Differential Adhesion of Normal and Inflamed Rat Colonic Mucosa by Charged Liposomes

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Purpose. To study the adhesion properties of charged liposomes to the healthy and inflamed (colitis-induced) rat intestinal epithelium.
Methods. Neutral, positively charged, and negatively charged liposomes were prepared and tagged. The cationic or anionic liposomes contained increasing amounts (13, 22, or 36 mol%) of either the cationic lipid dimethyl-dioctadecylammoniumbromide (DODAB) or the anionic lipid 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DSPG). Colitis was induced in rats by DNBS. Adhesion of the various types of liposomes was assessed in rat colon sacs. The effects of charge type, charge density (mol%), liposome size, and incubation time on the adhesion of the liposomes were compared in the inflamed and healthy epithelial tissues.
Results. Three times as many cationic liposomes adhered to the healthy colonic mucosa than neutral or anionic liposomes. However, anionic liposome adherence to the inflamed colonic mucosa was 2-fold that of either neutral or cationic liposomes (a finding that was verified by charged-dyes studies). Adherence was directly correlated with charge density. An inverse correlation was identified between cationic liposome size and healthy tissue adherence in short incubation periods. The adherence of cationic liposomes, which was also found to be time-dependent, decreased in healthy mucosa in the presence of high concentrations of aqueous Mg^{2+} rinse.
Conclusions. Anionic liposomes could be useful for the topical delivery of anti-inflammatory drugs in inflammatory bowel disease therapy.

KEY WORDS: charged liposomes; colonic epithelium; experimental colitis; inflammation; mucoadhesion.

INTRODUCTION

Oral administration offers a potential portal to the superficial layers of the gastrointestinal (GI) tract (local delivery). Due to the disputed extent of particle uptake from the intestinal lumen into the bloodstream, the most attractive use of particulate drug carriers is local drug treatment of intestinal diseases. A typical therapeutic opportunity is ulcerative colitis (UC), an inflammatory disease confined to the epithelium of the large intestine. Experience with drugs such as salicylates (5-aminosalicylic acid) (1) and steroids (budesonide) (2) have justified local therapy in the colon. Among many reasons mentioned in the etiology of the disease (3), oxidative stress is considered as a possible cause (4). The association between reactive oxygen species and UC pathophysiology has led us to suggest local antioxidant therapy in the area of the injured epithelium. Topical treatment was accomplished by direct cationization of the antioxidant enzymes superoxide dismutase (SOD) and catalase (5,6).

Despite the efficient attachment of cationized proteins to the colonic mucosa (7), a possible drawback is a partial loss of activity of the enzymes resulting from the chemical charge-modification. An alternative approach could be the entrapment of the active native protein(s) in adhesive particles. This study suggests the use of charged liposomes for that purpose. Lipid assemblies, such as microemulsions (8) and liposomes (9,10), have attracted considerable attention as potential drug carriers via the oral route, primarily due to the potential ability to target them to selected mucosal regions and to increase the intestinal absorption of lipophilic drugs (11).

Mucosal targeting by liposomes can be achieved by manipulating their surface properties. Surface polymerization and polymer-coated liposomes have been mentioned in the context of increased stability of orally administered liposomes (12,13). Liposomal targeting has been tested using covalently anchored collagen (14), lectins (15), or carbohydrates (9). Surface charge modification has been suggested as a common means of liposome localization to cells and a variety of body organs (16,17). Both cationic and anionic liposomes have been tested as targetable delivery systems, where the electrostatic interaction with the surface of cells was the leading cause for liposome attachment to the cell membrane, thus leading to their internalization (16,18).

The overall goal of this study was to compare the adhesive properties of charged liposomes in healthy and inflamed epithelium of the rat colon. More specifically, the study objectives were 1) to compare the adhesion properties of positive and negative liposomes in healthy and inflamed (colitis-induced) colonic epithelium of the rat, 2) to explore the effect of charge density and liposome dimensions on the attachment properties of cationic and anionic liposomes, and 3) to study the nature of the electrostatic attachment of the charged liposomes by using a competitive electrolyte.

