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# Transferrin as a Luminal Target for Negatively Charged Liposomes in the Inflamed Colonic Mucosa

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**Abstract:** The need for improved specificity in the local treatment of inflammatory bowel diseases (IBD) led us to use negatively charged liposomes to target the inflamed colonic epithelium. The purpose of the present study was to elucidate the cause for our previous observations that such liposomes accumulate, preferentially, in the inflamed mucosa of rats that were induced with experimental colitis, following luminal administration. Protein analysis (tandem mass spectrometry, verified by Western blot) of inflamed mucosal specimens, extracted at pH 3, 5 and 7, revealed an increased expression of transferrin (TF) at pH 3. Histological examination indicated that the TF was located at the luminal side of the inflamed epithelium. Negatively charged (but not neutral) liposomes adhered to both commercial and mucosal TF at low pH, but not at neutral pH. Moreover, preincubation of negatively charged liposomes with TF profoundly attenuated their adherence to the inflamed mucosa of the rat colon. It is concluded that, at a low pH, typical of the colon lumen in ulcerative colitis, TF mediates specific mucoadhesion of negatively charged liposomes to the inflamed mucosa. This observation could be useful in the rational design of specific drug vehicles aimed at IBD therapy after luminal administration.

**Keywords:** Negatively charged liposomes; specific attachment; transferrin; pH; induced colitis; mucosa

## Introduction

Inflammatory bowel diseases (IBD), characterized by chronic inflammation of the gut mucosa, are manifested as either Crohn's disease (CD) or ulcerative colitis (UC). While the latter is confined to the rectum (where it commonly initiates) and the colonic mucosa and submucosa, CD expands over large areas of all intestinal segments. Typically, UC forms crypt abscesses and mucosal ulceration. CD, on the other hand, involves full gut thickness. In addition to lowering life quality (malnutrition and chronic anemia, diarrhea and pain), distinctive complications include toxic megacolon, sepsis, thromboembolic disease and malignancy.[1,2] Despite significant progress in IBD systemic therapy, in particular after the introduction of biological drugs such as

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infliximab,[3,4] the disease commonly relapses. One medical approach requires high local doses of anti-inflammatory drugs (steroids and nonsteroidal anti-inflammatory drugs, such as 5-aminosalicylic acid derivatives),[5−7] commonly accompanied by severe adverse effects. This situation necessitates novel strategies to localize potent drugs in the closest proximity possible to the inflamed mucosal tissues.[8,9] Indeed, some interesting approaches for oral administration of drugs, active at the mucosal level, have been reported in the past decade.[9,10] Still, a major drawback of colonic delivery systems is their lack of specificity after the stage of successful colon arrival. They are competent of delaying the onset of drug release to caudate portions of the intestine, but they cannot really target the injured epithelium of the colon.

We have recently shown that targeting the inflamed mucosa can be accomplished with negatively charged liposomes.[11] Low and high molecular weight antioxidants that were delivered via anionic liposomes to the inflamed mucosa of experimental colitis-induced rats were more effective in attenuating the induced inflammation compared with aqueous solutions of the enzymes. This was due to the specific attachment of the negatively charged liposomes to the inflamed regions, which led to a prolonged residence time and a better uptake of the antioxidants into the inflamed epithelium. It was also observed that while positively charged liposomes exhibited enhanced adherence to both healthy and normal intestinal mucosa, negatively charged liposomes adhered, preferentially (and better than positively charged liposomes) to the inflamed (experimental colitis) epithelium of the rat colon.[12]

In this study we proposed two explanations that are not mutually exclusive, for this attachment of negatively charged liposomes to the inflamed mucosa: (a) the inflammation process is accompanied by an *in situ* secretion of positively charged proteins; (b) the inflammatory process is associated with a local reduction of pH. Several studies support each of these hypotheses. Although concise studies of the colon pH under conditions of active UC have not been reported, sporadic reports suggest that the pH in UC patients may drop below pH 2, compared with normal pH values of 5−6.5.[13−15] This could be attributed to malabsorption of short-chain fatty acids that are constantly generated by the microflora of the large bowel. Also, it is well documented that eosinophils and neutrophils accumulate at the site of CD and UC lesions, where they secrete cationic proteins such as eosinophil cationic proteins[16,17] and bactericidal/permeability-increasing proteins.[18] Moreover, short bactericidal proteins such as $\beta$-defensins and antimicrobial anti-proteases accumulate in the injured tissues and possibly contribute to the positive charge of the mucosal surface.[19] Indeed, we have already demonstrated that the inflamed epithelium of the rat colon was stained more intensively with the anionic dye Eosin B, compared with healthy epithelium stained with the same dye.[12]

The aims of this study were to (a) identify a typical positively charged protein in the inflamed colonic mucosa of the rat, (b) examine its capability to attract negatively charged liposomes, and (c) delineate the role of low pH in the attachment process.

