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Research Paper

Selective Adhesion of Nanoparticles to Inflamed Tissue in Gastric Ulcers

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Purpose. Gastrointestinal deposition of nanoparticles was examined after oral administration to mice suffering from an experimental gastric ulcer model. Local drug delivery could reduce side effects and would be a distinct improvement compared to existing therapeutic approaches, e.g. in the local therapy of Helicobacter pylori.
Methods. A gastric ulcer was induced to Swiss mice by acetic acid injection. Fluorescent polystyrene particles with a nominal size of 50, 200, and 750 nm were administered orally for 3 or 5 days and particle adhesion in the gastrointestinal tract analyzed.
Results. In the ulcerated regions, an enhanced particle adhesion was observed compared to healthy controls. A size dependency of the deposition was found which further increased with a prolonged treatment period. For 750 nm particles only fair adhesion was observed (control, 2.0±1.4%; ulcer, 4.5± 0.7% of daily administered particle mass), while already 200 nm particles showed higher binding (control, 2.9±1.3%; ulcer, 7.8±1.2%). Highest relative adhesion was found for 50 nm particles (control, 2.8±1.3%; ulcer, 10.0±1.5%). The targeting index of gastric ulcer *versus* healthy control was nearly constant around 2 after 3 days treatment, but increased distinctly for smaller particles after 5 days.
Conclusions. The use of sub-micron sized carriers holds promise for the targeted delivery of drugs to the ulcerated mucosal areas in the stomach.

KEY WORDS: bioadhesion; drug targeting; gastric epithelium; gastric ulcer; nanoparticles.

INTRODUCTION

A peptic ulcer may arise at various locations, namely stomach, duodenum, or esophagus being a mucosal defect which penetrates the muscularis mucosae and muscularis propria, produced by acid–pepsin aggression. Gastrointestinal bleeding is the most common manifestation of the disease. Tobacco smoking, blood group, spices and other factors that were suspected to cause ulcers, are actually of relatively minor importance in the development of peptic ulcers. A potential causative factor is chronic inflammation due to Helicobacter pylori that colonizes the antral mucosa (1). The immune system is unable to clear the infection, despite the appearance of antibodies. Thus, the bacterium can cause a chronic active gastritis, resulting in a defect in the regulation of gastrin production by that part of the stomach, and gastrin secretion can either be decreased resulting in hypo- or achlorhydria, or increased where it triggers the increase in acid that can contribute to the erosion of the mucosa and therefore ulcer formation. Since gastric ulcers caused by *H. pylori* have a higher risk to develop cancer, this therapeutic context is highly important (2).

The gastroduodenal response to chronic *H. pylori* infection is characterized by the infiltration of plasma cells, lymphocytes, neutrophils and monocytes into the mucosa (1). Eradication studies have shown that this inflammatory response represents a specific reaction to the presence of *H. pylori.* As well as stimulating specific local T and B cell responses and a systemic antibody response, *H. pylori* infection also induces a local pro-inflammatory cytokine response. Interleukin-8, which is expressed and secreted by gastric epithelial cells, may be an important host mediator inducing neutrophil migration and activation. Neutrophil activation and the production of reactive oxygen metabolites is induced directly by bacterial factors and indirectly via host-derived cytokines, products of complement activation and bioactive lipids. The gastric epithelium thus plays an active role in mucosal defence.

When *H. pylori* infection is present, the most effective treatments are combinations of 2 antibiotics (e.g. Clarithromycin, Amoxicillin, Tetracycline, Metronidazole) and a proton pump inhibitor, sometimes together with a bismuth compound. In complicated, treatment-resistant cases, more complex combinations of antibiotics may be used together with a proton pump inhibitor.

Actual therapeutic approaches rely on the administration of the before mentioned drugs which are available in standard formulations, e.g. tablet or capsule. Subsequently, drug is released in the stomach developing partially a local effect followed by a significant systemic availability after absorption from the small intestine which is in many cases therapeutically of limited interest and rather causes adverse effects than

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being of pharmacological value. In terms of a selective therapy, it appears to be desirable that drugs are only locally active with limited availability to other tissues. This may lower adverse effects and, more important, increase local drug efficiency.

