Interaction of a Self-Emulsifying Lipid Drug Delivery System with the Everted Rat Intestinal Mucosa as a Function of Droplet Size and Surface Charge

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Purpose. To investigate the interaction of positively charged self-emulsifying oil formulations (SEOF) following aqueous dilution as a function of resulting emulsion droplet charge and size with rat everted intestinal mucosa, adherent mucus layer and Peyer's patches, using cyclosporine A (CsA) as a lipophilic model drug.

Methods. Droplet size determination (TEM technique) and ζ-potential measurements were used to characterize the resulting emulsions. For the *ex vivo* interaction study, the well-known rat intestine everted sac technique was used in combination with confocal microscopy.

Results. The positively charged oil droplets formed by SEOF dilutions at ratios of 1/50 and 1/10 elicited the stronger interaction with the mucosal surface. The positive charge of the smaller droplets was more readily neutralized, and even reversed in aqueous solutions containing moderate subphysiological mucin concentrations. Parameters such as droplet size, negativity of the epithelial mucosa potential and presence of the mucus layer on the epithelial surface affected drug mucosa uptake and the adhesion of the positively charged droplets to the rat intestinal mucosa.

Conclusions. The enhanced electrostatic interactions of positively charged droplets with the mucosal surface are mostly responsible for the preferential uptake of CsA from the positively charged droplets as compared to negatively charged droplets irrespective of the experimental conditions used. The increased uptake of the CsA from the negatively charged oil droplets was consistent with the dilution extent, as expected, whereas in the positively charged droplets, an intermediate droplet size range was identified resulting in optimum drug uptake and clearly suggesting that drug uptake was not consistent with either dilution extent or droplet size.

KEY WORDS: positive charge; emulsion; self-emulsifying; everted intestine; mucin; Peyer's patches; cyclosporine A.

INTRODUCTION

Numerous potent lipophilic drugs exhibit low oral bioavailability due to their poor aqueous solubility properties. Ongoing efforts are being made to enhance the oral bioavailability of lipophilic drugs in order to increase their clinical efficacy. The most popular approach is the incorporation of the active lipophilic component into inert lipid or surfactant vehicles to improve oral absorption by increasing the solubility or dissolution rate of the drug (1).

Self-emulsifying drug delivery systems defined in the present paper as self-emulsifying oil formulations (SEOF) consist of isotropic mixtures of oil and surfactants and can improve oral availability of drugs poorly soluble in water. In contrast to emulsions, which are sensitive and metastable dispersed dosage forms, SEOFs are physically stable solutions and easy to manufacture. Taken in gelatin capsules these formulations disperse in the GI tract to form a fine emulsion, upon dilution with gastrointestinal fluids (2).

To the best of our knowledge, all SEOFs described in the literature were developed without regard to the surface charge properties of the resulting emulsion and usually produced dispersed oil droplets bearing a negative charge. Recently, a SEOF which results in positively charged dispersed oil droplets upon dilution with an aqueous phase was developed and characterized in an attempt to enhance interaction with mucosal surface and increase cellular uptake. This formulation elicited an increase in the oral bioavailability of progesterone in young female rats (3).

Barry and Eggenton (4) have shown that the intestinal cell interior is negative relative to mucosal fluid while the small intestine maintains a potential difference in the absence of actively transferred solutes. This potential is dependent upon the ionic environment of the tissue: replacement of mucosal sodium chloride by mannitol increases the negativity of the mucosal potential (5).

The epithelial surface of the G.I. tract is covered by a water-insoluble, free flowing and viscous gel: mucus which is also present in a viscous, soluble form in the lumen. Direct examination of *ex vivo* sections of rat stomach and duodenum suggested that the adherent mucus gel consists of a continuous layer of variable thickness, 5–200 μm and occasionally 400 μm (6). In the stomach and intestine the mucus layer sets up a stable gradient of pH and protects the surrounding tissue by lubricating the movements of the stomach and intestinal contents. The macromolecules of mucus carry a compendium of binding sites so that incident microorganisms, viruses and foreign macromolecules are blocked both mechanically and chemically and then recognized, trapped and eliminated. The principal component of the mucus is glycoprotein mucin which can be degraded into 500 kDa basic subunits by reduction of disulfide bonds (7).

It was already shown that a correlation exists between the rate of diffusion through mucus and the absorption rate for a range of compounds whose distribution coefficients did not act as good predictors of absorption. Removal of the mucus layer from the surface of the gastro-intestinal epithelium significantly increases the rate at which drug molecules are transferred across a section of everted rat gut *in vitro* (8).