## Experimental Section

Unless stated otherwise, all materials were purchased from Sigma (St. Louis, MO). Solvents were of analytical grade or higher. Water was deionized and ultrafiltered by reverse osmosis (Barnstead Nanopure, Waltham, MA). Mouse anti-myosin heavy chain (sc-58793) was purchased from Santa Cruz

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Biotechnology (Santa Cruz, CA). Chicken anti-mouse transferrin (TF) was purchased from Abcam (Cambridge, U.K.). Sheep anti-human transferrin was purchased from AbD Serotec (Martinsried, Germany). Rabbit anti-human TF (ab-9538) was purchased from Abcam. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and Santa Cruz Biotechnology (Santa Cruz, CA). Hydrogenated soybean phosphatidylcholine (HSPC) and phosphatidylglycerol (HSPG) were obtained from Lipoid KG (Ludwigshafen, Germany). The HSPC and HSPG mixture has an iodine value of 3.0, an acyl chain composition of 86% stearic acid (C18:0) and 13% palmitic acid (C16:0), and less than 1% of other acyl chains. Its solid-ordered (SO) to liquid-disordered (LD) phase transition temperature ($T_m$) is 52.5 °C.[20] 1,2-Dioleyl-*sn*-glycerol-3-phosphoethanolamine-*N*-carboxyfluorescein (PE-CF) was purchased from Avanti Polar Lipids (Alabaster, AL).

**Rats, Maintenance and Euthanasia.** Male Sprague−Dawley rats (200−250 g), obtained from the Animal Farm of Hadassah Medical Center and The Hebrew University of Jerusalem, 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 accordance with the Principles of Laboratory Animal Care (NIH publication #85-23, revised 1985). The joint ethics committee (IACUC) of the Hebrew University and Hadassah Medical Center approved the study protocol for animal welfare. The Hebrew University Animal Facility is an AAALAC International accredited institute (#1285). Anesthesia was performed by an intraperitoneal injection of a mixture of 100 mg/kg body weight of ketamine (Ketaset, 0.1 g/mL, Fort Dodge, IA) and xylazine HCl (20 mg/mL). Euthanasia of the anesthetized rats was carried out by chest wall puncturing.

**Induction of Experimental Colitis and Inflammation Assessement.** Twenty-four hours prior to colitis induction, rats were deprived of food, but allowed free access to water. Colitis was induced by a rectal slow instillation (over 20 s) of 30 mg of dinitrobenzensulfonic acid (DNBS) dissolved in 1 mL of ethanol 25% v/v under light anesthesia (isoflurane inhalation) through a flexible, perforated Foley catheter. Immediately following DNBS administration the catheter was removed and the rats were left in an inverted position for an additional 40 s. Three days after colitis induction (maximal onset of inflammation as identified in preliminary results) the rats were sacrificed and the colon that was exposed via laparotomy was split and spread wide open. Inflammation was assessed macroscopically by scoring the inflamed regions and by measuring the dimensions (length and weight) of the isolated colons. The control (healthy) group was treated with a normal saline solution according to the same procedure.[11,21,22]

**Protein Extraction and Identification from the Intestinal Epithelium.** Colon specimens (400 mg) from either DNBS induced or healthy rats were homogenized (Polytron PT 2100 benchtop rotor-stator homogenizer, Kinematica, NY) in 500 $\mu$L of phosphate buffer solutions at pH 3, 5, and 7 on ice. The lysates were centrifuged (4 °C), and the supernatant was collected and divided into two portions. One portion was stored at −70 °C for total protein content analysis (Bradford reagent, Bio-Rad, Hercules, CA), using BSA for the calibration curve.[23] The other was mixed with a Tris buffer (120 mM, pH = 6.8), containing 0.6% w/v sodium dodecyl sulfate (SDS), 30% w/v glycerol, 100 mg of bromophenol blue and 0.5 M $\beta$-mercaptoethanol and warmed to 95 °C (2 min). After centrifugation, the protein content in the supernatant was resolved (1 h) by gel permeation chromatography (12% polyacrylamide SDS−PAGE, 35 mA), stained (overnight) with Coomassie blue, rinsed (4 h) with a dilute methanolic solution of acetic acid followed by a rinse with water and dried. Protein analysis of the different mucosal specimens (extraction at pH 3, 5 and 7), which was performed on the dried gels by tandem mass spectrometry, revealed the expression of two major proteins: transferrin (TF) and albumin. This identification was verified by Western blot. The resolved proteins on the SDS−PAGE gels were electrophoretically transferred onto a nitrocellulose membrane. Nonspecific binding was blocked with 5% milk. To identify TF, the membranes were incubated with chicken anti-human TF polyclonal antibody, capable of binding rat TF followed by goat anti-chicken IgY (IgG) HRP (Figure 1).

**Detecting TF Location in the Rat Colonic Mucosa and Human Colon by Immunohistochemistry.** Mucosal specimens from the isolated colons (healthy or inflamed regions) were rinsed with a 4% w/v aqueous sucrose solution and fixated with 12% v/v formaldehyde in PBS. After a PBS rinse the fixated tissues were preserved in a 20% w/v sucrose solution until sectioning, prior to which the specimens were dehydrated (ethanol and PBS). Sectioning (10−12 $\mu$m) was performed by embedding in paraffin blocks. Nonspecific staining was blocked by BSA (in Triton-X-containing PBS). The primary antibody was sheep anti-human TF, and the secondary antibody was donkey anti-sheep IgG-HRP. Human biopsies were treated in the same manner. Immunostaining of human samples was performed using rabbit anti-human TF (1:200), followed by mouse anti-rabbit-HRP (1:500). Rat samples were stained with sheep anti-mouse transferrin (cross-reactive with rat, 1:100) followed by donkey anti-sheep IgG-HRP (1:100).

