Bioadhesion by Means of Specific Binding of Tomato Lectin

Claus-Michael Lehr,[1,2] Joke A. Bouwstra,[1] Wouter Kok,[1] Arthur B. J. Noach,[1] Albertus G. de Boer,[1] and Hans E. Junginger[1]

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The possibility of developing bioadhesive drug delivery systems on the basis of molecules which selectively bind to the small intestinal epithelium by specific, receptor-mediated mechanisms was investigated using a lectin isolated from tomato fruits (*Lycopersicum esculentum*). The tomato lectin (TL) was found to bind specifically onto both isolated, fixed pig enterocytes and monolayers of human Caco-2 cell cultures with a similar affinity. TL-coated polystyrene microspheres (0.98 μm) also showed specific binding to enterocytes *in vitro*. Lectin binding was found to be favored at neutral pH and to be reduced in an acidic environment. Crude pig gastric mucin, however showed a marked cross-reactivity *in vitro*, indicating that lectin binding to the cell surface *in vivo* might be inhibited by mucus.

KEY WORDS: bioadhesion; Caco-2 cells; pig enterocytes; microspheres; specific binding; tomato lectin.

INTRODUCTION

Most approaches in developing bioadhesive drug delivery systems for oral application are based on the use of mucoadhesive polymers (1,2). This concept, however, suffers from two disadvantages.

(i) Mucoadhesion relies on general physicochemical interactions (3), in which nonspecific surface energy thermodynamics play an important role (4,5). Surface properties of the two adhering phases involved—mucus gel layer and mucoadhesive hydrogels—may change in response to the composition of the interstitial liquid phase, i.e., the luminal fluid of the GI tract. The latter again might vary considerably in its colligative properties, such as pH, surface tension, ionic strength, etc., in both normal and pathological circumstances. Furthermore, as adhesion of mucoadhesive polymers is not selective for mucosal surfaces but may occur rather ubiquitously, interactions with food or other contents of the GI tract might inactivate those systems before they can reach their intended site of adhesion.

(ii) Although bioadhesion should provide for direct contact with the mucosal epithelium, mucoadhesion provides only contact with the mucus gel layer which covers the mucosal tissue surface of the GI tract. As the mucus gel layer is permanently renewed at a considerable turnover rate (6), the duration of bio(muco)adhesion is limited to a few hours only. This retention, however, appeared to be too short to delay or even control the GI transit of mucoadhesive drug delivery systems (7).

The use of bioadhesion in drug delivery might be enhanced if it can be achieved by means of *specific, receptor-mediated interactions,* which permit binding directly onto the mucosal cell surface rather than onto mucus. Fixation of foreign bodies, such as, e.g., bacteria, within the intestines of mammals does occur, and the ability to adhere is at least an advantage, if not a prerequisite, for bacterial infectivity (8). To account for these adhesion phenomena, physicochemical models, relying on van der Waals forces, Hamaker constants, free surface energy, DLVO theory, etc., have been proposed initially (9). However, there is growing evidence for specific effects (structural and chemical) in the context of bacterial adhesion (10). Specific surface recognition molecules expressed by adhesive bacteria—so-called adhesins—play an important role in this process (8). These can be associated with large polymeric structures in the form of filamentous appendages, the so-called fimbriae. Complementary structures on the host cell surface represent the receptors with which the bacteria ligands are able to interact. It is possible that bioadhesive structures are able to attach drugs or delivery systems onto the glycocalyx of mucosal cells within the human GI tract.

To be used for drug delivery purposes, candidate bioadhesive structures have to fulfill the following requirements: (i) be nontoxic and nonimmunogenic; (ii) be stable under the conditions of the GI tract; and (iii) bind specifically to surface structures of absorptive epithelial cells but—preferably—not to mucus.