Peyer's patches are the collections of lymphoid follicles, distributed within the lamina propria and submucosa of the small intestine. Peyer's patches perform a central role of antigen uptake and induction of the immune response (9). The insorption of small particles obviously occurs primarily via the M-cells of Peyer's patches (10).

In the present work, the interaction of SEOFs as a function of emulsion droplet charge and size with components of intesti-

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nal mucosa, adherent mucus layer and Peyer's patches, was investigated using rat everted intestinal sacs. A lipophilic compound-cyclosporine A (CsA) was used as a model drug.

MATERIALS AND METHODS

Materials

Ethyl oleate (EO) was purchased from Fluka AG (Buchs CG, Switzerland). Oleylamine (Armeen® OD) was obtained from Akzo Chemicals (Littleborough, UK). Tween 80, dithiotreitol (DTT), mucin, mannitol and PTA were obtained from Sigma (St. Louis, MO, USA). Cyclosporine A (CsA) was received from Balkfarm (Sofia, Bulgaria). The fluorescent probe Dio C_{18} (3) was ordered from Molecular Probes, Inc. (USA). Water was double distilled.

Experimental Methods

SEOF Preparation and Characterization

The SEOF tested consisted of (% w/w) polysorbate (Tween 80) 25, ethanol 30, oleylamine (OA) 3 and ethyl oleate to 100. The preparation of the various SEOFs consisted simply of weighing and mixing various components until a clear solution was obtained. This formulation was reported to result in positively charged emulsions following aqueous dilution in the presence of the cationic lipid OA whereas negatively charged emulsions were formed by omission of OA from the SEOF (3). CsA was dissolved in SEOF at a concentration of 100mg/ml.

The size of droplets was determined by transmission electron microscopy, using negative staining method: the emulsion at each dilution was stained with 1% solution of phosphotungstic acid (PTA) sodium salt at pH 7.5.

ξ-potential was measured in duplicate using a Coulter DELSA 440 at standard SEOF dilutions of 1:10,000. Scrapped mucus layers of everted sacs containing oil droplets for ξ-potential measurements after *ex vivo* study were dispersed in a minimal volume of distilled water and further diluted 1:1000 prior to measurement.

Mucin was dispersed in the water at the concentration of 5% using a Polytron® rotating homogenizer and the next dilutions were made from this stock solution.

Ex Vivo *Studies*

Male Sprague-Dawley rats (six animals per group) weighing 225–250g were anaesthetized with ether and intraperitoneally injected with ketamine (Imalgene 1000®) at a dose of 0.1ml/100g body weight. Duodenum and ileum (11) were removed, divided into two equal parts (for comparison purposes within the same rat) and everted. The rats were then sacrificed with an overdose of ether. The intestinal segments were immediately placed in ice-cooled saline or mannitol solution (12). For mucus detaching, the sacs were incubated in 0.1M solution of dithiotreitol (DTT) over 10 min. The mucus layer was then removed mechanically with a tissue moistened with cold saline and finally washed with saline.

The intestinal segments were placed in 10ml of CsA emulsion prepared *in situ* by dilution of SEOFs with saline (1/10, 1/50 or 1/100) or mannitol solution (1/10) (comprised of 25 mM NaCl and mannitol osmotically equivalent to an additional 125mM NaCl) and incubated for 10 min at 37°C. Some of the sacs were washed with two portions of 5ml saline after the incubation, and 5 cm segments from the central part of the sacs were scrapped with a scalpel.

The CsA was extracted from the scrapped tissues or residues with acetonitrile and determined by HPLC (13).

Statistical analysis of the *ex vivo* results was made using Wilcoxon matched pairs method.

The rats had free access to food on nights and were not allowed to eat during daytime till evening, when the experiments were carried out.

For confocal laser scanning microscopy experiments, intestinal specimens, freshly excised, were placed directly in the imaging chamber, epithelial side down and incubated for 10 min at 37°C in positively or negatively charged emulsions (1/50) containing the fluorescent dye Dio C_{18} (3) at the final concentration of 10 μM. After incubation the specimens were washed with saline in order to remove the emulsion excess and immediately examined microscopically without additional tissue processing. In addition, in the experiments with the Peyer's patches, everted sacs of ileum containing same number of lymphoid nodes were incubated either with positively or negatively charged emulsions following dilution of 1/50 with saline and containing the lipophilic fluorescent dye Dio C_{18} (at a final concentration of 10 μM). 5 μm slices were made in LEICA CM 3000 cryostat from ileum intestine segments with Peyer's patches following the usual incubation procedure.