It has been reported that small particles are efficiently taken up by macrophages (3), allowing for the specific targeting of inflamed tissue, namely in the therapy of intestinal inflammation (4–6). Thus, it may be expected that particle uptake into the disrupted barrier in gastric ulcerations could allow the accumulation of the particulate carrier system in the desired area. A subsequent increase in local drug concentration, which would be postulated for smaller particles compared with existing drug delivery systems, allows for a dose reduction. Besides, it has been described in literature that nanoparticles allow the enhanced drug penetration into bacteria after the adhesion to their cell surface (7,8). This may suggest an increased efficiency for a potential antibiotic therapy against *H. pylori* (9). One parameter of major importance for a particulate drug carrier system in this context is potentially the particle size.

This project evaluates the use of nanoparticles for their targeted deposition in inflamed tissue for a potential therapy of gastric ulcers. We examined qualitatively and quantitatively the adhesion behavior of polymeric carrier systems in the ulcerated tissue of the stomach and the influence of particle diameter on this deposition.

MATERIALS AND METHODS

MATERIALS

Acetic acid, *o*-dianisidine hydrochloride were obtained from Sigma-Aldrich (Deisenhofen, Germany), hexadecyltrimethylammonium bromide (HTAB) was obtained from Fluka (Deisenhofen, Germany). Astra blue, eosine G and nuclear fast red for the histological staining were purchased from Merck AG (Darmstadt, Germany).

Microspheres and Nanospheres

Polystyrene fluorescent micro- and nanoparticles with nominal sizes of 50, 200 and 750 nm were purchased from Polysciences Ltd. (Eppenheim, Germany). Particle sizes and zeta potential were confirmed using a Zetasizer II® (Table I). All particles were washed twice with distilled water in order to remove residual surfactants or antibacterial agents, separated by centrifugation and dispersed in saline solution (10 mM NaCl) for *in-vitro* characterization or phosphate buffer (pH 6.8) for the oral administration to mice.

Table I. Size and Zeta Potential of Particles Used for the *In-Vivo* Study

Theoretical size (nm)	Measured size (nm)	Zeta potential (mV)
50	57±3	−25±2
200	208±7	−22±6
750	740±11	−28±7

Animals

All animal experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, US). Male Swiss mice (average weight 30–35 g) were used in all experiments. Each group of treated animals contained at least six mice and was housed in standard cages. The gastric ulcer group was treated by the following procedure to effectuate an inflammation: After narcotizing mice underwent a ventral incision giving access to the stomach. Around 50 μL of acetic acid were injected into the gastric mucosa. The wound was closed and animals were housed for 24 h without treatment allowing for the development of the full gastric ulcer model. The control groups consisted of the same number of healthy mice. On day 2, a 0.1 mL particle suspension (12.5 mg particles/kg body weight) was administered orally with a blunted, curved needle to all mice for three or five consecutive days. Thereafter, no particles were administered in order to wash out unabsorbed particles from the stomach. 24 h after the last particle application the animals were sacrificed and the stomachs were resected.

Assessment of the Stomach Wet Weight/Body Weight Ratio

The stomach was opened and rinsed with phosphate buffer. The stomach segment including the major gross pathologic changes was weighed. The stomach/body weight ratio was calculated as an index of tissue edema (5).

Assessment of the Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was measured to ensure the full development of the ulceration. MPO activity is a reliable index of inflammation quantifying the infiltration of activated neutrophils into the inflamed tissue in intestinal inflammation. Also here, inflammation degree was analyzed by the MPO activity determination (10). Briefly, inflamed stomach specimens were minced in 1 mL of HTAB buffer (0.5% in 50 mM phosphate buffer) on ice and homogenized. The homogenate was sonicated for 10 s, freeze-thawed three times and centrifuged at 10,000 rpm for 3 min. MPO activity in the supernatant was measured spectrophotometrically. 0.1 mL of supernatant was added to 0.167 mg/mL *o*-dianisidine hydrochloride and 0.005% hydrogen peroxide. The change in absorbance at 460 nm was measured, with one unit of MPO activity being defined as the amount which degraded 1 μmol of peroxidase per minute at 25°C. All values of MPO activity were normalized to wet colon tissue weight.

Confocal Laser Scanning Microscopy (CLSM) for the Qualitative Localization of Particles

A Biorad MRC 1024 Laser Scanning Confocal Imaging System (Hemel Hempstead, UK), equipped with an argon ion laser (American Laser Corp., Salt Lake City, USA) and a Zeiss Axiovert 100 microscope (Carl Zeiss, Oberkochen, Germany), was used to qualitatively detect the fluorescent particles in the tissue sections. The laser was adjusted in the

green fluorescence mode which yielded an excitation wavelength at 488 nm.