Bioadhesive structures might be found among bacterial adhesins (11), synthetic polymers (12), or plant lectins. Especially with respect to safety aspects, the tomato (*Lycopersicon esculentum*) lectin (TL) is an interesting candidate, as it has been reported by Kilpatrick *et al.* (13) to bind to rat intestinal villi without inducing deleterious effects and to the rat duodenum after a concentrated lectin diet for 10 days. Further, the widespread consumption of raw tomatoes indicates the low toxicity potential of TL. The average ingestion of TL has been estimated from the tomato consumption in the United States to range between 100 and 200 mg a year. Exposure of some individuals might be considerably higher (14). The first studies toward an application of TL for drug delivery have been described by Woodley and Naisbett (15). The lectin showed adhesion to everted rat intestinal rings *in vitro* which could be specifically inhibited by tetra-(*N*-acetylglucosamine), $(GluNAc)_4$. After intragastric administration to rats, the GI transit of the radiolabeled lectin was slightly different from that of both a nonabsorbable (polyvinylpyrrolidone) and a proteinaceous (bovine serum albumin) radiolabeled control. Additional studies with rat everted intestinal sacs *in vitro* (16) demonstrated a significantly increased uptake of the lectin in comparison to the same controls as used in the transit studies. Uptake of TL was saturable and inhibited at 4°C, which suggested the mechanism of absorption to be adsorptive endocytosis. After oral administration, mice developed a measurable immune re-

[1] Center for Bio-Pharmaceutical Sciences, Leiden University, Leiden, The Netherlands.

[2] To whom correspondence should be addressed at University of Southern California, School of Pharmacy, 1985 Zonal Avenue, PSC 704, Los Angeles, California 90033.

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sponse against the entire lectin, suggesting absorption of the intact molecule.

The aim of this study was to investigate further the reported adhesion of TL to the intestinal mucosa, with emphasis on those aspects recognized as disadvantages of nonspecific bio(muco)adhesion of polymeric hydrogels. For that purpose, lectin binding to the aimed target cells, the enterocytes, was studied quantitatively.

MATERIALS AND METHODS

Purification of Tomato Lectin

TL was isolated from the locular fluid of ripe beef tomatoes by chromatofocusing using Polybuffer Exchanger and Polybuffer 96 (Pharmacia, Woerden, The Netherlands) as described by Kilpatrick *et al.* (17). To remove macromolecular impurities which might otherwise affect the running of the column, a fractionated $NH_4(SO_4)_2$ precipitation was performed first. The precipitates between 25 and 75% saturation showed the highest lectin activity and were pooled. Macromolecular buffer components (ampholytes) from the focusing buffer were removed by gel filtration over a Sephadex G100 column. Lectin activity was assayed by determining the hemagglutination titer using fresh human erythrocytes as described by Kilpatrick and Yeoman (18).

The purity of the obtained lectin was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, Protean-mini II, Bio-Rad, Veenendaal, NL) on freshly cast 10% gels run for 30 min at 200 V. Marker proteins in a range from 14 to 97 kDa (Bio-Rad) and purchased TL (Sigma, St. Louis, MO) were used as references.

Radiolabeling

A 100-μl aliquot of TL (10 mg/ml) in 50 m*M* phosphate-buffered saline (PBS; pH 7.4) was incubated with 0.5 mCi $Na^{125}I$ (IMS-30, Amersham, Buckinghamshire, GB) for 15 min at room temperature in an Eppendorf cup coated with 1 mg Iodogen (Sigma). Free label was removed using a disposable desalting column (Econo-Pac 10DG, Bio-Rad). Specific radioactivity of the labeled lectin was about 40,000 cpm/μg. Labeling efficiency was about 25%. Hemagglutination titer of the iodinated lectin was not reduced.