MATERIALS AND METHODS

Materials

Hydrogenated soybean phosphatidylcholine (HSPC) (iodine value 3) was obtained from Lipoid (Ludwigshafen, Germany). 1,2-dioleoyltrimethylammonium-propane (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-carboxyfluorescein (CF-PE), 3-β-[*N*-(*N',N'*- dimethylaminoethane)carbamoyl]-cholesterol (DC-Chol), and 1,2-distearoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DSPG) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, dioctadecyldimethylammonium bromide (DODAB), dinitrobenzenesulfonic acid (DNBS), hematoxylin, and eosin B were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

All other chemicals were of analytical grade unless otherwise stated in the text.

Liposome Preparation

Neutral Liposomes

Appropriate amounts of lipids (containing 16 mM HSPC and 12 mM cholesterol) and CF-PE (at a molar ratio of 1:200

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of the total lipids, for fluorescence labeling) were weighed, dissolved in *tert*-butanol, and lyophilized overnight. The lyophilized bed of lipids was hydrated using distilled water at 60°C, a temperature above the HSPC gel-to-liquid crystalline phase transition temperature (52°C), and sonicated by a probe sonicator (Microson, UL Laboratory Equipment, Farmingdale, NY, USA) till a translucent dispersion was achieved. The obtained liposomes were then freeze-thawed three times and lyophilized overnight. The lyophilized bed was then hydrated by L-histidine buffer (5 mM in normal saline), pH 6.5, to the appropriate volume. In this formulation, HSPC served as the main liposome-forming lipid (19) and was selected due to its high solid-ordered to liquid-disordered (SO↔LD) phase transition temperature (52°C), allowing it to stay in a solid-ordered state at body temperature. Cholesterol was added to improve the physical stability of the liposomes during preparation, storage, and in the body (20). Liposomes composed of HSPG and cholesterol have a record of good performance in some applications (21) because of their high chemical and physical stability, contributing to good drug retention with minimal leakage (19).

Cationic Liposomes

Cationic liposomes were prepared as described for the neutral liposomes, except for the addition of the cationic lipid DODAB at the desired level (13, 22, or 36 mol% of total lipids). In separate studies, cationic liposomes containing 22 mol% of DOTAP or 22 mol% of DC-cholesterol were also prepared.

Anionic Liposomes

Anionic liposomes were prepared as described for the neutral liposomes, except for the addition of the anionic lipid DSPG at the desired level (13, 22, or 36 mol% of total lipids).

Throughout the study, all liposomes were extruded at 65°C, 11 times through 800-nm pore-size polycarbonate filters using the LiposoFast syringe extruder (Avestin, Ottawa, ON, Canada) to prepare sized multilamellar liposomes (MLVs). In some cases, this was followed by extrusion through a 100-nm pore-size polycarbonate filter to prepare 100-nm unilamellar vesicles. The dimensions of the liposomes were analyzed by a submicrometer particle sizer (Coulter, Luton, UK). For each batch, a size-distribution curve was plotted (not shown). Thus, the average size of the large liposomes was 800 ± 50 nm and that of the small liposomes was 100 ± 27 nm (mean value ± SD). The liposomes maintained these measured dimensions throughout the course of the study.

Zeta Potential

Zeta potential of the liposomes was measured, after dilution (1/100) in a 0.01 M NaCl solution, by a Zetasizer 3000 HS system (Malvern, England).

Animals, Maintenance Anesthesia, and Euthanasia

Male Sprague Dawley rats (220–250 g) were obtained from the Animal Farm of Hadassah Medical Center at The Hebrew University of Jerusalem. They were kept under constant environmental conditions (22°C, 12-h light/dark cycles) and fed with standard laboratory chow and tap water. All animal studies were conducted in accord with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985). The Mutual Committee of Hadassah University Hospital and the Faculty of Medicine for Animal Welfare approved the study protocol. Anesthesia was performed by an intraperitoneal injection of 100 mg/kg body weight of ketamine (Ketaset, Fort Dodge, USA). Euthanasia of the anesthetized rats was carried out by chest wall puncturing.

