Influence of Chitosan Microspheres on the Transport of Prednisolone Sodium Phosphate Across HT-29 Cell Monolayers

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Purpose. The present study was performed to investigate the influence of chitosan microspheres on transport of the hydrophilic, antiinflammatory drug prednisolone sodium phosphate (PSP) across the epithelial barrier.

Methods. Microspheres were prepared using a precipitation method and loaded with PSP. Transport studies were performed in a diffusion cell chamber using the polarized human cell line HT-29_{B6}. Porcine small intestine and fluorescence-labeled microspheres were used to investigate penetration ability of microspheres.

Results. It was shown that transport of PSP drug solution was not saturable across the cell monolayers ($P = 8.68 \pm 8.24 \times 10^{-6}$ cm sec^{-1}) and no sodium dependency could be established. EGTA treatment resulted in an increased permeability ($P = 18.69 \pm 1.09 \times 10^{-6}$ cm sec^{-1}). After binding of prednisolone to chitosan microspheres its permeability was enhanced drastically compared with the drug solution ($P = 35.37 \pm 3.21 \times 10^{-6}$ cm sec^{-1}). This effect was prevented by EGTA treatment ($P = 15.11 \pm 2.57 \times 10^{-6}$ cm sec^{-1}). Furthermore the supporting effect of chitosan microspheres was impaired by pH and ion composition of the medium, whereas the effect remained unchanged in cells treated with bacterial lipopolysaccharides. *In vitro* incubation of fluorescence-labeled microspheres in the lumen of freshly excised intestine revealed a significant amount of the spheres in the submucosa.

Conclusions. Chitosan microspheres are a useful tool to improve the uptake of hydrophilic substances like PSP across epithelial layers. The effect is dependent on the integrity of the intercellular cell contact zones and the microparticles are able to pass the epithelial layer. Their potential benefit under inflammatory conditions like in inflammatory bowel disease, in order to establish high drug doses at the region of interest, remains to be shown.

KEY WORDS: chitosan microspheres; HT-29_{B6} cell monolayers; small intestine; prednisolone sodium phosphate; drug absorption.

INTRODUCTION

Steroids represent the most important therapeutic tool in managing acute symptoms of inflammatory bowel disease. However, their well known severe side-effects initiated the development of new delivery forms like rectal foams for topical application. A further improvement in therapy may be achieved by the use of microspheres, since they have been shown to exhibit a considerable affinity to inflammatory lesions (1–3). This affinity may lead to high local drug levels at the region of interest and enable a reduction in systemic drug concentrations that in turn results in reduced side-effects. However, most of the particles investigated so far exhibit potential adverse effects. Therefore, we recently developed microspheres based on chitosan (4).

Chitosan, a linear polymer of β-(1→4)-linked 2-amino-2-deoxy-D-glucopyranose, is a natural cationic polysaccharide derived by N-deacetylation of chitin, which is isolated from the exoskeleton of crustaceans such as crabs and shrimps. This biopolymer is one of the most useful materials from the viewpoint of effective utilization of natural resources. Owing to its biocompatibility (5) and biodegradability it has a variety of pharmaceutical and medical applications (6,7). Accordingly chitosan was employed for the preparation of microspheres.

The objective of the present study was to investigate the effects of chitosan microspheres on epithelial transport of prednisolone sodium phosphate, a hydrophilic steroidal antiinflammatory drug. HT-29_{B6}, a polarized human colonic cell line, and porcine small intestine were used as *in vitro* models. HT-29_{B6} cells represent a subclone of the permanent cell line HT-29 derived in 1975 from an adenocarcinoma of the human colon (9). Since the HT-29_{B6} subclone demonstrates a higher grade of differentiation than its stem cell line, it is comparable to other frequently used cell lines like Caco-2 or T84. HT-29_{B6} cultures have many properties in common with small intestinal epithelium, and are considered to be a relevant model for studying epithelial drug transport in the intestine (10). They possess well-developed microvilli and express some of the disaccharidases and peptidases that are typical for normal small intestinal villous cells.

Pigs have been widely used in the study of absorption and digestion, since large parts of the porcine GI-tract are anatomically close to that of humans (8). For this reason, GI tissues of these animals were used for the present study.

MATERIALS AND METHODS

Chemicals

Chitosan (MW 750,000) was provided by Fluka Bio-Chemika (Buchs, Switzerland). Acetic acid, sodium chloride, sodium dihydrogenphosphate, sodium hydrogenphosphate, sodium hydroxide, and sodium sulfate were obtained from Merck (Darmstadt, Germany). Polysorbate 80 and glycerol were purchased from ICI Specialty Chemicals (Essen, Germany). Lipopolysaccharide (LPS, E. coli Serotype 026:B6), N-methyl-D-glucamine (NMDG), ethylene-glycol-bis-[β-aminoethyl-ether]-N,N,N′,N′-tetraacetic acid (EGTA) and celite-adsorbed Texas Red were purchased from Sigma Chemicals Co. (Deisenhofen, Germany). Prednisolone sodium phosphate was obtained from Synopharm GmbH (Barsbüttel, Germany). Metylcellulose (Tylose® C 600) was received from Hoechst AG (Frankfurt, Germany). LIVE-DEAD kit was from Molecular Probes (Eugene, USA).