Isolation of Pig Enterocytes

Porcine small intestinal tissue was received from a local abbatoir and transported in oxygenated Krebs–Ringer buffer to the laboratory. A segment of about 10 cm was opened longitudinally and carefully rinsed with cold PBS. Enterocytes were isolated from the mucosa by incubating the tissue for 5 min in an isotonic 10 m*M* EDTA buffer as described by Knutton *et al.* (19). This method, originally developed for human intestinal tissue, worked well with porcine but not with rat intestinal tissue. Enterocytes were easily identified under the light microscope by their shape and size. After repeated washing and centrifugation (100*g*, 2 min), the isolated cells were fixed for 1 hr in 2% (v/v) glutaraldehyde/PBS. Fixed cells could be stored at 4°C in PBS (containing 0.2% NaN_3 as preservative) for several weeks and used for binding studies. No morphological changes were observed by light-microscopical inspection.

Enterocyte Radiobinding Assay (ERBA)

Binding of TL on pig enterocytes was quantified by incubating 50 μl of an enterocyte suspension (~10^6 cells/ml) in PBS containing 0.5% bovine serum albumin (BSA; Fraction V, Sigma) with 50 μl TL at varying concentrations, spiked with radiolabeled tracer. Incubation was carried out in 4 × 1-cm polystyrene RIA tubes (Greiner, Alphen a/d Rijn, NL) at room temperature. After 1 hr, 2 ml PBS was added, the cells were spun down (100*g* for 2 min), and the supernatant was decanted. Cells were washed one more time following the same procedure, which was found to be sufficient to remove all unbound radioactivity. Bound radioactivity was counted. Total binding was determined in triplicate for each TL concentration. Nonspecific binding was determined by incubation after adding 10 μl of a 10 m*M* $(GluNAc)_4$ solution in PBS to one RIA tube per each TL concentration. Cellular protein in aliquots of the enterocyte suspension without TL was determined by Coomassie assay.

pH Dependence and Mucus Cross-Reactivity

Aqueous buffer solutions were prepared according to the monograph "standard buffer solutions" in the USP XXI. For pH 2.0 to 5.8, a 0.2 *M* phthalate buffer was used; for pH 5.8 to 8.0, a 0.2 *M* phosphate buffer. All solutions were made isotonic by adding the necessary amount of glucose.

Crude pig gastric mucin was purchased from Sigma. To study the inhibition of TL binding onto enterocytes, ERBA was performed in the presence of various amounts of mucin.

Lectin Binding to Monolayers of Human Colon Carcinoma (Caco-2) Cells

Caco-2 cells, originating from a human colorectal carcinoma line, were obtained as a gift from Dr. G. van Meer (Department of Cellular Biology, Utrecht University Hospital, NL). Cells of passage number 78 were grown in sterile flat-bottom 96-well plates (Greiner) in 200 μl/well Dulbecco's modified Eagle's medium, containing 10% (w/v) fetal calf serum, 1% (w/v) nonessential amino acids, benzylpenicillin (100 U/ml), and streptomycin (100 μg/ml). All reagents were received from Sigma. Cultures were maintained in a humidified atmosphere (5% CO_2) at 37°C. The medium was changed every second day. After 2 weeks, microscopic inspection revealed that monolayers had been formed on the bottom of the wells. Binding studies were performed at room temperature by incubating the cells for 1 hr with 100 μl/well culture medium containing TL at given concentrations, spiked with traces of radiolabeled TL. After 3× washing, the cells were solubilized with 5 *M* KOH for gamma-counting and protein assay, respectively. Binding at each TL concentration was determined in triplicate. Nonspecific binding was determined in the presence of 1 m*M* $(GluNAc)_4$. One week later, the experiment was repeated with cells of the same growth which had been maintained in culture in another 96-well plate.

Lectin Coating of Microspheres

Fluorescently labeled polystyrene microspheres (0.98 μm, Polysciences, St. Goar, D) were coated with TL by passive adsorption according to the manufacturer's instructions. A 30-μl aliquot of a TL solution (1 mg/ml) was added to 0.5 ml of a 2.5% (m/v) microspheres suspension in 0.1 *M* borate buffer (pH 8.5) and incubated overnight under continuous slow mixing. Nonspecific binding sites were blocked with BSA. The presence of biologically active lectin on the bead surface was checked by hemagglutination assay after repeated washing. Control microspheres were coated with BSA to block nonspecific adsorption of cellular proteins.