Fluorescent samples were analyzed using an inverse LSM 410 laser scanning confocal microscope (Carl Zeiss, Jena, Germany) equipped with 488 nm argon ion laser. Settings such as neutral density filters, electronic gain, pinhole size, and background level were maintained at identical values during all experiments. Optical excitation was done by 488 nm line of argon laser and the fluorescent emission was observed above 515 nm for FITC. The same 488 nm line of laser was used for transmitted light differential interference control (DIC) according to Nomarski as an optical contrast enhancement method. The samples were optically scanned at 10 μm increments through the Z-axis and sequential images were computerized.

RESULTS

In Vitro Characterization of the Resulting Emulsions Following Aqueous Dilution of the SEOFs

Particle Size

Photon correlation spectroscopy (PCS), the most acceptable method for colloidal particles sizing, was not used in the present study because it requires infinite dilution of the sample before measurements. In such conditions, irrespective of the initial SEOF dilution, the particle size profiles of the resulting emulsions are similar. It was previously reported (3) that the particle size of self-emulsifying systems may change with the dilution. For this reason in the present work the particle size of the diluted SEOFs was evaluated by transmission electron microscopy without further infinite dilutions. The results depicted in Fig. 1 show that at the dilutions 1/10 and 1/50 a wide distribution of the droplet size was observed. A marked population of large droplets ranging in size from 0.5 to 1 μm