HEPES buffer solution used for the transport experiments consisted of 10 mM N-2-hydroxyethylpiperazine-N′-2-ethansulfonic acid (HEPES), 141 mM sodium chloride, 4 mM potassium chloride, 0.45 mM magnesium sulfate, 1.3 mM calcium chloride, 25 mM glucose, 0.9 mM potassium dihydrogenphos-

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phate, and an appropriate amount of sodium hydroxide or hydrochloric acid to adjust the pH. Tyrode buffer solution used for penetration studies consisted of 136.9 mM sodium chloride, 2.68 mM potassium chloride, 0.12 mM magnesium chloride, 1.8 mM calcium chloride, 5.55 mM glucose, 0.42 mM sodium dihydrogenphosphate, and 11.9 mM sodium hydrogencarbonate. Buffer substances were purchased from Sigma Chemical Co. (Deisenhofen, Germany).

All reagents and solvents were of analytical grade and used as received. Water was always used in demineralized form.

Preparation and Characterization of Microspheres

The method for obtaining microspheres is based on the precipitation of chitosan in solution following the addition of a sodium sulfate solution (4). For this purpose, a chitosan solution was prepared by dissolving 1.0 g chitosan in 400.0 ml acetic acid (2.0 % v/v) containing 1.0 % polysorbate 80. Ten ml of sodium sulfate solution (20.0 % w/v) was added dropwise (about 5 ml min^{-1}) to the polymer solution during stirring (RW 18, IKA-Werk, Staufen, Germany) and ultrasonication at 240 Watt (Sonorex Super RK 106, Bandelin electronic, Berlin, Germany). The formation of microspheres was indicated by the appearance of turbidity. After completion of addition of the sodium sulfate solution, stirring and sonication were continued for another 30 min. The resulting microspheres were separated by centrifugation for 15 min at 2500 g (GPR Centrifuge, Beckman Instruments, Palo Alto, California, USA). The supernatant was discarded and the obtained sediment was purified by being washed twice with water.

The microsphere size was measured by photon correlation spectrometry (PCS) using a BI-200 SM Goniometer Ver. 2.0 equipped with the digital correlator BI-2030 AT (Brookhaven Instruments Corp., Holtsville, New York, USA) and a 30 mW helium neon laser (Melles Griot, Cincinnati, California, USA) at 632.8 nm.

The surface charge of drug-loaded microspheres was determined by electrophoresis using a Lazer Zee Meter Model 501 (Penkem Inc., Bedford Hills, New York, USA). The determination was performed in HEPES buffer solutions between pH 6 and pH 8.

For the preparation of drug-loaded microspheres, a solution of prednisolone sodium phosphate at an appropriate concentration was added to the suspension of previously prepared microspheres. Usually, the final drug concentration of the resulting suspension was 30.83 $\times 10^{-2}$ mg ml^{-1}, whereas the microspheres concentration was kept at 0.8 mg ml^{-1}. The systems were allowed to equilibrate for 3 h at ambient temperature. Then the microspheres were separated from unbound drug by centrifugation and the drug content of supernatant was measured by UV spectrophotometry at 247 nm (Hitachi U-3000, Hitachi Ltd., Tokyo, Japan). The adsorption efficacy was calculated in relation to unbound drug (for further details see reference 4).

Texas Red labeled microspheres were prepared by a technique usually used for labeling of proteins such as antibodies with the red-emitting fluorophore (11). Therefore, about 1 mg celite-adsorbed Texas Red was added to 50 ml chilled microsphere suspension (4 mg ml^{-1}) during rapid mixing. The conjugation of Texas Red with microspheres was performed at 0°C at pH 9.0. The pH was adjusted with sodium hydroxide. The suspension was kept on ice and continuously stirred. After 4 hours, unbound as well as hydrolyzed Texas Red was removed by centrifugation for 15 min at 2500 g (GPR Centrifuge, Beckman Instruments, Palo Alto, California, USA). The supernatant was discarded and the obtained sediment was purified by being washed twice with water. The fluorescence-labeled spheres were stored in the dark until experiments were performed. As expected, average size and surface charge of microspheres was not affected by Texas red labeling since dye concentrations used were small to minimize potential side effects of dye loading.

Cell Lines and Culture Methods

The $HT29_{B6}$ subclon was a kind gift of Professor M. Fromm, University of Berlin, Germany. HT-29_{B6} cells were cultured at 37°C in Dulbecco's Modified Eagles Medium (DMEM, Gibco, Germany) containing 15 mM HEPES, 15 mM $NaHCO_3$, 2 mM glutamine and 10% heat inactivated fetal calf serum in a humidified atmosphere of 5% CO_2 and 95% O_2. To avoid bacterial and fungal contamination penicillin (50 I.E. ml^{-1}), streptomycin (50 μg ml^{-1}) and nystatin (2 μg ml^{-1}) were added. Cells were passaged every week and cells of passage 30–50 were used. For experimental purposes cells were seeded on polycarbonate filters with a pore diameter of 0.4 μm (Millipore, Bedford, Massachusetts, USA). Cells reached confluency after around 9 days.

