Bioadhesion of Lectin-Latex Conjugates to Rat Intestinal Mucosa

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Purpose. The specific interactions between three lectin-latex conjugates and different structures of rat intestinal mucosa have been studied *ex vivo*.

Methods. These systems were prepared by covalent coupling of different ligands, i.e., tomato lectin (TL), asparagus pea lectin (AL), *mycoplasma gallisepticum* lectin (ML), and bovine serum albumin (BSA) as control, to poly(styrene) latexes.

Results. Using mucosa samples without Peyer's patches (PP), the extent of interaction of all three lectin-latex conjugates with the mucosa decreased from duodenum to ileum, probably due to progressive diminution of the mucin concentration along the gastrointestinal tract. The following order of interaction of the conjugates with the mucus gel layer was observed: TL > ML = AL ($p < 0.05$). For each lectin, these results corresponded well to the concentration of its specific sugar in the mucus. Using intestinal samples with PP, an important increase of interaction of the conjugates with the mucosa was found for ML (about 25%) and AL (about 50%), whereas the interaction of TL decreased about 25%.

Conclusions. Photomicrographs with fluorescent latexes have confirmed the specificity of the ML- and AL-latex conjugates for the PP region and of the TL-latex conjugates for the mucus gel.

KEY WORDS: lectin; bioadhesion; mucin; Peyer's patches; nanoparticles.

INTRODUCTION

A possible approach to obtain a specific bioadhesive drug delivery system at intestinal level consists in coupling a nano- or microparticulate system as drug carrier to lectins (1). Lectins are proteins with the capacity to bind specifically to certain sugars, and therefore agglutinate cells and polysaccharides or glycoconjugates (2).

In fact, the binding of different lectins to intestinal villi has been reported (3–5). Binding is usually not homogeneous because the surface glyconconjugate expression differs along the gastro intestinal tract (6). Similarly, bacterial adhesins may be used for obtaining specific adherence of a drug delivery system, since some microorganisms colonize epithelial surfaces. This may be related to their ability to bind specifically to these surfaces (7). Generally, it was found that this attachment was mediated by bacterial cell surface appendages, such as fimbria, pili or flagella (8), i.e., bacterial adhesins, with specific carbohydrate-binding properties. For example, *Escherichia Coli* was reported to adhere to the lymphoid follicle epithelium of ileal Peyer's patches in rabbits (9) and *Vibrio cholerae* to the rabbit intestinal brush border membrane by a fucose-sensitive mechanism (10). Moreover, different Staphylococci were observed to adhere to the mucous gel layer but not to apparently mucin-free mucosae (11).

Recently, the use of tomato lectin (specific for N-acetylglucosamine) has been reported for increasing bioadhesion (12, 13). It was of interest to investigate the behaviour of lectins exhibiting other sugar specificities, including plant lectins (i.e., *Lotus tetragonolobus* lectin which is specific for fucose), and bacterial lectins (i.e., *Mycoplasma gallisepticum* lectin specific for sialic acid). Thus, it was the aim of this study to test the specificity of bioadhesion of three different lectin-latex conjugates to various structures of intestinal mucosa *ex vivo*, in order to predict their potential for drug delivery at specific sites of the intestine.

MATERIALS AND METHODS

Chemicals

Lycopersicon esculentum lectin (tomato lectin, TL), *Lotus tetragonolobus* lectin (asparagus pea lectin, AL), *Mycoplasma gallisepticum* lectin (bacterial adhesin, ML), bovine serum albumin (BSA), crude pig gastric mucin (PGM), α-L-fucose, type IV chitin and N-acetylneuraminic acid (sialic acid) were purchased from Sigma (St. Quentin-Fallavier, France).

Amino poly(styrene) latexes (Polybead® amino microspheres) with a size of 750 ± 6 nm (PAM-750), and fluorescent carboxylated poly(styrene) latexes (Fluoresbrite® carboxylate YG microspheres; YG = fluorescein dye) with a size of 830 ± 6 nm (FCM-1000) were obtained from Polyscience Inc. (Eppelheim, Germany). These latexes were chosen as a nanoparticulate model because of their well described physico-chemical properties. All other chemicals used were of reagent grade.