Induction of Experimental Colitis

Twenty-four hours prior to colitis induction, the rats were deprived of food but allowed free access to water. The water contained 10 mg/l of the laxatives sennoside-A and sennoside-B (X-Prep Liquid, Rafa Pharmaceuticals, Jerusalem, Israel) and sucrose (200 g/l). With the rats under light ether inhalation anesthesia, colitis was induced by intracolonic administration of 30 mg of dinitrobenzensulfonic acid (DNBS) dissolved in 1 ml of an ethanolic solution 25% (v/v) (5,22). The solution was instilled slowly during 20 s via a flexible, perforated foley catheter, which was then immediately removed, leaving the rats in an upside down position for another 40 s.

Inflammation Severity Characterization

Inflammation was quantified macroscopically and by monitoring myeloperoxidase (MPO) activity, as described elsewhere (23,24). Briefly, a specimen from the colonic mucosa was taken and homogenized on ice, then 0.5 ml of the homogenized tissue was centrifuged at 25,000*g* for 5 min. The pellet was dispersed in 0.5 ml of ice-cold 50 mM phosphate buffer, pH 6, containing 0.5 ml of hexadecyltrimethyl ammonium bromide (HTAB). The suspension was frozen–thawed twice, sonicated for 15 s, and centrifuged at 5000*g* for 5 min. Then, 0.1 ml of supernatant was added to 2.9 ml of phosphate buffer, pH 6, containing 0.167 mg/ml *o*-dianisidine hydrochloride and 5×10^{-4}% v/v of hydrogen peroxide. The rate of change in absorbance was determined at 460 nm during 30 s. Results were expressed as units of MPO activity per gram tissue weight.

Macroscopic evaluation was performed by scoring the damage observed on a 0–5 scale (Table I), as previously described (5). In our experimental setup, the whole length of the colon was inflamed, with intermittent ulcers. Inflamed tissues with a damage score of 3–5 were selected for the attachment studies in which the adherence of the liposomes was compared in healthy and inflamed tissues. The average value of MPO activity in healthy tissues was 20 ± 5 mU/g tissue

Table I. Damage Index Used for Macroscopic Scoring of Inflammation Severity in the Colonic Epithelium of the Colitis-Induced Rat

Score	Macroscopic observation
0	Naive control (normal epithelium)
1	Hyperemia or colonic wall thickening
2	Small inflamed area (≤0.5 cm)
3	Large inflamed area (>0.5 cm), or two or more small inflamed areas
4	Small ulcerated area (≤1 cm)
5	Large ulcerated area (>1 cm), or two or more small ulcerated areas

Table II. Effect of Liposome Lipid Composition on Liposome Zeta Potential

Liposome type	Lipid composition (mol%)	Zeta potential (mV)
Neutral	HSPC:Chol 57:43	–12
Cationic	HSPC:Chol:DOTAP 45:33:22	70
Cationic	HSPC:Chol:DC-Chol 45:33:22	68
Cationic	HSPC:Chol:DODAB 50:37:13	49
Cationic	HSPC:Chol:DODAB 45:33:22	64
Cationic	HSPC:Chol:DODAB 36:28:36	76
Anionic	HSPC:Chol:DSPG 50:37:13	–28
Anionic	HSPC:Chol:DSPG 45:33:22	–66

whereas that of the inflamed tissues averaged at 97 ± 10 mU/g tissue.

Colon Sac Preparation and Adherence Studies

The abdomen of either healthy (control) or colitis-induced rats was cut open, and a 10 cm length of from the distal colon was excised and rinsed with PBS, after which 5-cm-long sacs were prepared by tying one end with 3/0 silk suture, filling with 0.4 ml of liposomal suspension (diluted ×10 in PBS), and tying the other end. The rest of the colon was excised and kept for macroscopic scoring and MPO analysis. The sacs were incubated in 15 ml of PBS, containing 10 mM glucose, at 37°C, in a glass vial on a shaking bath for either 15 or 75 min. At the end of the incubation, the sacs were cut open, rinsed three times by immersion in PBS, and weighed. The tissues were homogenized with a Polytron (Kinematica GmbH, Berlin, Germany) in a solution containing isopropanol:borate buffer pH 9.0 (9:1). One milliliter of the homogenate was centrifuged at 14,000 rpm for 15 min. The level of fluorescence in the supernatant was then measured by a spectrofluorimeter (Perkin-Elmer LS-SB, Bucks, England): λ excitation = 495, λ emission = 525.