Quantitative Determination of Particle Deposition in the Stomach

The quantification was adapted from a similar method described elsewhere (4,11) After resection of the stomach, the tissue was rinsed carefully to remove food residues. Inflamed areas which were identified by the macroscopic damages of the mucosal tissue, were separated from the non-inflamed tissue and cut into small pieces. All tissue samples were lyophilized in the dark to avoid bleaching of the fluorescent dye. Samples of the entire gut were treated accordingly but homogenized prior to lyophilization in order to allow an complete fluorescence extraction.

Samples were incubated with 10 mL chloroform at 30°C in a shaking water bath for 24 h in the dark. The extraction procedure was repeated two times in order to dissolve the polystyrene completely. The polymer solutions were diluted with chloroform to a total volume of 100 mL and were analyzed for their fluorescence by fluorescence spectrophotoscopy (F-2000 Fluorescence Spectrophotoscope, Hitachi Ltd., Tokyo, Japan). Blank lyophilized particles were used as references. Untreated stomach samples with and without ground chow were used for background correction.

Statistical Analysis

The results were expressed as mean values±S.D. The statistical analysis of treatments was performed with ANOVA on ranks followed by Dunn's test, except when normality and equal variance were passed, it was followed by the Tukey test. In all cases, $P<0.05$ was considered to be statistically significant.

RESULTS

The experimental model in mice after the intramucosal administration of acetic acid establishes a stable ulcer for at least 10 days (12). Among other properties, this gastric ulcer model was considered appropriate due to its reproducibility. This permits the characterization of the *in-vivo* deposition of the particulate carrier system under the influence of gastritis symptoms.

After inducing the gastric ulcer, histological sections were performed to get visual evidence of the ulceration and to characterize the differences to healthy tissue (Fig. 1A, B). In histological analyses strong damages of the gastric tissue were observed, as well as a highly increased infiltration activity of immune related cells was found (1). MPO activity was determined to ensure and quantify the inflammation in the ulcerated gastric area (10,13). The average MPO activity in the areas with macroscopic damages was 0.69±0.07 U/mg tissue, while in the healthy control the activity was as low as 0.01±0.01 U/mg tissue. Additionally, it was observed that the stomach wet weight/body ratio increased by a factor of 1.9 compared to the healthy control group, which is known as an indicator for inflammation (13,14).

The behavior of the proposed nanoscale system was examined with respect to particle deposition and accumulation in the ulcerated gastric tissue after oral administration.