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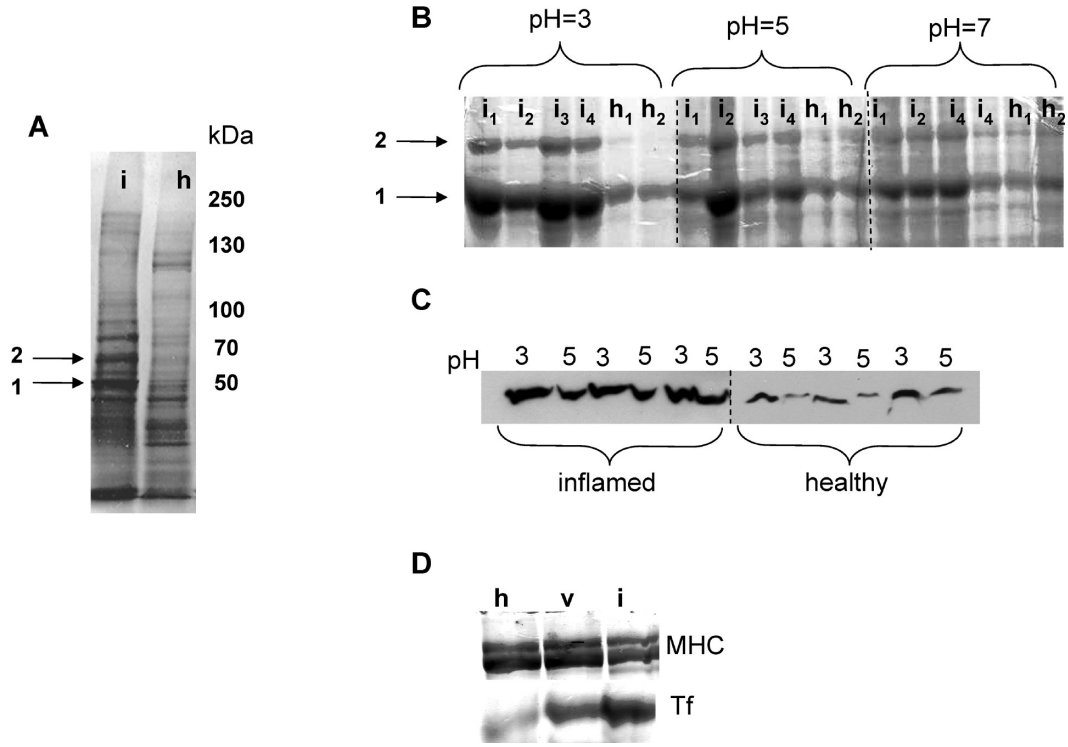


***Figure 1.*** (A) SDS−PAGE analysis of mucosal homogenates extracted in pH 3 from inflamed (experimental colitis, denoted i), or healthy (h) epithelium of the rat colon (band 1, albumin; band 2, TF). (B) Individual SDS−PAGE results (quadruplicates for inflamed and duplicates for healthy) of mucosal homogenates extracted from inflamed (experimental colitis, denoted i), or healthy (h) epithelium of the rat colon in three buffer solutions (pH 3, 5 and 7). Band 1: albumin. Band 2: TF. (C) Immunoblot analysis of the samples in panel B with anti-TF. 10 $\mu$g of protein was separated by SDS−PAGE, electrotransferred into nitrocellulose membrane and immunoblotted by anti-TF. (D) Immunoblot analysis of mucosal homogenates extracted from the colon of inflamed (i), healthy (h) or vehicle (v) treated rats with anti-TF, and anti-myosin heavy chain (MHC) as loading controls.

**Liposome Preparation and Characterization.** Liposomes were prepared by the ethanol injection method as follows: phospholipids (94 mg for the preparation of anionic liposomes and 68 mg for the preparation of neutral liposomes) were dissolved in 400 $\mu$L of ethanol, and warmed to 65 °C (above the temperature range of solid-to-liquid phase transition of the phospholipid). Warm PBS pH = 7.4 (3.6 mL, 65 °C) was added and ethanol residues were removed by dialysis (cutoff: 100 kDa) against PBS. Fluorescent labeling of the liposomes for the colon everted sacs experiments (see below) was accomplished by the inclusion of 1 mol % of the fluorescent phospholipid PE-CF (820 $\mu$g to the anionic liposomes and 640 $\mu$g to the neutral liposomes) in 4 mL of the liposome suspension in PBS. Downsizing to an average globule size of 400 nm was carried out by extrusion (LiposoFast syringe extruder, Avestin, Ottawa, ON, Canada) through a 0.4 $\mu$m polycarbonate filter. The liposomes' phospholipid concentration was determined by the modified Bartlett assay as described elsewhere.[24] Size distribution of the liposomes was analyzed by dynamic light scattering using the particle size analyzer ALV-NIBS/HPPS equipped with am ALV-5000/EPP multiple dynamic correlator (ALV-Laser Vertribsgesellschaft, GmbH, Langen Germany). Liposomal charge was measured by zeta potential analysis after dilution (1/100) in water using the Zetasizer 3000 HS (Malvern, U.K.). The composition of the liposomes and their properties are summarized in Table 1.