Tissue Staining by Charged Dyes

Colonic sacs, of either healthy or inflamed rat colonic tissues, were prepared as described above and filled, in separate studies, with 0.5 ml aqueous solutions (containing 2% v/v DMSO) of either the cationic dye, hematoxylin (1 mg/ml), or the anionic dye, eosin B (0.5 mg/ml). The sacs were incubated in 15 ml of PBS containing glucose (10 mM), at 37°C (glass vial) for 15 min in a shaking bath, after which the sacs were cut open, measured for their surface area (ruler), and rinsed (PBS) three times. The tissues incubated with eosin B were then homogenized in 3 ml of absolute methanol whereas the tissues incubated with hematoxylin were homogenized in 3 ml of absolute ethanol. The concentration of dyes in the tissue homogenates was measured spectrophotometrically (Uvikon 933, Kontron, Zurich, Switzerland) at 292 nm (hematoxylin) or 523nm (eosin B).

Statistical Analysis

The results were expressed as mean values ± SEM. The Kruskal-Wallis test (25) was used to identify differences among the results obtained. The Mann-Whitney test was then used to identify differences between the groups. A p value of less than 0.05 was considered to be significant.

RESULTS

The adherence of cationic, anionic, and neutral liposomes (22 mol% of cationic or anionic lipids in the formulations; liposome sizes were 800–1000 nm) to the epithelium of the healthy colon of the rat is shown in Fig. 1. The amount of liposomes attached to the colonic epithelium was calculated as the relative fluorescence detected in the tissue homogenates. In all cases (three different types of cationic lipids), the adherence of the cationic liposomes was better than the anionic or neutral ones (47 ± 5, 37 ± 4, 33.8 ± 4.2% fluorescence of initial amount per gram tissue wet weight ± SEM for liposomes containing DODAB, DOTAP, DC-Chol, respectively, compared with 10.5 ± 0.8 and 12 ± 2% fluorescence of initial amount per gram tissue wet weight ± SEM for liposomes containing DSPG and neutral liposomes, respectively).

The adherence of the cationic liposomes was charge density dependent (expressed as mol% of charged lipids) (Fig. 2). That is, the higher the amount of cationic lipid in the lipid mixture, the higher the adherence measured as observed in the case of DODAB (11 ± 1.4, 39.1 ± 1.8, 47 ± 5, 70 ± 2.5% fluorescence of initial amount per gram tissue wet weight ± SEM for 0,13, 22, and 36% DODAB-containing liposomes, respectively).

The effect of liposome size on liposome adhesion to the colonic mucosa was also evaluated. A significantly larger fraction of the 100-nm liposomes adhered to the colonic mucosa than the 800-nm MLVs at 15 min incubation time, as shown in Fig. 3A. However, after 75 min, the adhesion of the 800-nm liposomes was better (33.6 ± 1.7 and 36.9 ± 3.7% fluorescence of initial amount per gram tissue wet weight ± SEM for 100-nm liposomes at 15 and 75 min incubation, respectively; 20 ± 4.6 and 47 ± 5% fluorescence of initial amount per gram tissue wet weight ± SEM for 800-nm liposomes at 15 and 75 min incubation, respectively).

We also tested the relationship between the charge density, expressed as mol% of cationic lipid in the liposomal formulation, and the incubation time in the colonic sacs. Fig.

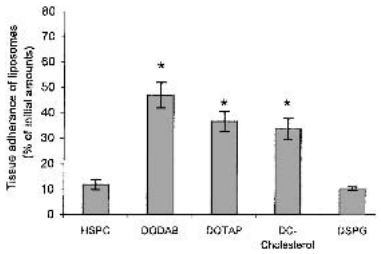

Fig. 1. Adsorption of three types of cationic (DODAB, DOTAP, and DC-Chol); neutral (HSPC); and anionic (DSPG) liposomes (800 ± 50 nm) to the healthy epithelium of the rat colon after 75 min of incubation in colon sacs. Ratio of all charged lipids was 22 mol%. (For details on exact liposome composition, see Table II.) Shown are the averages of six different studies ± SEM (*$p < 0.001$ when compared to HSPC-containing liposomes).