Integrity of Cell Monolayers

The integrity of the HT-29_{B6} monolayers was checked by measuring the transport of Rhodamine Green™ Dextran (MW 10.000 g mol^{-1}) across this monolayer. The percentage of transported amount was assayed by fluorescence detection (PTI Deltascan, Photomed, Wedel/ Holstein, Germany) using excitation and emission wavelengths of 504 nm and 527 nm, respectively. With increasing culture time the permeability decreased. The monolayers appeared to be impermeable to the hydrophilic fluorescent marker after 13 days in culture. At this time, only a small fraction (0.09 ± 0.08 %) was transported within 4 hours, whereas 48.03 ± 9.68 % of the marker was transported across the blank filters during the same time. This was taken as an indicator that the cell monolayer was confluent and intact. Consequently, cells were used between day 13 and day 16.

Cell Viability

Cell viability was checked using a fluorescence-based LIVE-DEAD kit. Briefly, cells were loaded with 2μM Calcein acetoxymethyl ester and 4μM ethidium homodimer. Live and dead cells could be differentiated, respectively, by their green and orange emitted light after illumination at 500–550 nm. The percentage of dead cells was calculated after counting a representative amount of cells. Under control conditions the percentage of dead cells was about 1–2%.

Transport Studies

A side-by-side diffusion cell setup (Fischer, Frankfurt, Germany) was used to determine drug transport across the HT-29_{B6} cell monolayers. The monolayers grown onto porous polycarbonate membranes (Millicell®-PCF, 4 μm pore size, Millipore, Bedford, Massachusetts, USA) were washed free of medium with HEPES buffer prior to placement between the

diffusion half-cells. The experiments were performed on 13- to 16-day-old epithelial cultures. To start the permeability experiments, 7 ml of HEPES buffer containing the compound of interest was placed in the donor chamber, whereas the receiver chamber was filled with 7 ml of pure buffer solution. Usually, the pH of the buffer was kept at 7.0 at a constant temperature of 37°C. The solutions were perfused with carbogen gas consisting of 95% oxygen and 5% carbon dioxide, and stirred continuously during transport experiments. At appropriate time points, samples (500 µl) were taken from the receiver phase (basolateral side), and replaced by an equal volume of fresh buffer. The samples were diluted with water and assayed by spectrophotometry (Beckman, Germany) at 247 nm.

To characterize the transport mechanism of prednisolone sodium phosphate across the epithelial monolayer the influence of several parameters was investigated. Therefore, the initial drug concentration was varied between $3.85 \cdot 10^{-2}$ and $61.66 \cdot 10^{-2}$ mg ml^{-1}, the pH of the used buffer solution was altered between pH 6.0 and pH 8.0, and sodium ions were replaced by N-methyl-D-glucamine (NMDG). Furthermore, effects of addition of 2.5 mM EGTA, and of LPS pretreatment (24 hours, 1.5 µg ml^{-1}, 37°C) on monolayers were investigated.

Experiments to investigate the effect of chitosan microspheres on drug transport were performed in the same way as described above except that drug solutions were replaced by microspheres suspensions. The drug content of the preparations was kept constant at $30.83 \cdot 10^{-2}$ mg ml^{-1} while the concentration of the used microsphere suspensions (0.2–3.1 mg ml^{-1}) and the pH (6.0–8.0) of the transport media were varied. Furthermore, the effect of the complete replacement of sodium ions by NMDG and the partial replacement by potassium ions (reduction of sodium to 91 mM with potassium simultaneously increasing to 54 mM) were investigated. In addition, a number of experiments was performed in the presence of 2.5 mM EGTA and with LPS pretreated (24 hours, 1.5 µg ml^{-1}, 37°C) monolayers.

Permeability Coefficient

The apparent permeability coefficient (P_{app}, cm sec^{-1}) was calculated according to the following equation:

$$P_{app} = \frac{V \cdot dC}{A \cdot C_0 \cdot dt}$$

where V is the volume (ml) of the diffusion chambers, A is the surface area of the cell monolayer (cm^2), C_0 is the initial drug concentration (mg ml^{-1}) in the donor chamber, and dC/dt is the change in drug concentration (mg ml^{-1}) per unit time (sec).

In Vitro Penetration Studies

The small intestine from freshly sacrificed pigs was rinsed with 0.9% sodium chloride solution to remove its contents. Then a suspension of 1% fluorescence-labeled microspheres in Tyrode buffer was filled into the lumen of intestine (pieces of about 0.1 m). Both ends of the piece of intestine were closed by tightly tying the ends with thread. The intestine sacks were placed into Tyrode buffer immediately after preparation, and the buffer was gassed with carbogen (5% CO_2 and 95% O_2). Experiments were performed at 37°C. After 2 hours, the intestine sacks were taken out of the buffer, opened on both ends, and the sphere suspensions were discarded. The tissue was washed with 0.9% sodium chloride solution in order to remove loosely adhering microspheres. The intestine was cut open, and small pieces were then embedded in 3% methylcellulose (Tylose® C 600) on a metal frame and immediately frozen in liquid nitrogen. Slices of 35 µm were cut from the frozen intestine using a microtome (Kryomikrotom Polycut, Leica, Bensheim, Germany). The slices were finally mounted on glass slides in 85% glycerol. All preparations were examined with a fluorescence microscope (Zeiss Axioskop, Oberkochen, Germany) fitted with a ×25 objective for oil immersion. The photographs were taken with a microscope camera (MC 100, Zeiss, Oberkochen, Germany) and Kodak T-Max p3200 film.