RESULTS

Lectin Purification

Lectin purification by chromatofocusing was feasible, but substantial amounts of TL remained bound to the polybuffer exchanger. Because of the unusually basic isoelectric point of the TL [8.8 and 10.0 as reported for two isolectins (14)], the lectin was eluted with the first fractions of the polybuffer. Hence, the separation had more in common with simple ion-exchange chromatography than with chromatofocusing. The separation of two isolectins as reported by Kilpatrick *et al.* (17) could not be reproduced. A second disadvantage of this method is the contamination of the lectin with the ampholytes from the polybuffer. These could not be removed by dialysis and made a second chromatographic step necessary. Achieved yields were between 10 and 20 mg TL from 6-kg beef tomatoes. Considerably higher yields, however, might be possible if more effective purification methods were available.

On a Coomassie stained gel, the isolated lectin appeared as a single band of the same molecular weight as the commercial reference lectin (Fig. 1). After SDS gel electrophoresis, TL was reproducibly found above the 97-kDa marker protein, even when the gel was run for 16 hr at 15 V. In contrast, Kilpatrick *et al.* (17) reported an apparent molecular weight of about 68,000 for TL from gel electrophoresis. A more reliable estimate for its molecular weight seems to be the value of 71,000 as determined by sedimentation equilibrium ultracentrifugation by Nachbar *et al.* (14).

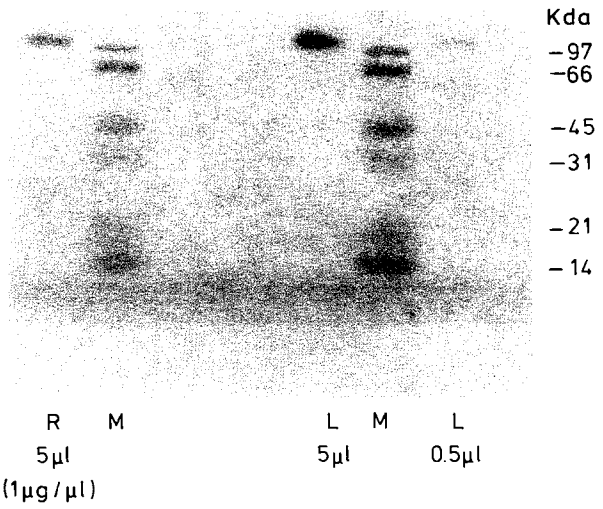

Fig. 1. SDS gel of tomato lectin isolated by chromatofocusing (L). R, commercial reference tomato lectin (Sigma); M, molecular weight markers as indicated on the right.

Lectin Binding to Isolated Pig Enterocytes and Monolayers of Human CaCo-2 Cells

Binding of TL to fixed pig enterocytes was found to be saturable and could be specifically inhibited by $(GluNAc)_4$. Equilibrium of the binding was reached after 15–30 min and did not change when incubation was prolonged up to 4 hr (not shown). Therefore an incubation period of 1 hr was chosen for all quantitative binding studies. Bound activity at the saturation maximum was between 1 and 5% of the total activity, which by approximation could be assumed to be equal to the free activity. Total binding was calculated as the mean of the triplicate determination per each TL concentration. Nonspecific binding in the presence of 1 m*M* $(GluNAc)_4$ was calculated by linear regression of the single data points. Specific binding was obtained as the difference between average total and nonspecific binding. Under the assumption of a one ligand-one receptor model, the data were fitted to the model equation (1) by nonlinear regression (Statgrafics, V 2.6, STSC Inc., Rockville, MD).

