. **HAMILTON DL, ROE WE, NIELSEN NO.** Effect of heat stable and heat labile *Escherichia coli* entrotoxins, cholera toxin and theophylline on unidirectional sodium and chloride fluxes in the proximal and distal jejunum of weanling swine. Can J Comp Med 1977; 41: 306–317.
14. **STALLA GK, STALLA J, VON WERDER K, MULLER OA, GERZER R, HOLLT V, JACOBS KH.** Nitroimidazole derivatives inhibit anterior pituitary cell function apparently by a direct effect on the catalytic subunit of the adenylate cyclase holoenzyme. Endocrinology 1989; 125: 699–706.
15. **ZENSER TV.** Inhibition of cholera toxin-stimulated intestinal epithelial cell adenylate cyclase by adenosine analogues. Proc Soc Exp Biol Med 1976; 152: 126–129.
16. **JOHNSON RA, YEUNG SM, STUBNER D, BUSHFIELD M, SHOSHANI I.** Cationic and structural requirements for P site-mediated inhibition of adenylate cyclase. Mol Pharmacol 1989; 35: 681–688.
17. **FORSYTH GW, GABRIEL SE.** α-Phenylcinnamate inhibits chloride conductance in jejunal brush border membrane vesicles. Biochim Biophys Acta 1989; 977: 19–25.
18. **WANGEMANN P, WITTNER M, DISTEFANO A, ENGLERT HC, SCHLATTER E, GREGER R.** Cl^- channel blockers in the thick ascending limb of the loop of Henle: structure activity relationship. Eur J Physiol 1986; 407: S128–S141.
19. **HUANG C, NELSON JT.** Geometric packing constraints in egg phosphatidylcholine vesicles. Proc Natl Acad Sci USA 1978; 75: 308–310.
20. **FAN CC, POWELL DW.** Calcium/calmodulin inhibition of coupled NaCl transport in membrane vesicles from rabbit ileal brush border. Proc Natl Acad Sci USA 1983; 80: 5248–5252.
21. **MAENZ DD, GABRIEL SE, FORSYTH GW.** Calcium transport affinity, ion competition and cholera toxin effects on cytosolic calcium concentration. J Membrane Biol 1987; 96: 243–249.