Statistical Analysis

All experimental protocols presented were repeated at least three times. Results are expressed as means ± standard deviation. Statistical differences between two mean values were evaluated using the unpaired Student's t-test (two-tailed). A difference between means was considered significant if the P value was less than 0.05.

RESULTS

Characterization of Chitosan Microspheres

Microspheres were produced by a precipitation method as previously described (4). The average size of the microspheres was 0.9 ± 0.2 µm, and their surface was positively charged in the pH range of interest (pH 6 / 8.48 ± 0.48 mV; pH 7.1/7.87 ± 1.51 mV; pH 8.1/6.03 ± 1.37 mV). Both parameters remained constant during storage for about 48 hours. Microspheres were loaded with prednisolone sodium phosphate (PSP) at a concentration of 0.3083 mg ml^{-1}. The plot of the adsorption efficiency of PSP against the concentration of microspheres in the aqueous phase indicated a saturable process (Figure 1). PSP

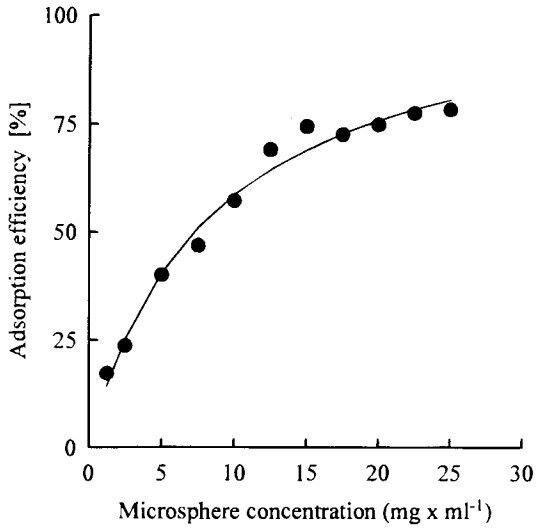


Fig. 1. Adsorption of prednisolone sodium phosphate onto chitosan microspheres. Adsorption efficiency is given by the percentage of bound drug to total drug. At a microsphere concentration of 8 mg × ml^{-1} about 50% initial drug concentration is adsorbed to microparticles.

loading did not affect average size of microspheres. About 53% of the employed drug was adsorbed by microspheres, if their concentration was maintained at 8 mg ml^{-1}. This microsphere concentration was used throughout the study.

Effect of Chitosan Microspheres on Drug Transport

Enhancing drug absorption using chitosan solutions is well known (12–14). In the present work a potential enhancement effect of microspheres on the uptake of the hydrophilic drug prednisolone sodium phosphate (PSP) was investigated. The time-transport profile of PSP across the HT-29 cell monolayers at pH 7.0 is shown in Figure 2 (filled squares). After 240 min about 3% of the initial amount of PSP in the donor chamber had arrived in the acceptor chamber. Permeability was found to be increased if PSP adsorbed to chitosan microspheres was used instead of pure drug solution (open circles). The enhancement of the drug transport by the microspheres became statistically significant after 60 min. Between 90 and 240 min PSP uptake increased continuously and linearly. After 240 min about 13% of the initial amount of drug was detected in the acceptor chamber. Thus, the drug transport was increased at least fourfold using chitosan microspheres. Control experiments demonstrated that cell viability was only minimally affected by chitosan microspheres (about 5±0.8% dead cells).

Characterization of Transport Pathways

In general, drugs can be transported by different routes across the intestinal epithelium (e.g., passive diffusion limited transcellular or paracellular transport vs. carrier mediated either primary or secondary active transport vs. transcytosis). The next series of experiments addressed the question of the transport mechanisms for PSP and for PSP-loaded microspheres.

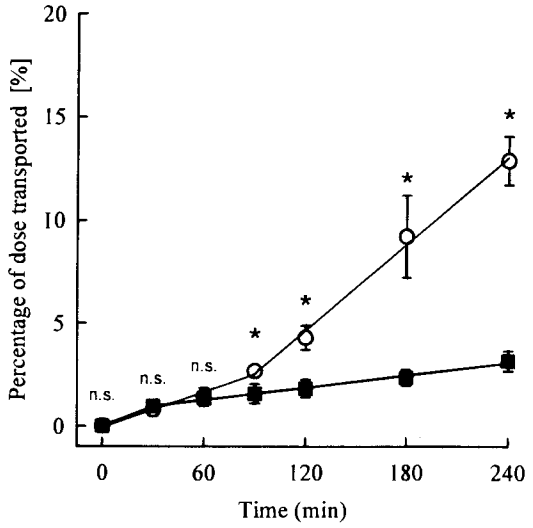


Fig. 2. Enhanced transport of PSP across HT-29_{B6} cell monolayers by using chitosan microspheres; open circles show the PSP transport using PSP-loaded microspheres, microsphere suspension had a concentration of 8 mg ml^{-1}; filled squares represent the pure drug solution. Drug content was kept at 30.83×10^{-2} mg ml^{-1} in each experiment. The effect starts at an incubation time of about 60 min and then increases continuously; data points are means ± SD. * indicates significance with $P < 0.05$, n.s. means not significant.