$$B = \frac{B_{max} \cdot F}{F + K_d} \quad (1)$$

In Eq. (1), F is the concentration of free lectin (μg/ml), and B the amount of lectin bound per mass unit cellular protein (μg/mg). K_d is an apparent affinity constant, indicating the lectin concentration at which 50% of the binding places are occupied. Figure 2 shows the data from one representative single experiment, which demonstrate that this model provides a reasonable fit. Table I shows the calculated model parameters assuming a molecular weight of 71,000 for TL (14). The data represent an average from four independent experiments with pig enterocytes. One experiment was performed with fresh enterocytes prior to fixation. Viability of cells about 2 hr after isolation was approximately 50% as estimated from trypan blue staining. Because binding did not appear to be significantly changed by fixation, cells were

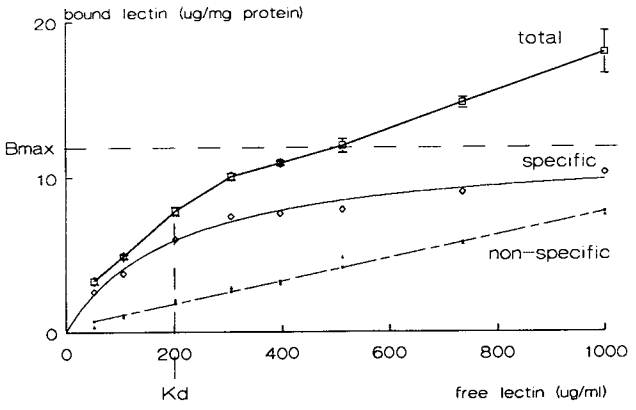

Fig. 2. Total, nonspecific and specific binding of TL to isolated pig enterocytes as found in one representative experiment. Data for specific binding were fitted according to Eq. (1).

Table I. Affinity Constants k_d and Lectin Binding Capacity B_{max} for Binding of TL to Isolated, Fixed Pig Enterocytes and Monolayers of Human Caco-2 Cells (Mean ± SD; n = 4)

	k_d (10^{-6} M)	B_{max} (10^{-9} mol/mg cell protein)
Islated, fixed pig enterocytes	4.0 ± 1.0	166 ± 18
Monolayers of human CaCo-2 cells	3.0 ± 1.2	54 ± 11

routinely fixed for all other experiments. As the protein content of the enterocytes was about 15 mg/10^7 cells, the total number of lectin molecules maximally bound per cell was about 1.5×10^7. For lectin binding to monolayers of human Caco-2 cells, affinity was comparable, but binding capacity was about three times reduced. There was no significant difference in binding in cells kept in culture for 2 or for 3 weeks.

pH Dependence of Lectin Binding and Cross-Reactivity with Mucus

The effect of pH on lectin binding was studied by ERBA at a lectin concentration of 250 μg/ml ($\sim K_d$). Figure 3 shows the percentage of total and specific binding in the pH range between 2 and 8 (mean ± SD; N = 3). Specific binding in PBS (pH 7.4) was used as a reference value and assumed to be 100%. Binding was favored at neutral pH and considerably decreased in an acidic milieu. There was, however, also an effect of the qualitative composition of the two buffer systems used, as indicated by the discrepancy in binding at the overlapping extrema at pH 5.8.

Cross-reactivity with pig gastric mucin could be estimated from an indirect (logit-log) Hill plot of the data according to Eq. (2). B_I is the amount of lectin specifically bound in presence of mucin at concentration I, and B_0 is the amount of lectin specifically bound without mucin. $IC_{50\%}$ is the mucin concentration which inhibits specific binding by 50% at equilibrium and can be determined from the plot as the intercept on the abscissa (20).

$$\log\left[\frac{B_I/B_0}{1 - B_I/B_0}\right] = -n \cdot \log I + n \cdot \log IC_{50} \qquad (2)$$

A plot of the data from two independent experiments (each in triplicate) is shown in Fig. 4. The Hill coefficient or "slope factor" $-n$ was 0.70 ± 0.05 (mean ± SE), i.e., slightly different from unity. This indicates that mucus inhibition of lectin binding is complex. Possibly, the effect of mucin on the viscosity of the test medium also affected the binding studies. Higher mucin concentrations could not be studied, as the system tended to gel. Nonetheless, IC_{50} could be estimated to be approximately 2.2 mg/ml mucin.