Preparation and Characterization of Lectin-Latex Conjugates

Two types of covalent coupling methods for the preparation of the lectin-latex conjugates were used (1): the carbodiimide activation for the carboxylated latex (FCM-1000) and a two-stages glutaraldehyde activation for the amino latex (PAM-750). Briefly, for the carbodiimide activation, 20 mg of 1-(3-dimethyl aminopropyl)-3-ethyl carbodiimide hydrochloride were added to 12.5 mg carboxylate latex (FCM-1000) in PBS, and the suspension was stirred for 3.5 hours. For the glutaraldehyde activation, 2 mL of a solution with 8% glutaraldehyde in PBS were dropped to the washed amino latex and stirred for 4 hours. After removing the exceeding activator the ligand (300 μg ML, AL, or BSA, 250 μg TL) was added, and the linkage was made by incubation overnight at room temperature. The conjugates were centrifuged to remove the exceeding ligand and then treated firstly with ethanolamine to block unreacted groups on the particles and, secondly, with a solution of BSA to block any remaining sites on the particles for non-specific protein binding. Finally, the conjugates were resuspended in 1 mL PBS with 10 mg/mL BSA as a stabilizer and 1 mg/mL sodium azide as a preservative.

The particle size of the latexes was measured by photon correlation spectroscopy on a Coulter® submicron particle analyzer N4MD (Coultronics, Margency, France). The latex con-

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centration was determined by turbidimetry (14) on a Perkin Elmer Lambda 5 spectrophotometer, and the amount of fixed protein (lectins and BSA) by HPLC analysis of the remaining bulk concentration of ligand after coupling (1).

Ex Vivo Studies on Rat Intestinal Mucosa

Fresh small intestine mucosa of sacrificed male Wistar rats (IFFA-Credo, Lyon, France) was excised, rinsed with physiological salt solution (NaCl 0.9%) and cut into segments of 5 cm length. Ten segments were prepared of the intestine of each animal: the first segment was taken from the duodenum, segments 2 to 4 were defined as jejunum and the last six segments as ileum. Two different groups of mucosa samples were prepared: segments (from duodenum, jejunum and ileum) without Peyer's patches (PP), i.e., situated between PP, and segments (from jejunum and ileum) only with one PP each, placed in the center of each sample.

Each segment was opened lengthwise along the mesentery, and the serosa side was glued onto a glass slide. An aluminium plate with a slit of 2 cm^2 in the center was then placed on the mucosa sample, as described earlier (16). The ligand-latex conjugates were diluted in PBS (composition: NaCl 8.0 g/L, KH_2PO_4 0.2 g/L, $Na_2HPO_4 \cdot 12\ H_2O$ 2.9 g/L, KCl 0.2 g/L; pH = 7.4; ionic strength 0.154 M) to a concentration of 10 μg of fixed ligand per mL of incubation medium, which represented a latex bulk concentration of about 0.9 g/L. Then, 1 mL of each ligand-latex conjugate was put into contact with the 2 cm^2 of intestinal mucosa for 30 minutes. The suspensions were then sucked off and the mucosa samples were rinsed with 5 mL physiological saline to eliminate the non-attached conjugates. Two types of investigation were prepared by this method.

Firstly, for the quantitative studies of adhesion, which were performed with the lectin-PAM-750 conjugates, the mucus layer including the adsorbed conjugates was scraped off the mucous membrane with a microspatule and dispersed in 10 mL of a solution with 1% sodium hydroxide and 2% sodium dodecyl sulphate. The samples were then sonicated during 2 hours and left overnight at room temperature until the mucus was completely dissolved. The amount of adhesion of the conjugates was then determined from turbidimetric assay at 450 nm (16).

Secondly, qualitative analysis of the specificity of adhesion of the lectin-latex conjugates for certain mucosa structures was made by means of fluorescence microscopy. Fluorescent ligand-FCM-1000 conjugates ($\lambda_{max.}$ excitation = 458 nm, $\lambda_{max.}$ emission = 540 nm) were put in contact with the mucosa as mentioned above. After sucking off the non-attached particles and rinsing the mucosa samples with 5 mL NaCl 0.9%, the different intestinal segments were analyzed with a LEITZ Diaplan Microscope (Wetzlar, Germany) combined with an UV source. Photomicrographs were taken by means of a WILD MPS 45/51 Photoautomat (Heerbrugg, Switzerland).