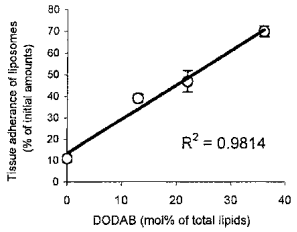

Fig. 2. Effect of increasing amounts of DODAB (expressed in mol% of total lipids in the cationic liposomes) on the attachment of cationic liposomes (800 ± 50 nm) to the healthy epithelium of the rat colon as measured after 75 min of incubation in colon sacs. Shown are the averages of six different studies ± SEM.

3B demonstrates that charge density has a greater effect on liposomal attachment to the tissue than does incubation duration. Fig. 3 shows that the effect of incubation time on adhesion was more pronounced in the case of larger liposomes and in those liposomes that contained lesser amounts of DODAB (low cationic charge density).

The nature of the electrostatic interaction between the colonic mucosa and the cationic liposomes (DODAB 22 mol%) was further examined by co-incubation (competition) with elevated concentrations of magnesium chloride. The results, which are summarized in Fig. 4, show that an increase in the concentration of $MgCl_2$ (in the range 0.25–1.0 M) had only a limited effect on the cationic liposome adherence to the colonic mucosa (ratio between DODAB and $MgCl_2$ concentrations ranging from 1/3000 to 1/12500), indicating that the polyvalency of the cationic liposomes is by far advantageous on bivalent ions, similar to what has been observed

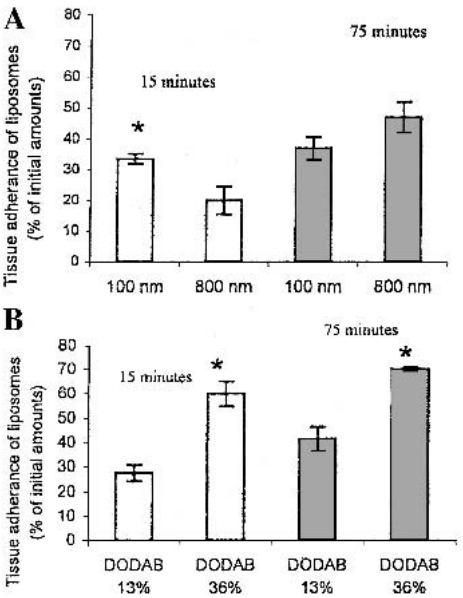

Fig. 3. A. Effect of incubation time and liposome size on the adherence of cationic (DODAB, 22 mol% of total lipids) liposomes to the healthy epithelium of the rat colon as measured in colon sacs (*p < 0.005 when compared to 800 nm group B). Effect of incubation time and charge density on the adherence of cationic (DODAB, 13 or 36 mol% of total lipids) liposomes (800 ± 50 nm) to the healthy epithelium of the rat colon as measured in colon sacs. Shown are the averages of six different studies ± SEM (*p < 0.005 when compared to liposomes containing 13 mol% DODAB).

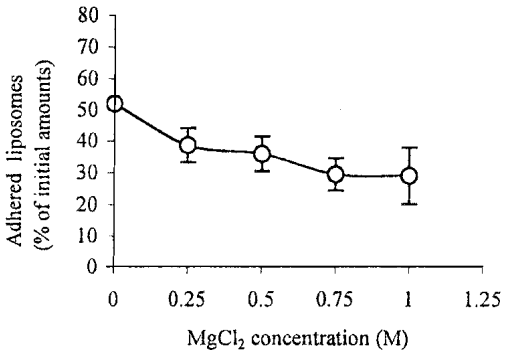

Fig. 4. Effect of increasing concentrations of $MgCl_2$ on the attachment of cationic liposomes (DODAB, 22 mol% of total lipids) to the epithelium of the healthy rat colon after 15 min of incubation in colon sacs. Shown are the averages of four different studies ± SEM.

with respect to the effect of salts on the interaction between nucleic acids and cationic liposomes (Even-Chen and Barenholz, in preparation).