Bioadhesive Properties of Lectin-Coated Microspheres

Lectin-coated microspheres spontaneously adhered to enterocytes (PBS; pH 7.4) and resisted repeated washing and centrifugation as visible under the fluorescence microscope (Fig. 5). The insert shows a single enterocyte with adhering microspheres at higher magnification. In contrast, control-microspheres coated with BSA did not adhere (Fig. 6). The total number of microspheres visible in the preparation is decreased, as most of them were removed by washing. Binding of lectin-coated microspheres was shown to be specific by inhibition with 1 mM $(GluNAc)_4$ (Fig. 7). Binding was also clearly reduced, however, in the presence of 0.2 mg/ml pig gastric mucin (Fig. 8). Remarkably, the isolated cells showed a tendency to aggregate in such a medium.

DISCUSSION

The observed adhesion of fluorescence-labeled polystyrene microspheres to pig enterocytes *in vitro* shows that, in principle, TL can impart specific bioadhesive properties to a potential drug delivery system.

The developed ERBA technique offers the possibility to quantify lectin binding onto a relevant substrate. Both affinity and capacity for TL binding on fixed enterocytes are of the same scale as previously reported by Feller *et al.* (21) for a low-affinity, high-capacity receptor for concanavalin A (ConA) on human fibroblasts. TL binding to both pig small intestinal and human Caco-2 cells showed the same affinity, indicative of a common lectin receptor. Further, for the lec-

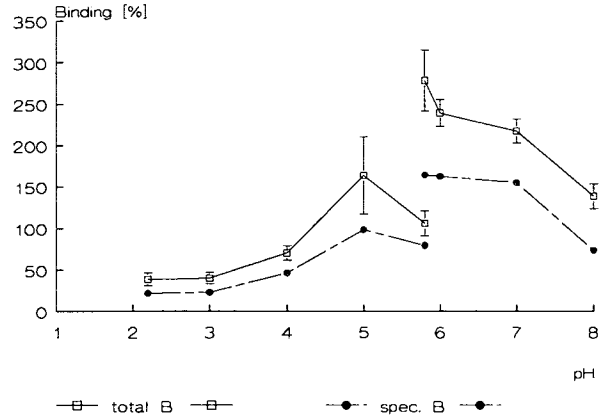

Fig. 3. pH dependence of TL binding to isolated pig enterocytes in two different buffer systems (see text). Reference was specific binding as measured in PBS (pH 7.4), set equal to 100%.

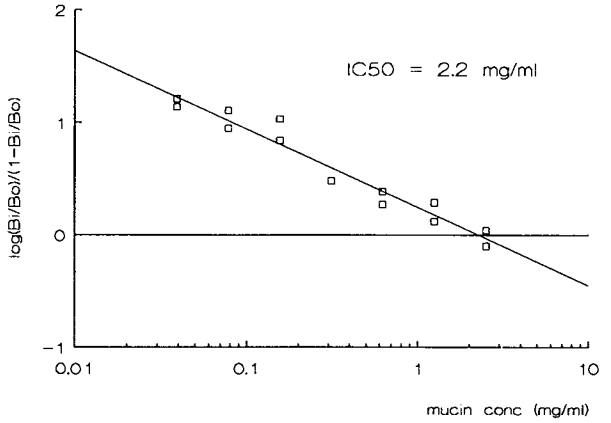

Fig. 4. Indirect Hill (logit-log) plot of specific TL binding to pig enterocytes inhibited by various concentrations of pig crude gastric mucin (pooled data from two experiments).