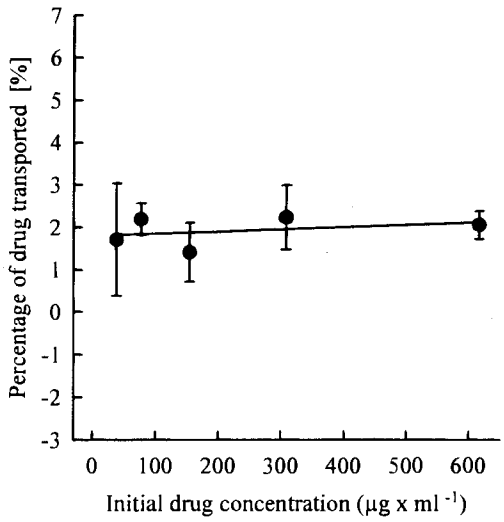


Fig. 3. Effect of different concentrations of prednisolone sodium phosphate on the drug transport represented as percentage of drug being transported after 240 min; data points are means ± SD.

Effect of Drug/Microsphere Concentration on Drug Transport

First the effect of different initial concentrations of PSP on the amount of drug transport was determined. Experiments were carried out over a wide concentration range (0.0385 mg ml^{-1} and 0.6166 mg ml^{-1}) for a time period of 240 min. Results show that the percentage of the drug being transported per unit time was independent of the initial concentration (Figure 3).

Secondly the effect of varying microsphere concentrations at a constant PSP concentration (0.3083 mg ml^{-1}) on drug transport was studied. The rate of PSP transport was nearly independent on the microsphere concentration used. Consequently, the lowest particle concentration used was sufficient to achieve a marked enhancement of PSP transport (Figure 4).

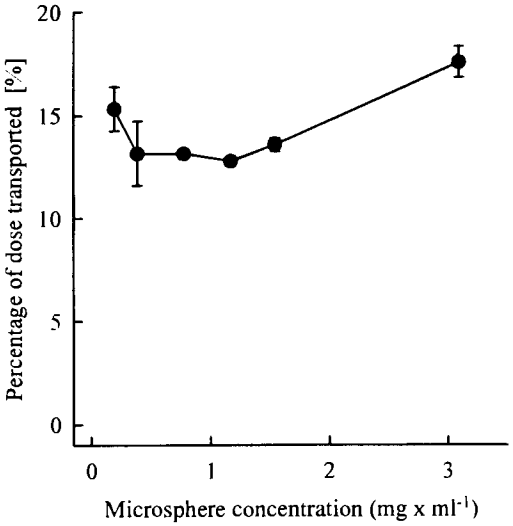


Fig. 4. Influence of microsphere concentration in suspension on the transport of prednisolone sodium phosphate across HT-29_{B6} cell monolayers at a constant drug concentration (0.3083 mg ml^{-1}) determined after an incubation of 240 min; data points are means ± SD.

Table I. Effect of Sodium and Potassium on the Permeability (P_{app}, Determined at 240 min) of HT-29_{B6} Cell Monolayers to PSP Drug Solution and PSP Loaded Microspheres

	Permeability ($\times 10^{-6}$, cm · sec^{-1})		
	Drug solution[a]	Microsphere suspension[a,b]	
with sodium	8.68 ± 8.24	35.37 ± 3.21	(0.01)[d]
sodium replaced by NMDG in acceptor phase	7.94 ± 6.14 (n.s.)[c]	—	
sodium replaced by NMDG in donor phase	9.85 ± 2.42 (n.s.)[c]	—	
sodium replaced by NMDG in acceptor and donor phase	8.90 ± 4.28 (n.s.)[c]	18.05 ± 2.59 (0.005)[c]	(0.05)[d]
50 mM sodium replaced by potassium in donor phase	—	14.31 ± 0.24 (0.001)[c]	

Note: Values are means ± SD.
[a] The drug content was kept at 30.83 · 10^{-2} mg ml^{-1} in each experiment.
[b] The microsphere suspension had a concentration of 8 mg ml^{-1}.
[c] Levels of significance between standard conditions (pH7, 141 mM sodium) and sodium replacement.
[d] Levels of significance between drug solution and microsphere suspension.

Higher particle concentrations had only a moderate additional facilitating effect on drug transport.

Effect of Sodium Concentration on Drug/Microsphere Transport

The following experiments addressed a potential sodium dependency of PSP and PSP-loaded microsphere transport. Therefore, the sodium gradient was reduced by replacement of sodium by N-methyl-D-glucamine (NMDG)(15). Neither partial replacement of sodium by NMDG in the donor or in the acceptor phase nor total sodium depletion affected the transport of PSP across the monolayers (Table I) suggesting that no sodium dependent carrier system is involved in the process of drug absorption. The high variability of permeability of experiments with pure drug solution resulted most probably from the low amount of drug being transported. In case of microsphere suspensions, drug transport across the monolayer was effected by extracellular sodium concentration. (Table I). Replacing sodium by NMDG led to a statistically significant decrease of permeability.

Additionally the effect of membrane depolarization on the efficiency of the microsphere supported drug transport was tested. Depolarization of apical membrane by replacing 50mM sodium by potassium decreased the drug permeability significantly and nearly diminished the enhancing effect of microspheres on PSP transport (Table I).