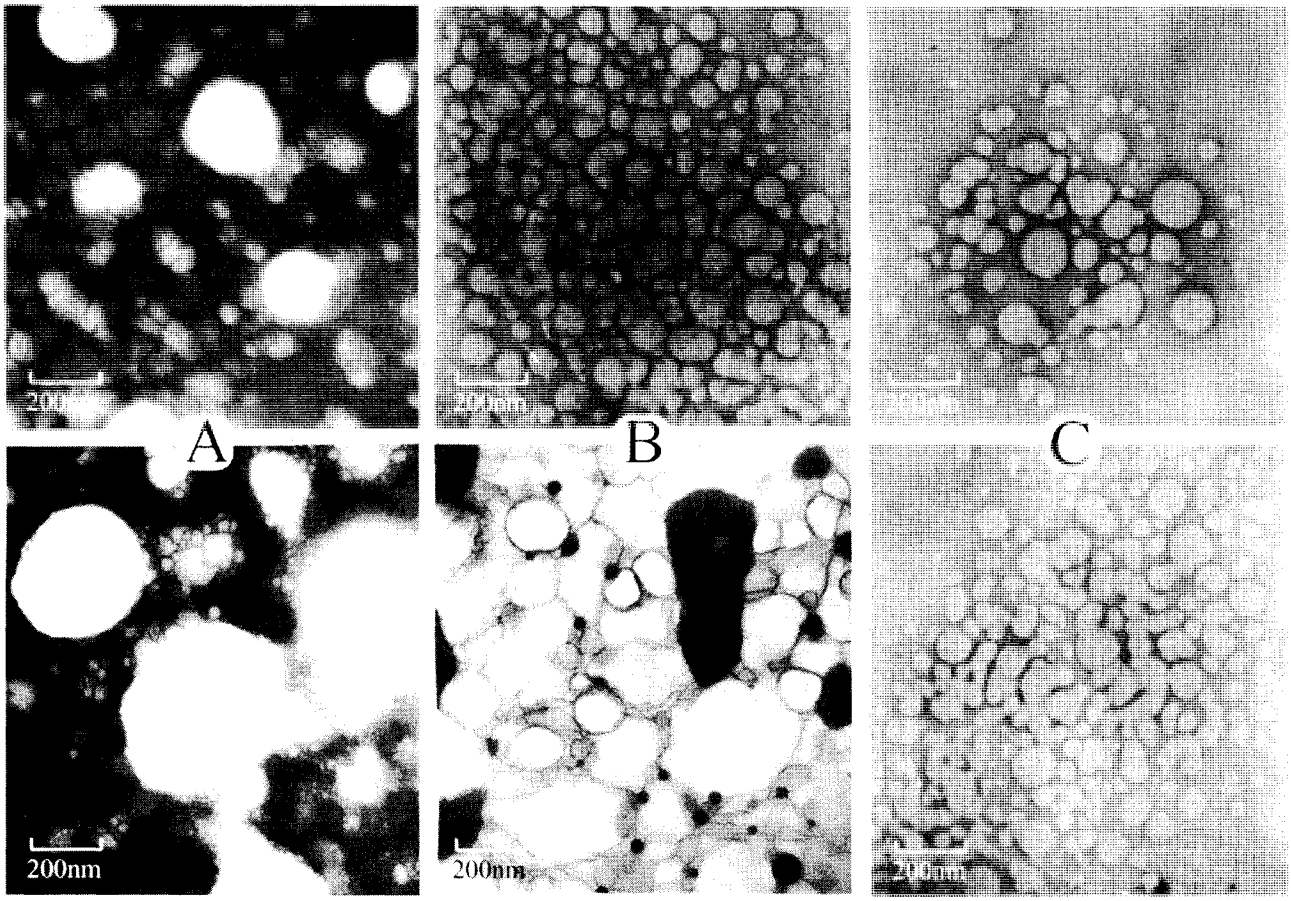

Fig. 1. Transmission electron microscopy photographs following negative staining with sodium phosphotungstate of SEOF containing 10% cyclosporine, 3% oleylamine and diluted with saline. 1/10 (A); 1/50 (B); 1/100 (C).

at 1/10 dilution and from 0.2 to 0.5 μm at 1/50 dilution was detected together with a large population of the smaller droplets in the size range of about 0.01–0.1 μm. On the contrary, at the dilution 1/100 the droplet size population was more homogenous within the range of 10–100 nm (Fig. 1). The same behaviour was observed for both types of emulsions irrespective of the oil droplet charge nature.

ζ-Potential Measurements

ζ-potential of the emulsions prepared by different initial SEOFs' dilutions in water was $+41.1 \pm 4.6$ and -10.0 ± 0.5 mV for positively and negatively charged emulsions respectively. The ζ-potential values measured were not affected by the extent of emulsion aqueous dilution. However, the values of measurements in the mucin solutions depended on the dilution at which the initial emulsion was formed. The positive charge of the oil droplets formed by 1/100 dilution was markedly reduced and even neutralized more easily than the charge of the droplets at the dilution 1/10 and 1/50. The ratio of mucin to SEOF and not mucin aqueous concentrations was kept constant in order to comparatively evaluate the neutralization ability of mucin while all the parameters remained unchanged except the droplet size of the diluted SEOF. It could be observed that the ζ-potential of the large droplets (i.e., low dilution rate) was moderately reduced with increasingly smaller concentrations of mucin per ml of SEOF (Fig. 2). The results were highly reproducible and the standard deviation ranges of the zeta potential values were low (± 0.5 - 2.0). A good correlation of the reduction of ζ-potential with decreasing droplet size was noted only at subphysiological mucin concentrations (less than 1%).

Precipitation Study

CsA is relatively soluble in ethanol and practically insoluble in water, so it may have precipitated following SEOF aqueous dilution when ethanol partitioned from the oil phase in favor of the aqueous phase. To validate that the oil droplets continued to be the prevalent source of the CsA after SEOF dilution, any possible precipitation which could occur was separated by repeated centrifugations and successive saline washings, and processed for CsA identification and quantitative determination by HPLC. It was found that only 3–5% of the initial CsA precipitated after each aqueous dilution irrespective of the ratio extent. Obviously, the major amount of CsA was provided by the oil droplets.

Everted Sac Results

The concentration of CsA in the intestinal scrapped mucosa after the incubation at 37°C was significantly higher with positively charged emulsions prepared by SEOF dilutions of 1/10

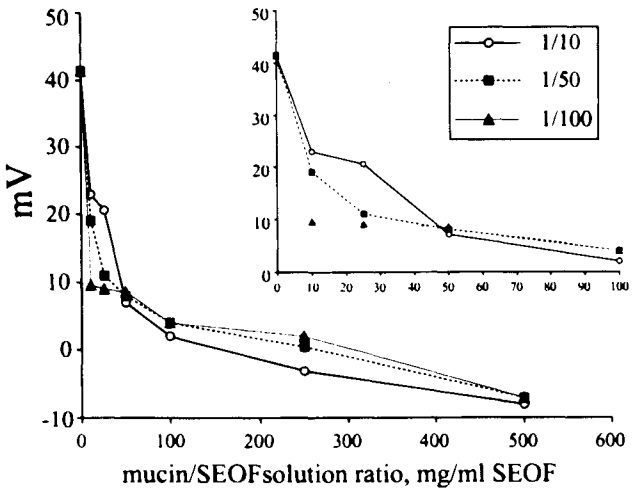

Fig. 2. Influence of the mucin/SEOF ratio extent on the ζ-potential of the resulting emulsions following dilution of 1/10, 1/50, 1/100 with saline. (Mucin was dissolved in the aqueous phase prior to self-emulsification).

and 1/50 than with the negatively charged emulsions ($p = 0.05$) in the duodenum or ileum. At the dilution of 1/100 there was no significant difference between the two emulsions (Fig. 3).