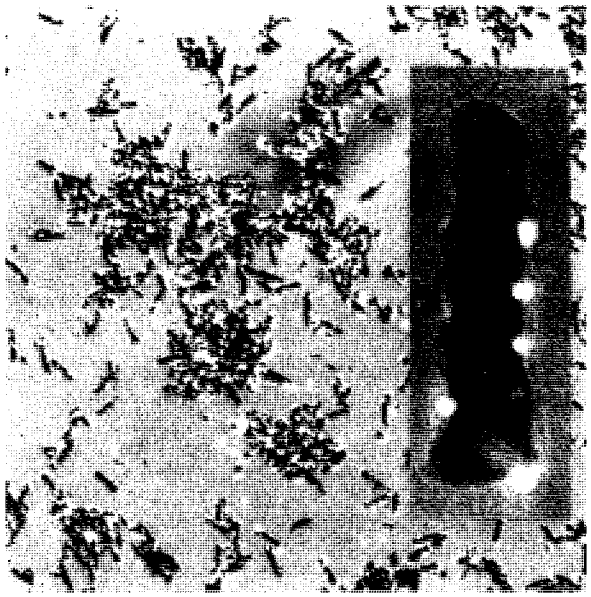

Fig. 5. TL-coated, fluorescence-labeled polystyrene microspheres adhere to isolated, fixed pig enterocytes. Inset shows a single enterocyte at a higher magnification.

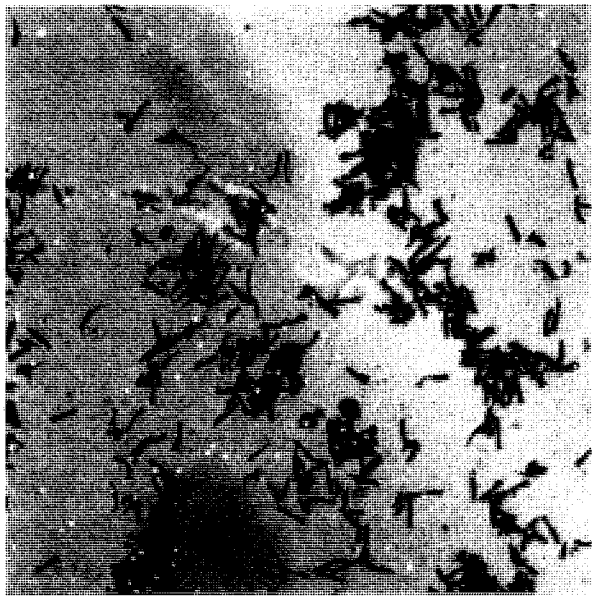

Fig. 7. Binding of TL-coated microspheres is inhibited by 1 m*M* $(GluNAc)_4$.

tin binding it did not matter whether the cells were living or dead, fixed or not fixed. Binding capacity of cultured Caco-2 cells was clearly reduced in comparison to loosely suspended pig enterocytes. Caco-2 cells are known to differentiate spontaneously in culture into polarized, columnar cells and to form confluent monolayers which structurally and functionally resemble the small intestinal epithelium, with tight junctions (22). It is likely that, in these binding studies, the lectin had access only to the apical, and not to the basolateral, side of the Caco-2 cells, whereas the isolated pig enterocytes were freely accessible. Because the number of binding sites was increased with increasing surface exposure of the cells, the receptor is not assumed to be exclusively located at the brush border membrane. This is further supported by the localization of lectin-coated microspheres bound on enterocytes which did not show any preference for the brush border membrane.

The TL receptor on enterocytes is not yet identified. Blotting experiments with electrophoretically separated rat small intestinal brush border proteins reported by Naisbett and Woodley (23) demonstrated specific TL binding to many high molecular weight components. The existence of a second, high-affinity receptor saturable at lectin concentrations lower than 50 μg/ml is also possible. Such a receptor was

Fig. 6. Microspheres coated with BSA do not adhere.