***Table 1.*** The Composition of the Anionic and Neutral Liposomes and Their Physical Characteristics[a]

| | negative | | neutral | |
|---|---|---|---|---|
| liposomes | mol % | mM | mol% | mM |
| HSPC | 45 | 16 | 57 | 16 |
| HSPG | 22 | 8 | | |
| cholesterol | 33 | 12 | 43 | 12 |
| zeta potential (mV) | −35 | | −4 | |
| mean particle size (nm)[b] | 415 | | 420 | |
| lipid concn (mM) | 24.8 | | 21.2 | |

[a] When needed, fluorescence labeling was accomplished by the addition of the phospholipid CF-PE in a molar ratio of 0.18 of total lipid content. [b] Unimodal distribution, SD ≤ 5% of mean.

**Liposome Binding to TF (of Human Source and of Rat Mucosa).** TF (human, 98%, Sigma) was dissolved in 800 $\mu$L (7.5 $\mu$g/mL) of PBS buffer at a pH of 3 or 5. 200 $\mu$L of liposomes (Table 1) in the corresponding pH was added and incubated for 1 h, 37 °C. The liposome-bound TF was

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separated from the free fraction by ultracentrifugation (42000*g*, 45 min, 4 °C); the liposomal pellet was dissolved in reduced (+5% $\beta$-mercaptoethanol) Laemmli sample buffer and boiled (5 min). Supernatants were collected and precipitated with 10% trichloroacetic acid, and centrifuged (14000*g*, 30 min, 4 °C). The pellet of the nonbound TF was washed twice with cold absolute ethanol and boiled for 5 min in reduced Laemmli sample buffer. Analysis was done by Western blotting using sheep anti-human TF. Binding of liposomes to rat mucosal TF was performed in a similar manner. Rat mucosal homogenate equivalent to 80 $\mu$g of protein was mixed with the liposomes in PBS of pH 3, 5, or 7. Western blotting was performed using a nitrocellulose membrane, which was then blocked with 5% nonfat milk in PBS-T (PBS containing 0.05% Tween 20, pH 7.4) before incubation with a primary antibody. Membranes were then stained with chicken anti-mouse TF which cross-reacted with rat TF.

**Liposome Binding to the Intact Mucosa.** Everted sacs (1 cm in length)[25] were prepared from the colon of either DNBS induced or healthy rats and incubated (75 min, 37 °C) in 1 mL of PBS solutions, pH 3 or pH 5, containing 100 $\mu$L of charged or neutral fluorescently labeled liposomes (Table 1). The sacs were then washed briefly and homogenized, on ice, in 3 mL of acidic isopropanol. The homogenates were centrifuged (25000*g*, 30 min, 4 °C), and the clear supernatant was collected for analysis. After the addition of a borate buffer (pH = 9), fluorescence was measured (485 nm excitation, 521 nm emission) and concentrations were calculated from a suitable calibration curve. Each study was repeated four times.

To verify that endogenous TF was involved in the specific attachment of the negatively charged liposomes to the colonic mucosa, 900 $\mu$L of external TF was added (7.5 $\mu$g/mL, in PBS of either pH 3 or pH 5) to the incubation (37 °C, 75 min) media of the everted sacs with the two types of fluorescently labeled liposomes. The tissues were then rinsed with the corresponding buffer to wash away unbound liposomes. Binding was evaluated as described above.

## Results

**TF Expression in the Inflamed Colonic Mucosa was Enriched at Low pH.** Our work hypothesis was that inflammatory-specific attraction between negatively charged liposomes and the colonic mucosa could be conducted through extracellular components, such as charged proteins. We further assumed that such proteins should possess a low isoelectric point, which may alter their water solubility upon pH drop from neutral to acidic. To ascertain this we investigated tissue protein profiles of healthy and inflamed mucosal specimens, extracted from scraped epithelium of the rat colon by detergent-free PBS solutions at pH 3 performed immediately after euthanasia. The extraction was performed 3 days after colitis induction, a time period required for the induced inflammation to peak and for the local irritation caused by the ethanol vehicle to subside.