The experiments at dilutions 1/10 and 1/50 were carried out with and without washing of the sacs after incubation in order to detect probable droplet penetration through the mucus. The experiments were repeated without mucus layer (treated by DTT). In all cases the CsA amounts were much lower (one order of magnitude) when the sacs were washed before the scraping (Fig. 4). In the emulsions prepared by SEOF dilution 1/50, the amounts of CsA in the intestinal mucosa were significantly higher with positively charged emulsions than with the negatively charged emulsions *before and after* the washing irrespective of the presence or absence of the mucus (Fig. 4). It should be pointed out that in the unwashed intestinal segment experiments, the uptake of CsA from the positively-charged emulsion was 5 to 6 fold higher than with the negatively charged emulsion suggesting the occurrence of a strong interaction between the positively-charged emulsion droplets and the negatively binding sites of the mucus. Similarly, in the emulsions prepared by SEOF dilution 1/10, the amounts of CsA in the intestinal mucosa of everted sacs treated with DDT (without mucus layer) were significantly higher with the positively charged emulsions *before and after* the washing. However, in the sacs where the mucus layer was not removed a significant difference was observed only in the *unwashed* sacs (data not shown). Probably because of the large size of the resulting oil droplets those which were not neutralized by the mucus were unable to permeate through the mucus macromolecular network and reach the enterocyte apical membrane. They were therefore removed with the mucus during the washing process.

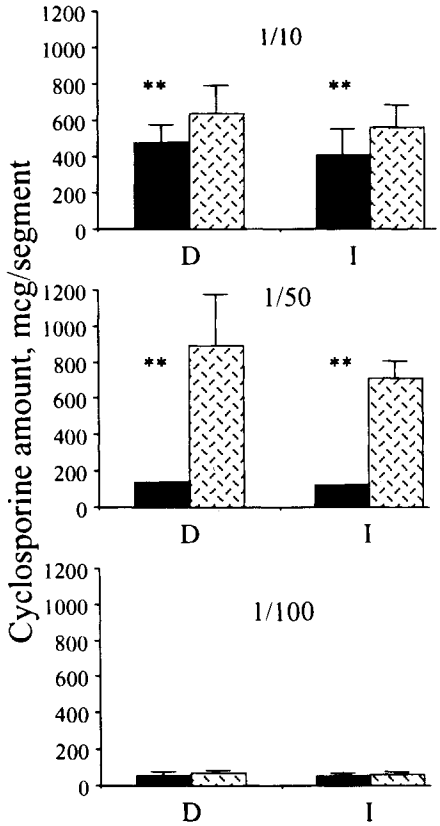

Fig. 3. Effect of the SEOF dilution extent ratio on mucosal CsA uptake in the duodenum (D) and ileum (I) as a function of the resulting emulsion charge. **$p < 0.05$, ■ negative, ▨ positive.

It can be seen from Figs. 5 and 6A that a marked superficial interaction of the positively charged emulsion as compared to the negatively charged emulsion occurred. This fluorescent difference was much more distinct with the duodenum than with the ileum. (Figs. 5 and 6A). Furthermore, in Peyer's patches both emulsions were shown to penetrate (Fig. 6B) with a qualitative trend in favor of the positively charged emulsion.

DISCUSSION

The present work pro[illegible]hat interactions occurred between emulsion droplets and mucosal surface: positively and negatively charged droplets adhered to the rat intestinal mucosa. However, the positively charged droplets formed by SEOF dilutions 1/50 and 1/10 exhibited an enhanced affinity to the mucosal surface resulting in a higher uptake of CsA into the mucosa (Fig. 3). The preferential affinity of the positively charged droplets to the mucosal surface compared to the negatively charged droplets was also evidenced by confocal microscopy (Figs. 5, and 6). It can be deduced from the results presented in Fig. 5 that the fluorescent probe dissolved in the positively charged oil droplets adhered markedly to the intestinal mucosa of the duodenum and even penetrated deeper in the crypts (up to the depth of 144 μm from the surface) while the fluorescent negatively charged oil droplets only reached the 72 μm depth from the surface (Fig. 5). A similar phenomenon was observed in the ileum (data not shown).