Effect of pH on Drug Transport

Next, the possibility of a potential effect of pH on the transport rates for PSP and for PSP-loaded microspheres was investigated, since pH-changes may alter permeability by affecting the polarity of cells. Furthermore, H^+-dependent carrier systems have been described in the luminal membrane of intestinal epithelia (16). Both acidic and alkaline pH had no significant effect on the permeability of PSP in pure drug solution. The microsphere suspension exhibited the greatest permeability at pH 7.0 (P_{app} (at 240 min) = 35.37 ± 3.21 [$\times 10^{-6}$, cm sec^{-1}]), whereas under alkaline pH the permeability decreased significantly (P_{app} (at 240 min) = 8.59 ± 0.48 [$\times 10^{-6}$, cm sec^{-1}]).

Effect of Coadministration of EGTA on Permeability

The functions of gap junctions in stabilizing monolayer integrity is highly dependent on the presence of calcium. By chelating calcium the importance of the paracellular pathway for drug absorption can be studied. We used the calcium chelator EGTA because of its higher calcium affinity than other chelators like citrate or ethylenediaminetetraacetic acid (EDTA). Control experiments demonstrated that cell viability was not affected after a 4 h incubation period with EGTA containing buffer (< 2% dead cells).

Exposure of monolayers to EGTA resulted in an increased absorption of PSP in pure drug solution (Figure 5a) as indicated by the upward shift of the time-transport profile, which was significant over the whole time period of 240 min. In contrast, the drug transport mediated by chitosan microsperes was decreased after exposure of monolayers to EGTA (Figure 5b). The time-transport profile shifted significantly downward after about 120 min. Interestingly, the different effects of EGTA under both conditions resulted in an approximatively overlay of the time-transport profiles for PSP and PSP-loaded microspheres.

Effect of the Exposure of Monolayers to LPS

Next, the effect of microspheres on an inflammatory status was tested. For this purpose HT-29_{B6} cells were incubated with bacterial lipopolysaccharides (LPS). This has been shown to induce secretion of cytokines like tumour necrosis factor or interleucins (17), known to be involved in inflammatory processes. Since LPS has been speculated to facilitate a physical disruption of the mucosal barrier and since loss of epithelial integrity (as measured by decreased electrical resistance) after prolonged exposure to LPS was reported (18), we checked the mucosal integrity and epithelial viability following a 24 hour incubation with LPS. No increased permeability of rhodamin dextrans was observed suggesting a still intact monolayer. Furthermore, cell viability remained unchanged compared to unstimulated cells (about 1–2% dead cells).

After LPS treatment drug transport using microspheres was reduced while there was no effect on drug transport using pure drug solution (Table II). However, the effect of microsphere enhanced PSP transport was still observed suggesting that microspheres represent a useful carrier even under inflammatory conditions.

Localization of Microspheres

Finally, to further characterize the transport mechanisms for the microspheres fluorescence-labeled microspheres were

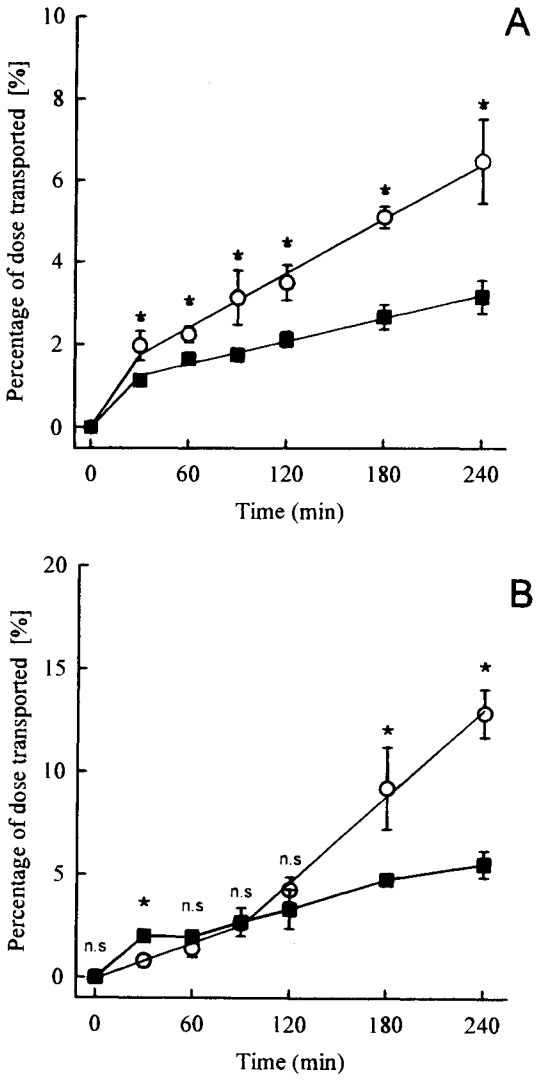


Fig. 5. (A) Influence of EGTA on the transport of PSP across HT-29_{B6} cell monolayers using a pure drug solution; drug transport in the presence of 2.5 mM EGTA is given by the open circles; filled squares are the controls using pure drug solution without addition of EGTA; data points are means ± SD. * indicates significance with $P < 0.05$. (B) Influence of EGTA on the transport of PSP across $HT29_{B6}$ cell monolayers using PSP loaded chitosan microspheres; drug transport in the presence of 2.5 mM EGTA is given by the filled squares; open circles represent the transport using chitosan microspheres suspension without addition of EGTA; data points are means ± SD. * indicates significance with $P < 0.05$, n.s. means not significant.

incubated with isolated porcine small intestine. As shown in Figure 6, the microspheres were able to cross the intestinal epithelial barrier and are visible in the core of the villi (lamina propria) and in large amounts in the submocusal layer below the villi after 2 h incubation time. There was almost no fluorescence found within the epithelial cells of the villi after this time.