At pH 3, several proteins were eluted from the inflamed tissues to a higher extent than from the healthy tissues. The most prominent protein bands (labeled as **1** and **2**, in Figure 1A) were analyzed by mass spectrometry and identified as albumin (**1**) and TF (**2**). This experiment was repeated with a larger pool of colitis and healthy specimens followed by extraction at different pH conditions (3, 5 and 7). We observed that TF levels in the extracts expressed a clear pH dependency. At pH 3, TF was extracted more profoundly in colitis than in the healthy tissue. At pH 7 this difference was diminished (Figure 1B). To specifically evaluate the level of TF in the mucosal specimens, the gels were subjected to immunoblotting analysis with anti-TF antibodies, an analysis which verified that mucosal extracts from the inflamed tissues were enriched with TF (Figure 1C). To refute a possible artifact that could have been caused by the normalizing approach (total protein) of those specimens assayed by SDS−PAGE, whole intestinal tissue (including the muscularis and serosa layers) was homogenized and the TF expression was compared to that of myosin heavy chain (MHC), a muscle protein whose expression is unchanged by the induced inflammation. The results, shown in Figure 1D, verified the sole effect of inflammation on TF elevation in the colonic mucosa. Collectively, our results show that TF is more accessible for extraction from colitis-induced tissues than from healthy rat colon at low pH. Similar experiments with antibodies against albumin did not yield a reproducible enrichment in inflamed tissues (not shown).

**TF Location in the Inflamed Mucosa.** The cause of this profound expression of TF in the mucosal tissue of the inflamed colon was investigated. TF is a prominent protein in the circulation, and is expected to inhabit mucosal capillaries. Thus, a possible reason for its expression in mucosal scrapes could be a TF leakage during the inflammation propagation. This assumption was tested by immunohistochemistry analysis of TF in histological preparations taken from the colonic mucosa of either colitis induced or healthy (control) rats. Although much of the TF was probably washed away during preparation of the tissue specimens, a specific staining for TF was detected in some capillaries (Figure 2A). Interestingly, TF was also detected in the luminal aspect of the crypts in those specimens taken from the inflamed mucosa.

We were concerned that the immunoreactivity of anti-TF observed in the mucus layer was a result of a nonspecific binding to the negatively charged mucus. Therefore, we performed control experiments, including staining with only a secondary antibody (Figure S1) in the Supporting Information, panels B and D). However, because this observation could not be perceived in normal tissues (Figure 2A, panel C of Figure S1 in the Supporting Information), it was postulated that, during the inflammatory response, TF leaked

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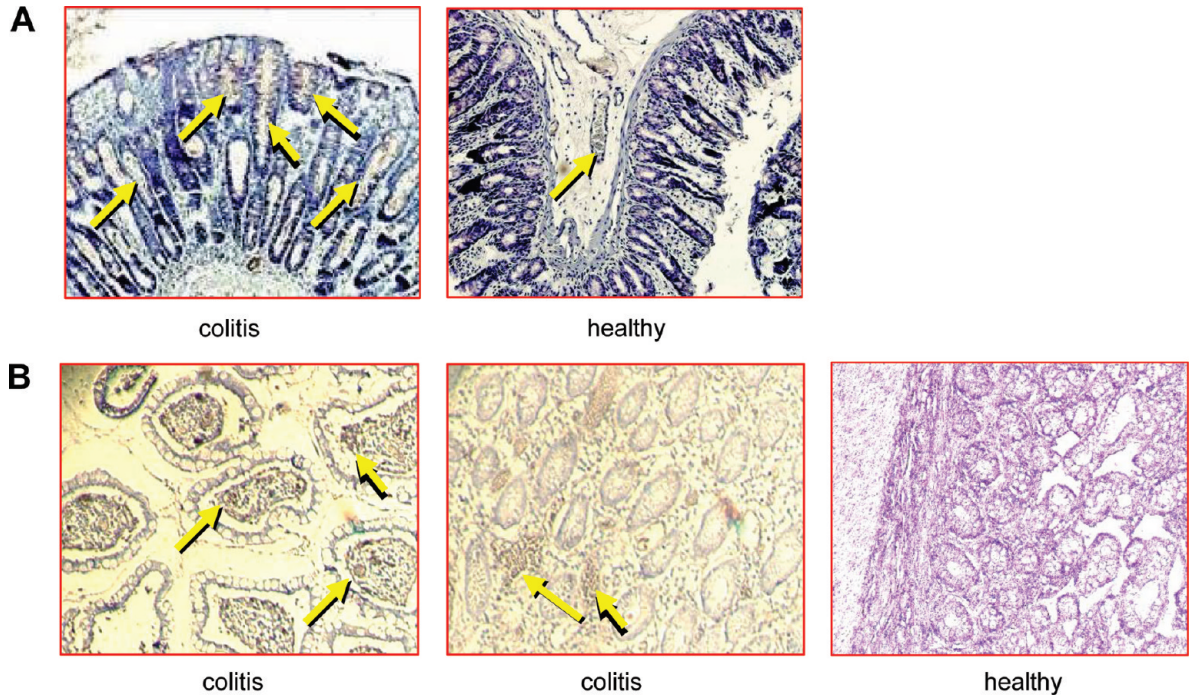


***Figure 2.*** (A) Immunohistochemistry analysis of endogenous TF (marked with yellow arrows) in specimens taken from healthy (right) and inflamed (left) colonic mucosa of the rat. (B) Immunohistochemistry analysis of endogenous TF (marked with yellow arrows) in colon specimens taken from healthy (right) and ulcerative colitis patients (two left panels).

from the circulation and adhered to the apical side of the colonic mucosa.