It was observed that mucin is important in the mucosal adhesion of dosage oral formulations, which may lead to prolonged G.I. transition (8). This hydrated, negatively charged glycoprotein network may present a significant barrier through obstruction and electrostatic effects, especially for colloidal particles (14).

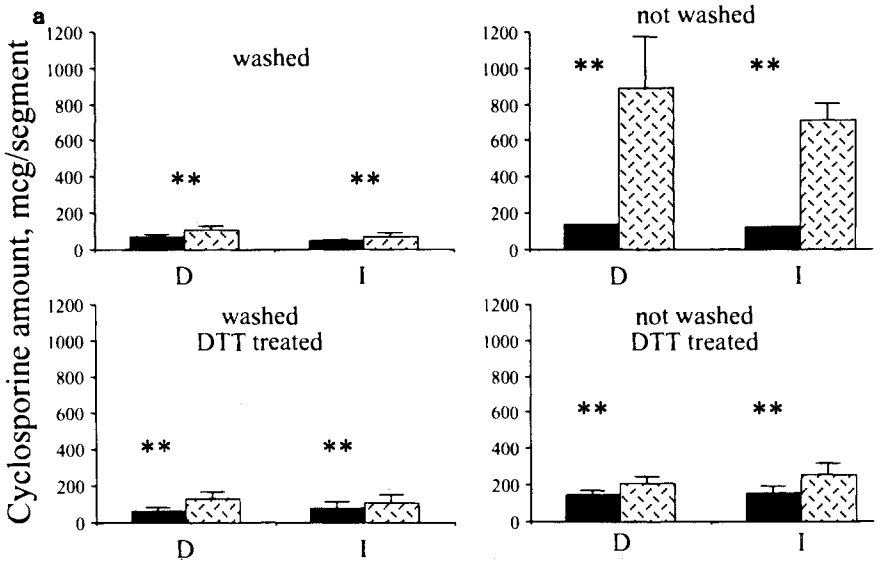

Fig. 4. Effect of mucus removal (DTT treatment) and mucosa washing on the CsA mucosal uptake in the duodenum and ileum at the SEOF dilution 1/50, **p < 0.05, ■ negative, ▨ positive.

The enhanced interaction of positively charged droplets with the mucosal surface may be explained in part by their electrostatic attraction to negatively charged sites existing in the mucin gel. The reduction of the mucosal potential to the more negative values due to the replacement of NaCl by mannitol increased the uptake of CsA from the positively-charged oil droplets by +25 ± 17%** and +27 ± 23%*% in the duodenum and ileum respectively (**p < 0.05; *p < 0.01). Whereas the CsA uptake from the negatively charged oil droplets in the duodenum and ileum decreased by −20 ± 23%* and −30 ± 24%* respectively. It is interesting that the effect of mucosal potential alteration could be detected even in the presence of the mucus layer which on its own contains various negatively charged sites that are able to bind positively charged particles.

The droplet size seems to be one of the principal factors affecting uptake of the CsA from the positively charged droplets. The charge of the small droplets (SEOF dilution of 1/100) could be neutralized by very low concentrations of the mucin gel, formed by spontaneous solubilization of the superficial mucin macromolecules facilitated by stirring during the incubation. The charge of the droplets at the dilution 1/10 (extremely