DISCUSSION

The present study utilized a new recently developed type of microspheres (4) using chitosan, a natural biopolymer from crustaceans. Although the usage of chitosan is not new in pharmacology and medicine this represents the first approach to employ chitosan microspheres as a carrier for antiinflammatory drugs. The formation of spheres resulted from the electrostatic interaction between the positively charged chitosan and the negatively charged sulfate ions (4). The product is a polyion-counterion complex, which is poorly soluble in aqueous systems. After purification of the obtained preparations, prednisolone sodium phosphate (PSP) was adsorbed onto the surface of the microspheres by incubation in a solution of this drug. The resulting suspensions consisted of prednisolone-loaded microspheres as well as of unbound drug in equilibrium with each other.

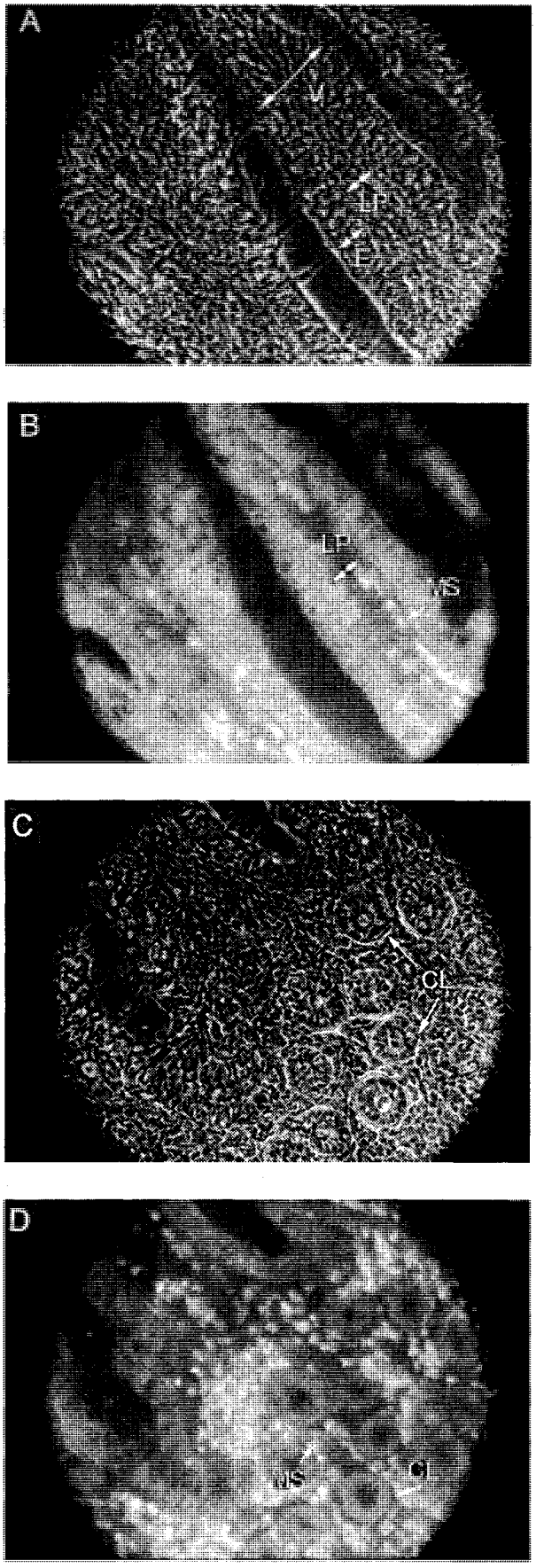
Table II. Effect of an Exposure (24 h, 37°C) of the $HT-29_{B6}$ Cell Monolayers to Lipopolysaccharide (LPS, 1.5 μg ml^{-1}) on the Permeability (P_{app}, Determined at 240 min) of PSP Drug Solution and PSP Loaded Microspheres

	Permeability [$\times 10^{-6}$, cm sec^{-1}]		
	Drug solution[a]	Microsphere suspension[a,b]	
pH 7.0	8.68 ± 8.24	35.37 ± 3.21	(0.01)[d]
LPS, pH 7.0	6.67 ± 3.41 (n.s.)[c]	23.72 ± 1.24 (0.005)[c]	(0.005)[d]

Note: Values are means ± SD.
[a] The drug content was kept at $30.83 \cdot 10^{-2}$ mg ml^{-1} in each experiment.
[b] The microsphere suspension had a concentration of 8 mg ml^{-1}.
[c] Levels of significance between standard pH 7.0 conditions and LPS-treatment.
[d] Levels of significance between drug solution and microsphere suspension.

The most striking result of the present study was that the transport of PSP across intestinal cells is drastically enhanced by the use of chitosan microspheres. This effect was time-dependent and started about 1 hour after incubation. To elucidate the involved mechanisms, the uptake of PSP alone and adsorbed to microspheres was investigated.