When the attachment of the various types of liposomes was measured in inflamed and healthy tissues, it was found that anionic liposomes adhered better to the inflamed colon than did cationic liposomes (17.8 ± 1, 8.5 ± 1.4, 7.1 ± 0.3% fluorescence of initial amount per gram tissue wet weight ± SEM for liposomes containing DSPG, DODAB, and HSPC, respectively) (Fig. 5). Moreover, this attachment to the colitis-induced epithelium was charge-density dependent. Better attachments were observed (6.5 ± 2.1, 7.5 ± 1.9, 11.3 ± 1.9, and 14.5 ± 1.5% fluorescence of initial amount per gram tissue wet weight ± SEM for 0,13,22, and 36% DSPG-containing liposomes, respectively) (inset of Fig. 5) with higher amounts of DSPG in the liposomal lipid mixture. The attachment of the neutral liposomes was found to be similar in both healthy and colitis-induced epithelium. The attraction between the inflamed epithelium and negatively charged groups was verified by the studies involving charged dyes. Figure 6A shows that

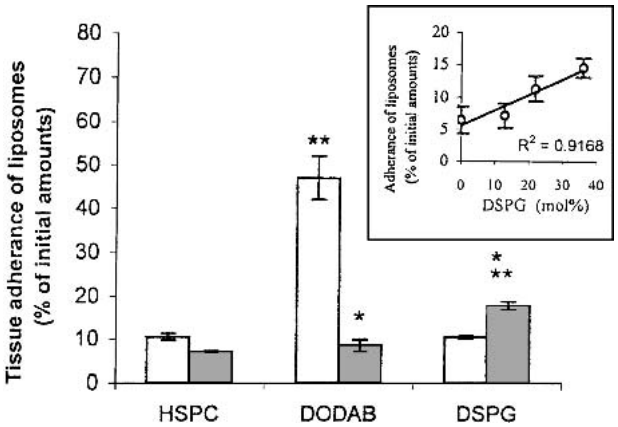

Fig. 5. Adherence of neutral, cationic (DODAB, 22 mol% of total lipids) and anionic (DSPG, 22 mol% of total lipids) liposomes (800 ± 50 nm) to healthy (white columns) and inflamed (gray columns) epithelium of the rat colon, as measured 75 min after incubation in colon sacs (*p < 0.001 when compared to healthy group; **p < 0.001 when compared to HSPC group). Inset: The effect of charge density (as expressed by the mol% of DSPG of total lipids) on the adherence of the anionic liposome to the epithelium of the inflamed colon of the rat. Shown are the averages of six different studies ± SEM.

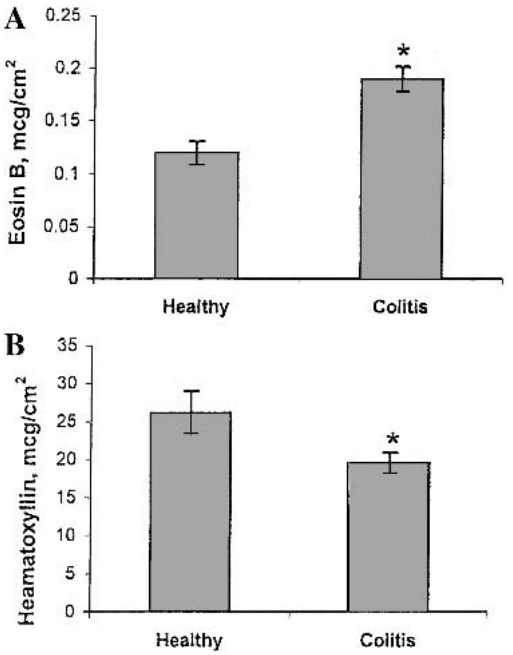

Fig. 6. Attachment of eosin B (plot A) and hematoxylin (plot B) to the healthy and inflamed colonic epithelium of the rat. Shown are the averages of three different studies ± SEM (*$p < 0.05$ when compared to healthy group).

the anionic dye eosin B adhered significantly better to the inflamed colonic epithelium than to the healthy tissue (0.12 ± 0.011 and 0.19 ± 0.012 $\mu g/cm^2$ for healthy and inflamed tissues, respectively). The cationic dye hematoxylin, on the other hand, adhered significantly better to healthy tissues than to inflamed ones (Fig. 6B) (26.2 ± 2.8 and 19.6 ± 1.4 $\mu g/cm^2$ for healthy and inflamed tissues, respectively).