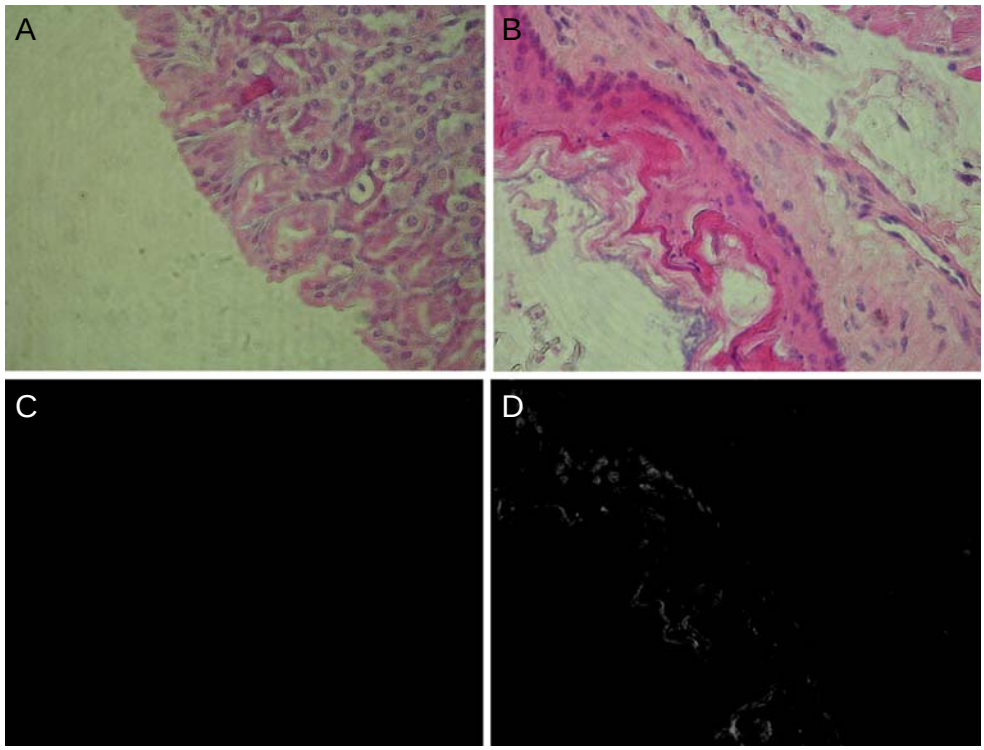

Fig. 1. Microscopical images of a stomach section through a tissue sample of the healthy control group (**A**) and of the ulcer group (**B**) showing a typical ulceration of the gastric tissue. CLSM images of gastric cross-sections from healthy control (**C**) and ulcer group after administration of 50 nm particles (**D**; magnification ×40).

Qualitatively, an increased adherence of all three sizes of particles was obtained in ulcerated tissue. A very slight increase of particle deposition compared to the healthy control was observed in the inflamed tissue with macroscopic visible edema, while a distinctly higher particle accumulation was found inside ulcerated tissue sections (Fig. 1C, D).

In order to examine quantitatively the gastric adhesion after oral administration, the particle deposition in different tissue samples from the stomach was quantified by fluorescence spectrophotoscopy (Figs. 2 and 3). Significant differences were determined between healthy and damaged regions of the stomach, for particle sizes after a daily administration for 3 days. When prolonging the treatment to 5 days, the selectivity of adhesion increased distinctly. 750 nm particles showed a constant twofold higher percentage of particle binding in gastric ulcer compared to the healthy control independently from the treatment period. 200 nm particles underwent a slight increase from a twofold higher binding to ulcerated tissue to a 2.7 fold increase when treatment was prolonged from 3 to 5 days. The highest deposition in inflamed tissue was observed for the 50 nm particles, showing a 2.3 and 3.5 fold increase of particle binding for 3 or 5 days treatment, respectively. It is remarkable that comparing the adhesion to ulcerated tissue or areas without macroscopic damages led to an enhanced particle accumulation in the ulcerated regions but a less pronounced increase in binding to the latter ones which was not significantly higher than healthy controls (data not shown). Subsequently, removing the mucus from the gastric mucosa decreased the total fluorescence signal in the tissue down to 68.6±9.9% for 50 nm particles which shows that still around two third of the adhering particles penetrate specifically the ulcerated region.

Residual adhesion was determined in the intestine, upper parts and colon, respectively. Generally, a fecal excretion at levels higher than 80% of the initial dose was observed, independently from particle size (Fig. 4).

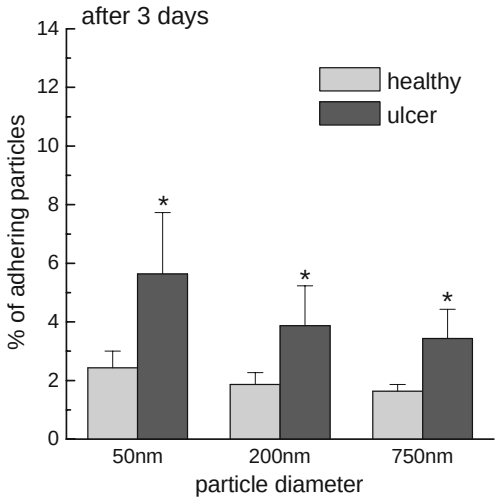

Fig. 2. Quantitative determination of particle deposition in the gastric ulcer group comparing to healthy controls after 3 days consecutive particle administration. Results are shown in percent of administered particle mass as mean values±SD. *$P<0.05$ compared to healthy controls at the equivalent particle diameter.

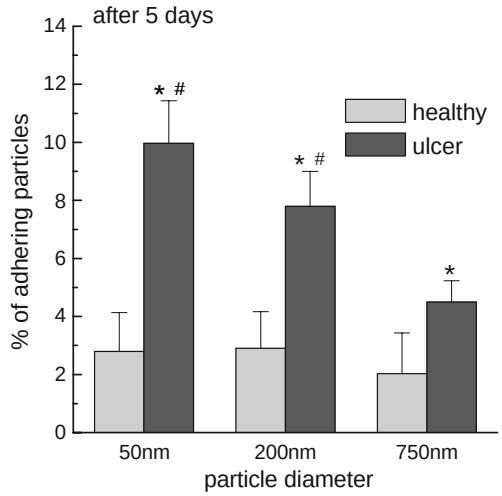

Fig. 3. Quantitative determination of particle deposition in the gastric ulcer group comparing to healthy controls after 5 days consecutive particle administration. Results are shown in percent of administered particle mass as mean values±SD. *$P<0.05$ compared to healthy controls at the equivalent particle diameter. #$P<0.05$ compared to ulcer group at 750 nm particles.