Care was taken to avoid ulcerated tissues during the histological analysis. Still, it cannot be excluded that the ethanol fraction of the DNBS solution affected the permeability of the mucosa and facilitated TF leakage. To elucidate this, mucosal location of TF in human biopsies taken from IBD patients was explored. In tangential cuts through the microvilli, TF was clearly observed inside the crypts and on top of the epithelial tissue between the crypts (Figure 2B, left and center panels). These observations reinforce the suggestion that when inflammation propagates, TF is released into the gut lumen during the course of colitis.

**TF Binds to Negatively Charged Liposomes at Acidic Conditions.** The isoelectric point of TF (5.6−5.8) implies that at physiological pH it is slightly negatively charged. In inflammation, however, when the pH drops below its isoelectric point, TF acquires a net positive charge. Under such conditions, with TF located in the mucosal crypts (Figure 2), the protein could serve as an inflammation-specific target for a negatively charged drug delivery system. The differential capacity of TF to bind negatively charged or neutral liposomes, at elevated pH values, was measured in the next stage of the study. It was found that, at pH 3, TF was completely bound to the anionic liposomes (Figure 3A). Binding to the neutral liposomes was also observed, albeit with a lesser efficiency (Figure 3B). Increasing the pH of the buffer caused a gradual loss of the binding capacity.

It is noteworthy that purified human TF may bind differently to negatively charged liposomes than the endogenous TF of the rat mucosa. Indeed, as demonstrated in Figure 3, the attachment of TF from homogenates of the inflamed mucosa of the rat colon to negatively charged liposomes was more pH-dependent than that of commercial TF. At pH 3 it was completely bound to the negatively charged liposomes, while at pH 5 binding diminished. As for neutral liposomes, neither at pH 3 nor at pH 5 could binding be detected. In summary, these results indicate that, at low pH values typical of colitis, strong attachment between TF and the negatively charged liposomes occurs.

Lastly, two corroborative studies were conducted. In the first one, the preferential adherence of anionic and neutral liposomes was compared in whole tissue preparations (everted sacs) from colitis induced rats at pH 3 and pH 5. Figure 4 shows that the mucosal adherence of both types of liposomes was better at the lower pH and that anionic liposomes adhered better than the neutral ones at all pH conditions. Although binding decreased as the pH was increased, the uncharged liposomes exhibited negligible binding at pH = 5, while the anionic preparations were still active. The role of TF in the preferential adherence was elucidated in an experiment in which the two types of liposomes were incubated with TF prior to their incubation with the inflamed mucosa (induced colitis) of the rat colon at pH 3 and pH 5. The results of this study, summarized in Figure 5, demonstrate that preincubation blocked the negatively charged liposomes' access to the inflamed mucosa but not the access of the neutral liposomes, indicating that neutralizing the negative sites at the surface of the anionic liposomes prevented them from binding to the inflamed colonic epithelium.

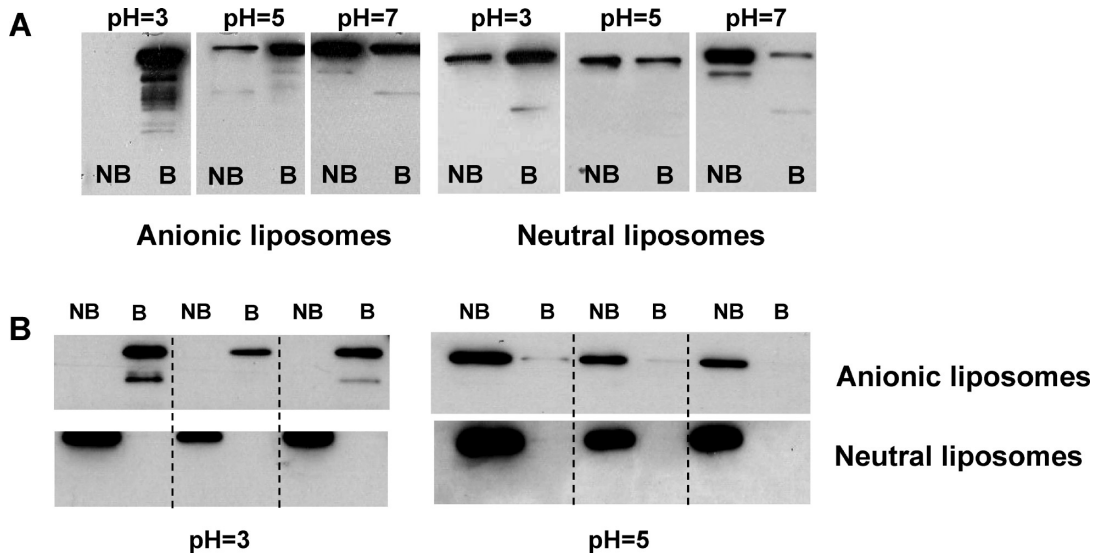


***Figure 3.*** (A) Immunoblot analysis for purified TF of free (nonbounded = NB) and liposomal (anionic or neutral) bound (labeled as "B") TF after incubation in buffer solutions of elevated pH values. (B) Same as in panel A, except the TF is from rat mucosa. Shown are three independent repetitions, analyzed together by Western blotting.