DISCUSSION

In his review on oral particulate uptake, Florence concluded that the uptake and translocation of particulate drug delivery systems in the intestinal epithelium "are neither exceptional nor unusual; whether the process will be mastered or achieve therapeutic drug delivery has yet to be shown conclusively" (26). Indeed, the most important potential of oral particulate drug carriers has been shown to be at the level of the intestinal mucosa as the extent of particulate uptake is still questionable and involves mostly adsorption and some absorption processes (27). Thus, much activity has been focused in the area of oral, mucosal immunization, using biodegradable particles (28). This niche is attractive because with small amounts of drugs that are active at the mucosal surface or mucosal organelles (e.g., gut-associated lymphoid tissue), a successful therapy could be accomplished. Another interesting opportunity is the topical treatment of intestinal diseases. A typical example is inflammation processes at the mucosal level, namely ulcerative colitis and Crohn's disease. If rationally designed and successfully brought to the site of injury, particles loaded with anti-inflammatory agents could be docked and release their cargo at the site of the disease.

In this study, we examined the possibility to use electrical surface charge as a docking tool to bring liposomes in the vicinity of the inflamed epithelium of the rat intestine. Polymerized, microencapsulated, and polymer-coated liposomes (9), as well as other lipid particulate carriers such as microemulsions, have all been tested in the context of oral delivery (11). Interestingly, we found differences in the attachment properties of charged liposomes when examined in healthy or inflamed mucosal tissues of the rat colon. Positively charged liposomes adhered to the healthy mucosa significantly better than anionic or neutral liposomes. Three different positively charged lipids were mixed with the zwitterionic lipid HSPC and the chargeless cholesterol to form cationic liposomes: DOTAP, DODAB (both having a single quaternary amine as the cationic group), and DC-cholesterol (having a single tertiary amine as the cationic group). The three were found to possess similar zeta potential values (Table I). Consequently, the mucosal attachment properties of the three types of cationic liposomes to healthy epithelium were similar (Fig. 1), with DODAB being superior, and increased with increasing level of cationization (Fig. 2). This finding indicates that the attachment depended primarily on the charge density of the cationic liposomes. Barbour and Hopwood (29) found that cationized ferritin attached rapidly to the tips of the colonic microvilli and even penetrated the cells by endocytosis. However, the cationized protein also adhered to the mucus lining of the colonic epithelium by reacting with the negatively charged sialic acid. These findings were verified recently by our previous study, in which the adherence properties of FITC-labeled cationized bovine serum albumin (BSA) to the various fractions of colonic crypt epithelium of the rat were compared to those of FITC-labeled native BSA. It was found that cationization of BSA facilitated the protein adherence to the colon epithelium in a crypt-depth dependent manner, with the largest extent of adherence in the tips of the crypts (7). Moreover, in accord with Barbour and Hopwood's findings, cationized BSA first attached to the mucus layer covering the colonic epithelium (5).

To overcome partially the mucus barrier for electrostatic charged macromolecules (such as proteins), it is planned to entrap them in liposomes. The first stage, presented in this report, examined how the type of charge, its density, and the liposome size/lamellarity affect their adhesion to either healthy or inflamed epithelium of the rat colon. One hundred–nanometer unilamellar vesicles adhered faster and to a larger extent than 800-nm vesicles. However, the difference in adhesion intensity diminished with contact time (slightly better adhesion results for 800-nm vesicles was reached within 75 min, Fig. 3). A straightforward explanation for these adhesion property differences is the improved distribution, accessibility, and diffusion of the smaller liposomes in the crypt area within a short period of time. Still, it should be noted that topical treatment requires prolonged residence time at the crypt area. Therefore, large liposomes may be favored, unless the site of action is in the colonocyte cytoplasm. This issue is being investigated now in our laboratory using liposomal superoxide dismutase (SOD) as an anti-inflammatory probe. Figure 3B shows that diminishing the difference in mucoadhesion with time occurred when the cationic liposomes contained relatively high mol% of cationic lipid (36 mol% DODAB). At lower DODAB concentrations (13 mol%), longer incubation times are required to improve the extent of liposome attachment. This finding could be imperative for situations in which there is a need to reduce the relative amount of cationic lipids due to toxicity considerations, and it

demonstrates that the attachment intensity is controllable by manipulating charge density and residence time, both in healthy tissue and in inflamed tissue.