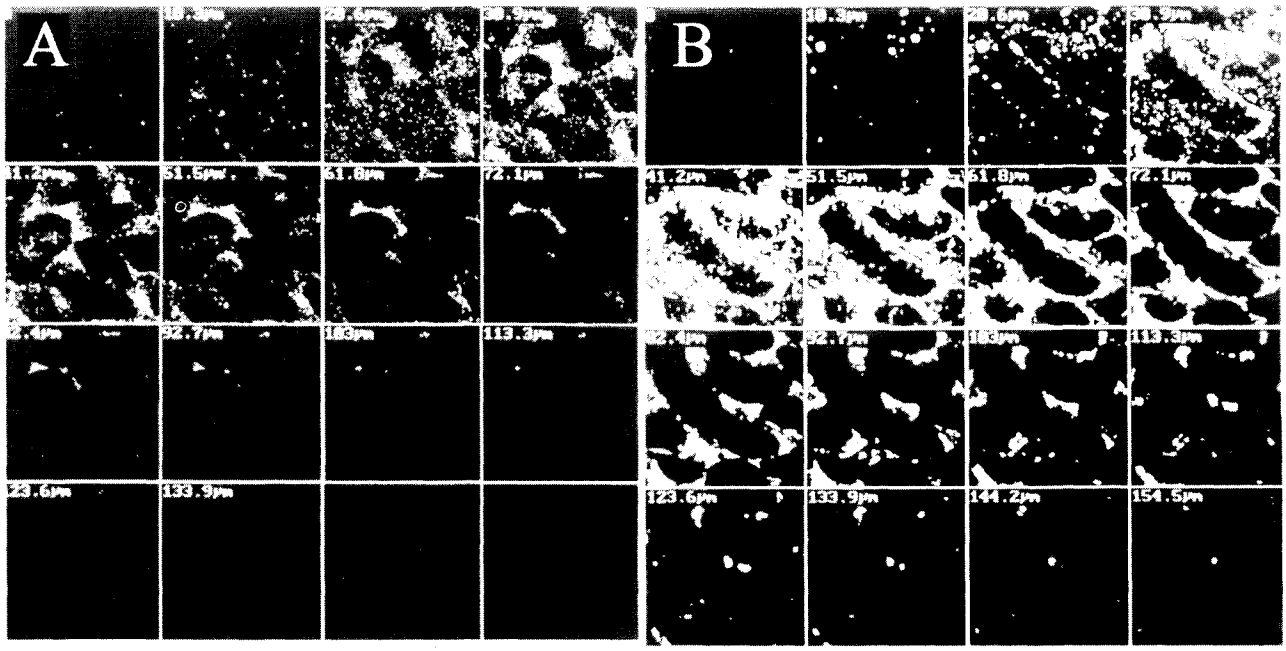

Fig. 5. Confocal laser scanning microscopy photographs of fluorescent duodenum samples incubated (at 37°C, 10 min) with resulting negatively (A) or positively (B) charged emulsions following saline dilution (1/50) of SEOF containing the fluorescent dye Dio C_{18} (10 μM). The samples were optically scanned at 10 μm increments through the Z-axis.

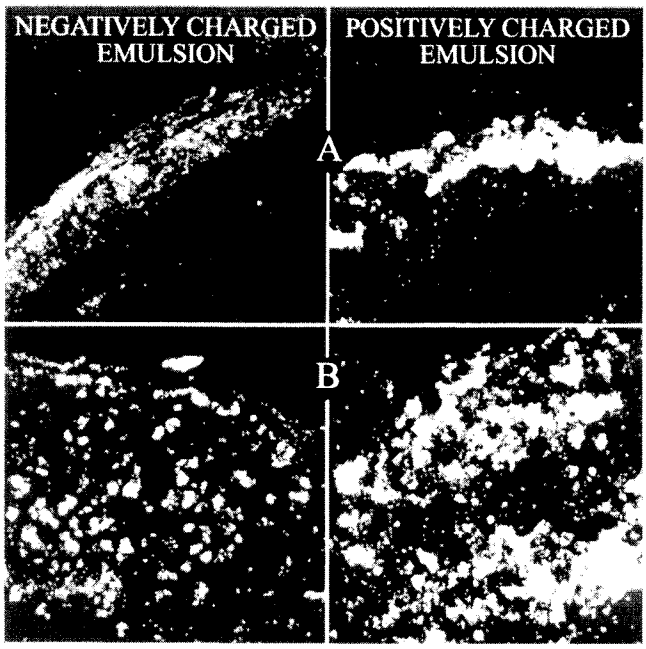

Fig. 6. Confocal laser scanning microscopy photographs of fluorescent ileum (A) and Peyer patch (B) samples incubated (at 37°C, 10 min) with resulting negatively and positively charged emulsion following saline dilution (1/50) of SEOF containing the fluorescent dye Dio C_{18} (10 μM).

wide distribution of droplet size including very large droplet populations) persisted at higher concentrations of mucin than at the dilution 1/50 (Fig. 2). At mucin concentrations typically found *in vivo*, 2–5% (9,14), the charge of all positive droplets irrespective of the size was neutralized and even reversed (Fig. 2). In order to be sure that the interaction of the positively charged droplets with mucus reverses their charge, ζ-potentials of the emulsions in the mucus aqueous dispersion (scrapings) and washings were measured and founded to be -28 ± 1.5 and -25 ± 0.8 mV respectively. The above findings confirm the conclusion that the positive charge of the larger droplets does not persist after the interaction with mucin in the physiological conditions very close to mucosal surface. Thus, this neutralization should not affect the potential of the positively charged emulsion droplets to promote oral bioavailability of the poorly absorbed active molecules, provided the neutralization occurs adjacent to the mucosa surface.