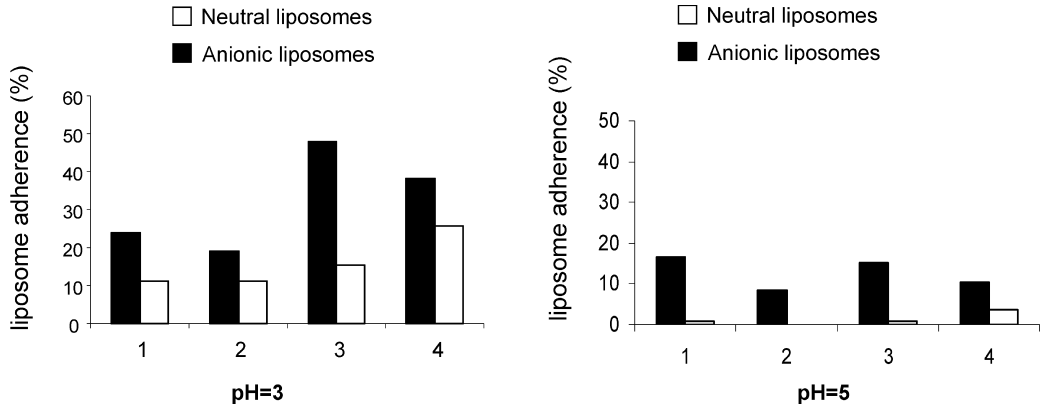


***Figure 4.*** Adherence of anionic and neutral liposomes to the inflamed mucosa of everted sac preparations of the rat colon at pH 3 and pH 5. Shown are the individual results of four different experiments.

## Discussion

Local treatment of mucosal disorders in the alimentary canal has been the objective of countless reports in the past 50 years, primarily in the context of UC, where the activity of some drugs (amino-salicylates, steroids) is correlated with their concentration in the colonic mucosa.[26] Irritable bowel syndrome (IBS) is a motility disorder which has recently been conceived as an immunological disorder, associated with a dysfunctional relationship between the indigenous flora and the host.[27] IBS could also merit from targeting relevant drugs to the colon mucosa. However, colonic delivery suffers from a major technological constraint: inability to target the mucosa after colon arrival.[8,9] One approach to overcome this problem is to use particulate drug carriers. Having large surface areas and minute dimensions (nanoscale is favorable) they possess improved capability to adsorb and accumulate in the mucus lining and reside at the site of attachment for prolonged periods of time, limited only by mucus turnover rates. If small enough and designed properly, the particles can diffuse into the mucosa and even penetrate into the broken tissue. Nakase and co-workers have already shown that polylactic microspheres were uptaken by macrophages of the inflamed mucosa in colitis induced rodent models.[28,29] Lamprecht and co-workers identified a significant deposition of poly(lactic-*co*-glycolic acid) nanoparticles

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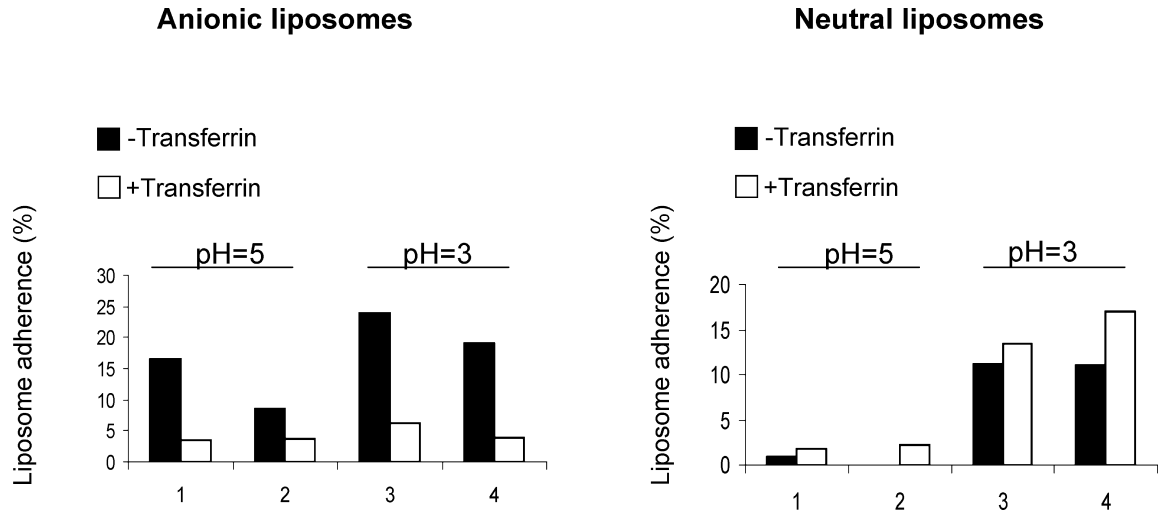


***Figure 5.*** The effect of preincubation of negatively charged and neutral liposomes with TF on their adherence to inflamed mucosa of the rat colon. Shown are the individual results of four different experiments.

in the mucosa of DNBS induced rats after systemic administration, probably due to the retention effect of the inflamed tissue.[30,31]