The results suggest that PSP is preferentially taken up passively via a diffusion-limited process. First of all, the transport of PSP was not saturable since increasing drug concentrations resulted in an increased drug flux. Carrier-mediated transport should be saturable as the amount of carriers is limited. However, carrier-mediated transport cannot be excluded totally since at the lowest PSP concentration used all carriers may be saturated. Diffusion-limited passive transport processes may occur transcellularly or paracellularly. Usually lipophilic substances are absorbed via the transcellular route, whereas hydrophilic agents are transported paracellularly. Since PSP is hydrophilic in nature, paracellular transport would have to be expected. Transport via the paracellular route can be enhanced using EGTA which interrupts the integrity of the junctional complex by chelating calcium, whereas transport of lipophilic agents remained unchanged (19). EGTA indeed enhanced the permeability of the epithelial barrier for PSP, which is consistent with paracellular transport. Furthermore, the PSP transport was not effected by the Na^+ gradient. In contrast to this, most carriers for amino acids, glucose, etc., in the apical membrane of intestinal cells are coupled to this inward-directed sodium gradient, which is established by the action of the Na^+/K^+-ATPase. From these findings it can be concluded that PSP seems to be trans-



ported via a passive non-saturable route, most probably via the paracellular pathway. However, it is not established that PSP is exclusively transported by that way. It is of importance to realize that not all active carriers are sodium-dependent, and that even hydrophilic drugs may be partly transported by the transcellular route (20).

How can be explained the supporting effect of chitosan microspheres on PSP uptake? A number of reasons might be responsible for this improvement in drug uptake.

By taking the known mucoadhesivety of chitosan into consideration (21), the increased permeability may be explained as a consequence of the adhesion of the microspheres to the cell surface. Consequently, the barrier effect of the unstirred water layer is reduced and, moreover, the contact time of the drug with the cell surface is increased, which leads to a facilitated delivery of PSP into the apical cell membrane of the monolayer. However, the tissue sections using fluorescence-labeled microspheres suggest that the particles do not simply adhere to the epithelial surface. Instead, they cross the epithelial barrier.

Recently it was shown that chitosan interacts with cytoskeletal F-actin (22), thereby changing the F-actin distribution. Since F-actin is associated with the proteins in the tight junctions (23), the simultaneous widening of the intercellular space may occur. The resulting opening of tight junctions would lead to an increased paracellular permeability. In addition, chitosan reacts as chelator for metal ions (7). The depletion of calcium from the electronegative site of the membrane, which requires coordination with cations for dimensional stability (24), also leads to opening of tight junctions. Likewise, artificial opening of the tight junctions using EGTA diminished the supportive effect of microspheres. This observation suggests two conclusions. First, it indicated that the tight junctions play a major role in the effect of chitosan microspheres on intestinal drug transport. Second, an intact junctional complex is required for the effect of microspheres suggesting that the junctional complex serves as a docking station for the microspheres possibly via calcium ions since such metal ion chelating properties of chitosan have been discribed previously (25). The docking process seems to rely on ion-ion interactions as indicated by the effect of pH or membrane-potential. Both, changing the surface charges of microspheres by changing the pH or changing the charge of the epithelial membrane by depolarization, had the same inhibiting effect. Additionally, this gating process may also be responsible for the temporal delay of the promoting effect of chitosan microparticles on PSP transport (Figure 2).

The transcytotic route also has to be taken into consideration for chitosan microsphere-facilitated transport. Uptake of particulate materials by the GI tract was already shown (26).

Fig. 6. Internalization of chitosan microspheres (**MS**) by porcine gut villous cells. The small porcine intestine was incubated for 2 h in the presence of fluorescence-labeled microspheres, as described in "Materials and Methods"; Upper half shows phase contrast pictures, lower half shows fluorescence pictures of the same histological slices; A, B: Apical part of intestinal villi (**V**): **LP**—lamina propria; **E**—epithelium); C, D: Basal part of intestinal villi and submucosa with crypts of Lieberkuhn (**CL**); As shown in Fig. 6B, the microspheres (**MS**) are present in the core of the villi; As shown in Fig. 6D, a massive accumulation of microspheres is occuring in the submucosa.

Similar to the stimulation of macrophage function by chitosan (27), the uptake of chitosan microspheres by phagocytosis of enterocytes becomes a possiblitity. However, if existent this route seems to play only a minor role at high microsphere concentrations since at high microsphere concentrations the percentage of PSP transported was slightly increased (Figure 4). It is possible that the transcellular route becomes significant at these high concentrations. On the other hand, in tissue sections almost no fluorescence was observed within epithelial cells making this route less attractive.

Interestingly, the higher flux obtained with the microspheres is preserved even after LPS treatment suggesting that these microparticles represent a highly promising delivery system for inflammatory intestinal diseases such as colitis ulcerosa and Crohns disease. Nevertheless, it must be noticed that the LPS treatment cannot serve as an inflammatory model and it is of limited utility since intestinal bowel disease is characterized by the complex interaction of immunocompetitive cells with epithelial and submucosal tissue. This necessitates further studies on human inflammatory tissues.

In summary, these experiments demonstrate that chitosan microspheres can improve transport of prednisolone sodium phosphate across the epithelial monolayer of HT-29_{B6} cells, probably by gating the transport of PSP through the tight junctions since the paracellular pathway seemed to be the main route of drug uptake. The transport was dependent on the ionic environment and was maximal at neutral pH. The increased drug transport rate was unchanged after treatment with LPS. Taken together, our results indicate that chitosan microspheres are a suitable carrier system for enhancing intestinal membrane transport of hydrophilic drugs like prednisolone sodium phosphate.

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