It can therefore be deduced from the data presented that the penetration of droplets through the mucus layer is also affected by their size. It was shown earlier that mucus represents a diffusion barrier, slightly more permeable for cations than for anions (15). Moreover, mucin not only obstructs the diffusion of micelles but also affects their aggregation state (14). The droplet size seems to affect differently the uptake of the CsA as a function of the oil droplet surface charge. It can be clearly seen from Fig. 3 that the uptake of CsA from the negatively charged emulsion is consistent with the dilution rate. Obviously, by diluting from 1/10 to 1/100, the concentration of the CsA in the incubation medium decreased by a factor of ten. Thus, the uptake of CsA amount from the 1/100 dilution is approximately a tenth of the amount of the dilution 1/10 (Fig. 3). However, the uptake of CsA from the positively charged emulsion is not consistent either with droplet size or with dilution rate. An optimum droplet size range exists (1/50 dilution) where maximum drug uptake is likely to occur (Fig. 3). At SEOF dilution 1/50 the overall droplet size was smaller than at dilution 1/10. Although these smaller droplets were not neutralized immediately, they were able in part to penetrate through the outer mucus layer to such an extent that even after washing a significant difference between positively and negatively charged emulsions persisted (Fig. 4). It is also noteworthy that at this dilution (1/50) the amount of CsA detected in the mucosa of the sacs incubated with the positively charged emulsion was about 3 times higher in the segments in which the mucus layer was not washed and removed (Fig. 4), thus demonstrating the occurrence of a deeper penetration of the droplets through the mucus layer. It can be deduced that in the present study 1/50 SEOF dilution with droplets of about 0.1–0.5 μm size was optimal for the interaction with the rat intestinal mucosa. Also in this study, the interaction of larger drops formed in a similar manner at 1/10 SEOF dilution was rather superficial (occurred at the mucus interface), so that a gentle washing procedure removed the excess of positively charged droplets bound to the outer mucus layer (data not shown). This was also the case with the dilution 1/50, after washing, the CsA uptake both in duodenum and ileum decreased markedly as compared to unwashed, but the significant difference in favor of the positively charged emulsion was maintained either in the presence or absence of mucin (Fig. 4).

The confocal microscopy examination revealed the accumulation of the positively and negatively charged droplets in the region of Peyer's patches after the 10 min contact. However, there was a qualitatively visible difference between the uptake of negatively and positively charged droplets by Peyer's patches (Fig. 6). The rapid uptake of the particles into the Peyer's patches is well correlated with the results published in previous studies (10). Furthermore, Yanagawa and Coll (16) found that concentrations of CsA in lymph-duct were significantly higher from negatively charged emulsion compared with those of conventional CsA. But to our knowledge the uptake of emulsion droplets by the Peyer's patches has not been presented in the literature until now. Furthermore, from the results depicted in Fig. 6A it can be clearly observed that the interaction of the oil droplets with the mucus is a surface phenomena. No visual support of any possible oil droplet penetration within the mucosa can be provided at this time.

The SEOF used in the present study was originally formulated for improving oral bioavailability of lipophilic drugs. This formulation enhanced the oral bioavailability of progesterone in young female rats (3). It is well known that an increased dissolution rate of poorly water soluble drugs may result in higher absorption. Many efforts have been made to reduce colloidal particles in order to increase the available surface area for drug partitioning and dissolution. Such a trend is verified for negatively charged particles only. To the best of our knowledge oral bioavailability as a function of the emulsion droplet charge has not been investigated with the exception of our previous work (3). In this study, we have shown that the positive charge of the smallest droplets is neutralized more readily, so that the larger droplets can undergo the enhanced interaction with the mucosal wall. Furthermore, the largest droplets did not easily penetrate the mucin layer resulting in smaller drug uptake than the intermediate droplet size. Thus, two different

phenomena occurred in the mucosa in the presence of mucus, the adhesion and subsequent penetration of the oil droplet through the mucus. In other words, in the case of positively charged SEOFs, the uptake of drug from mucosa is not consistent with droplet size.

The overall results indicated that an optimal size range prevails, paving the way towards improved design and development of positively charged self-emulsifying delivery systems when optimal experimental conditions have been identified.

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