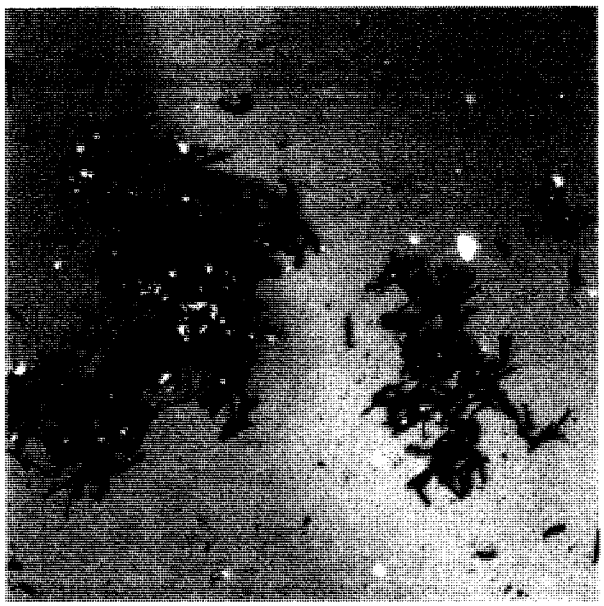

Fig. 8. Binding of TL-coated microspheres is also reduced in presence of 0.2 mg/ml crude pig gastric mucin.

identified by Feller *et al.* (21) for ConA on human fibroblasts. The observed saturation maximum of lectin uptake into everted rat gut sacs at a concentration of 15 μg/ml (24) suggests yet another, high-affinity receptor for TL on intestinal cells.

Recently, Callaghan *et al.* (25) reported the identification of a TL-binding 60–90 × 10^3 M_r glycoprotein from rat, dog, and pig gastric cell membranes, which was localized on the luminal face of tubulovesicles and canaliculi of parietal cells, involved in the gastric acid production. Additional structural investigations revealed a 35 × 10^3 M_r core protein, glycosylated with lectin binding *N*-glycans. Although this receptor glycoprotein is not likely to be the same one that provides binding of TL to enterocytes, these authors also noted strong lectin binding to mucous cells and mucins. Mucin cross-reactivity was evident in this study both from ERBA and from mucin-inhibited binding of lectin-coated microspheres. An indirect Hill plot of the data from the inhibition experiments indicated complex interactions; in a mucin-containing medium, the isolated enterocytes tended to aggregate. Both indicate that the inhibition of TL binding by mucus glycoproteins also involves nonspecific components, such as viscosity effects. Conclusions about the practical relevance of this cross-reactivity with mucus for the application of TL in bioadhesive drug delivery systems will be possible only after additional experiments *in vivo*. It is likely, however, that TL will act—although by specific mechanisms—as a mucoadhesive, i.e., it will not bind exclusively to the cell surface. However, TL has been demonstrated to be absorbed from the GI tract *in vivo* (13,24), which proves that it must have reached and crossed the mucosa.

In spite of the identified mucus cross-reactivity, TL still possesses some features which make it interesting for drug delivery purposes. Apart from possible toxic side effects, the application of many other lectins *in vivo* appears a priori to be restricted by the interaction with common monosaccharides in food carbohydrates. This could also be a problem for the application of mannose-sensitive bacterial adhesins recently reported by Caston *et al.* (11). Although experimental data are lacking, specific food interactions with TL can be expected to be less pronounced, as poly-(*N*-acetylglucosamines) are relatively scarce in food. Another advantage of TL in comparison to the "classic" mucoadhesive polymer Polycarbophil is its favored binding at neutral pH, which makes TL better suited for small intestinal applications. The reduced bioadhesiveness in an acidic milieu is one possible explanation for the modest effects on GI transit after intragastric administration of TL reported earlier (15). Further possibilities, other than bioadhesion alone, however, might arise if the endocytotic uptake of this lectin can be tapped for drug delivery in future.

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