The preferential accumulation of negatively charged liposomes in the inflamed mucosa of the experimentally induced colitis rat model,[11,12] prompted us to perform the present study, in which we identified the expression of relatively large amounts of TF in the mucosal crypts of the inflamed rat colon. Under mild acidic conditions, typical of colitis,[13−15] this iron-carrier, circulation-born protein, is positively charged. Indeed, we were successful in eluting it from inflamed colonic tissues at pH 3, and to a lesser extent, at pH 7 (Figure 1). This unexpected finding, in which TF was found in the inflamed mucosa, could be explained by inflammation-driven bleeding of microvasculature. In severe cases, a resulted anemia could induce a hepatic *de novo* generation of TF. Another explanation, for the TF expression in the luminal aspect of the mucosa, is the build up of edema at the site of inflammation. This accumulation enriches, probably, the inflamed tissue with TF-containing exudates, which are later entrapped in the mucosal crypts, as observed in both rat and human specimens (Figure 2). It is well documented that gut permeability is increased during inflammation.[32] Moreover, it has been suggested that interference with epithelium integrity may initiate, independently, an inflammatory response,[33] which, in turn, could causes a TF leakage. A model can be delineated in which mucosal TF acquires a positive charge during the course of inflammation accompanied by lumen acidification. The increased mucosal permeability allows TF diffusion across the epithelium into the mucus gel layer, at which place the protein charge prevents further voyage into the gut lumen, causing TF to remain adjacent to the negatively charged epithelium. It should be realized, however, that staining TF at the mucosal crypts might be artifactual due to improved fixation at this anatomic site. Still, our results demonstrate the potential of TF as an apical marker in the colon epithelium, for drug carriers in gut inflammation. Therefore, it is highly likely that at pH = 3, resembling a typical luminal pH at inflammation, transferrin is extracted better from the mucus onto which it had been adsorbed, after being secreted from the inflamed colon epithelium.

Does luminal TF have a physiological role in the colon? Organic and inorganic iron are absorbed in the duodenum. Inorganic iron is absorbed by the DMT1 transporter, while iron−heme complexes are transported by the HCP1 transporters.[34] The role of TF in iron absorption is not clear. A recent study demonstrated the existence of luminal TF in the duodenum of both rats and humans, thus a specific mechanism that shuttles TF to the lumen cannot be underestimated. Moreover, it has been demonstrated that luminal TF is biologically active and retrieves iron via interaction with its receptor.[35] It is tempting to speculate that the TF observed in the colon crypts in this study plays a biological

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function relevant to IBD, for example, sequestering the bleeding-born iron that had been released to the lumen of the gut.

The negative charge of the liposomes used in this study was caused by the phosphate diester group of phosphatidylglycerol, p$K_a$ $\sim$ 2.5−3.0, and therefore the liposome surface is charged at all three pHs used (3, 5, and 7) while the type and magnitude of TF's charge will be highly dependent on the local pH, due to TF's isoelectric point at pH 5.6−5.8. The lower the local pH, the larger the level of TF binding to the anionic liposomes. This resembles the effect of pH on binding of myoglobin to PG containing liposomes.[36] At acidic pH, the liposomes bound tightly to the TF (Figure 3), resembling their adherence to the inflamed mucosa (Figure 4A), supporting the rationale of using negatively charged liposomes for the purpose of targeting the colon epithelium in UC. The lower the pH, the tighter the TF interaction and association with the liposomes, suggesting that this protein is instrumental in anchoring negatively charged liposomes to the sites of inflammation at the low pH that usually occurs during inflammation.

Further elucidation of the exact role of TF in facilitating mucoadhesion of negative liposomes requires its depletion from the gut tissues. This could be accomplished experimentally by ligating the hepatic vein.[37] However, this procedure is not specific and is limited in experimental duration. Alternatively, we tested whether preincubation of liposomes with TF affected their mucosal binding in the rat colon. The decrease in binding of anionic, but not neutral liposomes, indicates that the electrostatic interaction with TF could effectively mask the ability of anionic liposomes to interact with the mucosa (Figure 4).

Overall, our findings demonstrate the potential of TF as a molecular luminal target for ingested negatively charged dosage forms for the topical treatment of inflammatory bowel diseases. Such application obviously requires colon-specific arrival of TF-targeted delivery systems. With the recent developments in colonic vehicles (for a review see ref 9), some of them under investigation in our group,[38] it is likely that this approach is feasible.

## Abbreviations Used

CD, Crohn's disease; PE-CF, 1,2-dioleyl-*sn*-glycerol-3-phosphoethanolamine-*N*-carboxyfluorescein; DNBS, dinitrobenzene sulfonic acid; HRP, horseradish peroxidase; HSPC, hydrogenated soy phosphatidylcholine; HSPG, hydrogenated soy phosphoglycerol; IBD, inflammatory bowel diseases; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TF, transferrin; UC, ulcerative colitis.

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**Supporting Information Available:** A figure depicting 10 $\mu$m sections of paraffin-embedded inflamed rat mucosa and normal rat mucosa stained with anti-transferrin antibody followed by a HRP-conjugated secondary antibody. This material is available free of charge via the Internet at http://pubs.acs.org.

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