A targeting index was defined for better comparison of the ratio between particle deposition in gastric ulcer (P_{ulcer}) and control group ($P_{control}$). The decrease in particle diameter led to an enhanced accumulation in animals suffering from gastric ulcer, but particle size had a negligible influence on the adhesion in the healthy controls. Since particle adhesion to the inflamed tissue after 3 days did not exhibit significant differences between the different sizes, the targeting index remains around a constant value of 2. A significant increase of the targeting index was however observed with decreasing particle diameter and for the longer treatment period, i.e. 5 days (Fig. 5).

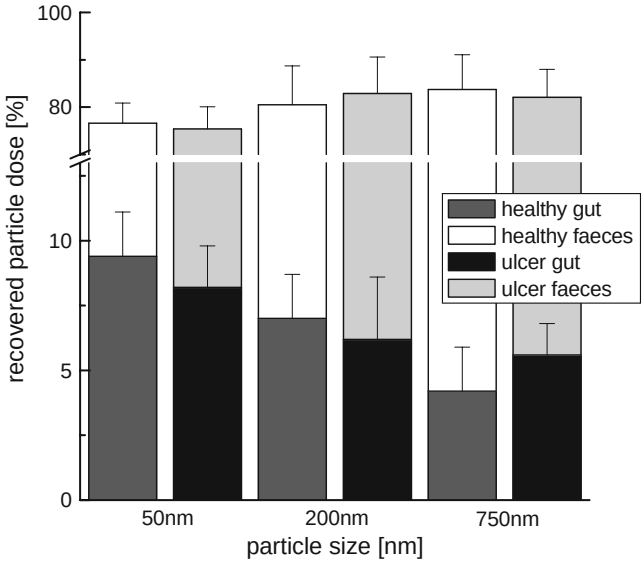

Fig. 4. Quantitative determination of particle deposition in the gut and particle recovery from faeces after 5 days consecutive particle administration. Results are shown in percent of administered particle mass as mean values±SD.

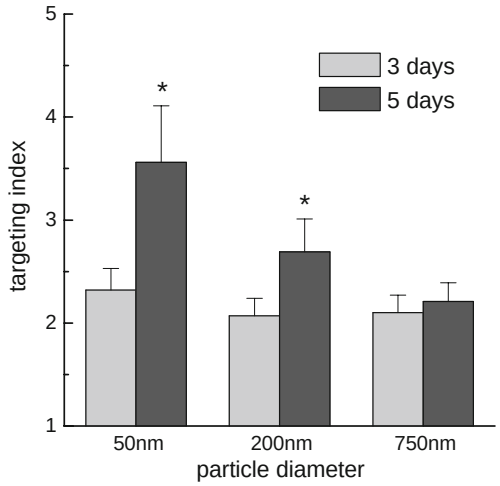

Fig. 5. Targeting indices for the different particle diameters after quantitative determination of fluorescence signals from the resected gastric tissues.

DISCUSSION

A size-dependent particle deposition in the gastrointestinal tract of healthy subjects was reported from diverse studies (15–17) as well as bioadhesion depending on the particle surface properties (18). However, data on adhesion phenomena in ulcerated gastric tissue are currently not available. One related study discovered the specific adhesion of small particles in ulcerated regions of inflamed tissues of the gastrointestinal tract (4). Smaller particles were found to be more adhesive which underlines the interest in testing nanoparticles for their adhesion in this study here.

Two principal mechanisms have been found for the particle adhesion in the ulcerated tissues. First, small particles are taken up more easily by immune related cells, namely macrophages in the area of active inflammation. Particles in the micrometer range, which have been reported to be taken up by macrophages less effectively (3), were defined as the upper limit, thus concentrating here on nanoscale particles.