The observation that increase in the charge density increased the attachment of the cationized liposomes could be explained by Fig. 4, which summarizes the effect of increasing concentrations of $MgCl_2$ on the attachment of cationic liposomes to the colonic epithelium. A similar study, which was performed recently with cationized BSA, showed an almost complete (80% of total cationized BSA amount) displacement of the protein by increasing concentrations of $MgCl_2$ (7). The fact that such a displacement could not be observed with cationized liposomes suggests that, apart from total charge, the charge distribution is equally important in the attachment process. Liposomes are supermolecular assemblies in which the small lipid molecules interact with each other in a way governed by the ratio between their hydrophobic/hydrophilic cross section (30,31), usually due to electrostatic and thermodynamic considerations, and the molecules carrying net charge are evenly distributed in the membrane of the assembly. Therefore, charge is evenly distributed on the surface of the cationic liposomes, a phenomenon that does not occur in charged proteins. Chemically modified BSA has less organized structure and therefore is more vulnerable to competition by small charged molecules such as Mg^{+2}. In addition, it is possible that electrostatics is not the sole factor involved in attachment and tissue uptake of the DODAB-containing liposomes and that lipid–lipid interactions should also be considered.

The most interesting finding was the one observed with liposomal attachment to injured tissues. When the healthy epithelium was replaced with inflamed epithelium in the colonic sac preparations, anionic liposomes adhered better to the surface of the mucosa (Fig. 5). Anionic liposomes adhered better to DNBS-induced epithelium than both cationic and neutral liposomes in a charge [relative amount of DSPG (see insert of Fig. 5)] -dependent manner. A validation study was performed with two charged dyes (eosin B and hematoxylin). Its results verified the findings of the experiments with the liposomal attachment (Fig. 6). It should be realized that these differences might be changed if the loaded drug is highly ionized. This matter is now being investigated. The observation that anionic liposomes adhere better to inflamed epithelium of the colitis-induced rats contradicts previous results of ours in which we found that cationized catalase and cationized SOD were more active against experimental colitis than native enzymes (negatively charged at physiological pH) (5). This contradiction could be explained by the conditions under which the studies were conducted. Though the pH of the lumen of the colitis-induced rats is acidic (32) (meaning that the native enzymes were not charged negatively in the diseased state), the incubation medium in the current *in vitro* studies with the colonic sacs was maintained at pH = 7.4 (PBS) for the entire duration of the attachment study. This point is now being investigated in our laboratory, together with the assumption that the negatively charged mucus lining covering the colonic mucosa is responsible for the attachment of positively charged antioxidant enzymes. It is noteworthy, however, that although the anionic liposomes were superior to the cationic liposomes in their attachment properties to the inflamed epithelium, the latter adhered better than the neutral liposomes.

In the past, Nagashima suggested that a possible cause for the affinity of the negatively charged aluminum sucrose sulfate product, sucralfate, to the ulcerated mucosa of the rat stomach was the high concentration of positively charged proteins in the inflamed regions (33). Prominent infiltration of eosinophils in the intestine lamina propria of IBD patients has been reported (34). Some of the patients showed persistently high concentrations of eosinophil cationic protein (ECP) (35,36). This increased release of ECP to the colorectal perfusion fluid was suggested to be an indicator for eosinophil involvement in the local disease (37). Our results strengthen this premise and provide a rationale for the design of particulate drug carriers for the topical treatment of the injured mucosa in IBD. It should be recognized, however, that the lumen contents would, most likely, interfere with liposomal drugs attachment to the epithelial surface. A supportive drug platform may be required. If successfully designed, this will open a new area in topical treatments of IBD.

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