Second, the strong mucus production in the gastrointestinal tract, especially in the stomach favors particle adhesion to the mucus. Smaller particles can better attach to mucus layers due to their relatively small mass. Besides, inflammation state in the gastrointestinal tract comes with an increased mucus production and results in a higher quantity of particle attachment. The relatively high turnover of the mucus explains the rather limited amount of particles adhering and remaining in the healthy stomach in this experimental setup after 24 h.

Since generally the mucus layer is thicker in the stomach than in other intestinal regions, the general level of particle adhesion from the healthy group is high. Differences from ulcerated tissues to the healthy control are therefore less visible due to the generally enhanced adhesion phenomena to the thick mucus layer. In consequence, experimental therapy requires a certain duration in order to visualize the selectivity of adhesion.

Another aspect to discuss is that polystyrene nanoparticles used in this study are hydrophobic and have an negative zeta potential. As it was described recently (19) such particles are rather sticking to the mucus than diffusing across. It might be suggested that ulcerated regions have a lower mucus amount or a different mucus density allowing for a modified penetration pattern. This could be due to the fact that ulcerated tissue areas do not have significant mucus production due to the eroded tissue area, subsequently allowing the penetration of a distinct number of particles. However, surface properties having a significant influence of the mucoadhesion and penetration still need to be analyzed.

The mucoadhesion of nanoparticles occurring in healthy as well as in tissues with ulcerations, thus assimilating the level of adhesion to some extent is limiting the selectivity of the particle deposition and appears therefore to be the major limiting factor of this therapeutic approach. Consequently the selectivity is less expressed in gastric ulcer targeting compared the experimental colitis therapy based on particulate systems (4,5). In the healthy colonic tissue the mucus level is relatively low, alterations in mucus amount and turnover by the inflammation state have a greater impact on particles adherence (20).

Charge interactions are reported to further enhance binding to the ulcerated tissue, especially in the stomach (21). Negatively charged particles may adhere more readily, since it has been reported that ulcerated tissues contain high concentrations of positively charged proteins that increase the affinity to negatively charged substances. This is furthermore supported by the observation of the slight non-significant increase of particle adhesion in the non-ulcerated regions of gastric tissues in the ulcer group. Thus, this could be another reason for the enhanced attachment to the inflamed mucus areas, it needs however to be clarified whether the particles exhibit significant negative charges also under *in-vivo* conditions.

An optimal particle size for the design of a drug delivery system in this context seems to be difficult. Indeed, the smallest diameter leads to the highest adhesion however, particle size reduction also enhances premature drug leakage during the gastrointestinal transit, especially in the nanometer range (22). While hydrophilic drugs are rather released immediately from nanoparticles, the encapsulation of lipophilic drugs into small particles allows for a more sustained release of the drug due to their interaction with the polymer matrix. However, at particle diameters as low as 50 nm, these principal strategies of encapsulation appear to be inefficient. This still will remain a major challenge which needs to be solved, although recent particle design approaches appear to be promising for overcoming this problem of premature drug leakage (23,24). Another issue is that the small particles can upon their uptake and transport in the body built the basis for toxicological incidents. However, due to the possible use of biocompatible and biodegradable polymers for the design of such delivery systems the risk may be considered as low.

Beside the therapeutic relevance of this outcome, there is another aspect which may be discussed in this context. In line with results from earlier studies on inflammatory bowel disease where nanoparticles selectively adhered to inflamed tissue (4) we observe similarly, the adhesion of particles to the ulcerated gastric tissue.

Consequently, it seems likely that there exists a phenomenon equivalent to the 'Enhanced Permeability and Retention' effect (EPR), the passive targeting after intravenous administration based on the increased endothelial permeability in inflamed tissues and tumors. Such an increased permeability and the subsequent particle adhesion was observed here similarly for the gastric epithelium as well as in other studies on the inflamed epithelial tissue of the lower intestine (4,6).

Thus, the existence of an epithelial 'Enhanced Permeability and Retention' effect (epEPR), passively targeting towards inflamed and ulcerated tissues in the gastrointestinal tract, could be concluded.

CONCLUSIONS

Polymeric particulate carrier systems proved *in-vivo*, to be a promising approach to the selective bioadhesion to inflamed areas in gastric ulcers. The particle deposition was significantly increased in mice suffering from gastric ulcers compared to the equivalent tissue areas of a healthy control group, although effects were less dramatic than those observed in colitis. Size dependent deposition of particles in gastric inflamed tissue could be of particular importance in the design of new carrier systems for the chronic and resistant *H. pylori* infection where local drug delivery might contribute an essential plus.

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