RESULTS AND DISCUSSION

Lectin Fixation on the Latexes

The main physico-chemical characteristics of the ligand-latex conjugates are listed in Table I. The surface concentration of ligand was typically higher than 1.5 mg/m^2 and the coupling efficiency was 22–27% of the ligand bulk concentration. The quantity of adsorbed BSA, used to cover the remaining free particle surface (data not shown), ranged between 18.9 and 20.6 mg/m^2 for PAM-750 conjugates. For FCM-1000 conjugates, this concentration ranged between 18.9 and 21.0 mg/m^2.

Table I. Physico-chemical Parameters of the Ligand-Latex Conjugates (mean ± sd, n = 4): Particle Size, Latex Concentration, Concentration of Fixed Lectin (Difference Between Initial and Remaining Ligand Bulk Concentration) and Calculated Surface Concentration of Fixed Ligand Per Surface of Latex

	Particle Size (nm)	Latex Concentration (mg/mL)	Concentration of Fixed Ligand (μ/mL)	Surface Conc. of Ligand (mg/m^2)
Controls				
BSA-PAM-750	854 ± 51	6.32 ± 0.21	65.23 ± 1.54	1.54
BSA-FCM-1000	914 ± 59	6.60 ± 0.23	70.06 ± 2.33	1.70
Lectin-Latex Conjugates				
TL-PAM-750	846 ± 48	6.21 ± 0.30	70.02 ± 1.48	1.67
AL-PAM-750	826 ± 39	674 ± 0.27	71.43 ± 2.42	1.53
ML-PAM-750	837 ± 45	6.53 ± 0.19	76.11 ± 2.29	1.71
TL-FCM-1000	924 ± 47	6.82 ± 0.32	79.17 ± 1.49	1.88
AL-FCM-1000	930 ± 63	6.54 ± 0.21	75.47 ± 2.41	1.88
ML-FCM-1000	956 ± 71	6.79 ± 0.25	81.38 ± 2.59	2.01

The *in vitro* activity of these conjugates and their specificity for certain sugar moieties have been shown in a previous work (16). In this study, the different conjugates were incubated with pig gastric mucin (PGM) chosen as a model glycoprotein. Precipitated mucin-particles complexes were eliminated by centrifugation and the remaining PGM in the supernatant was determined by HPLC. Typically, the interactions between conjugates and PGM ranged between 10 and 14% of bulk mucin concentration. On the contrary, only 2% of PGM bulk concentration interacted with control conjugates. In order to verify that the specificity of lectins was maintained after covalent coupling, competitive inhibition studies were performed by incubating the AL- ML- and TL-conjugates with PGM in presence of a series of sugars including non specific sugars. Interactions expressed as % of the control were only 58%, 49.7% and 61.4% when AL-ML- and TL-latex were incubated with L-fucose (50 mM), sialic acid (20 mM) and a saturated chitin solution (poly N-acetylglucosamine), respectively. Interactions were always close to 100% of the control when the latex-conjugates were incubated with non specific sugars. Moreover, BSA control conjugates interactions with PGM were not influenced by the presence of sugars. Therefore, the respective sugar-specificities of the lectin-latex conjugates were preserved after covalent coupling to the particles.

Interactions Between Lectin-Latex Conjugates and Intestinal Mucosa Without Peyer's Patches

The amounts of ligand-PAM-750 conjugates, which have interacted with the intestinal mucosa samples without PP, are displayed in Figure 1. By comparing the different lectins tested, TL-PAM-750 conjugates showed always the higher interaction with all intestinal segments compared with AL- and ML-PAM-750 conjugates which had both similar results ($p < 0.05$). These observations agreed with the carbohydrate composition of

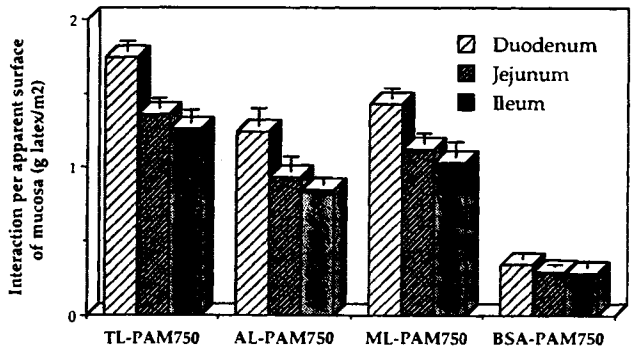

Fig. 1. Ligand-PAM750 conjugates interaction with intestinal segments without Peyer's patches. Control: BSA-PAM750 conjugates.

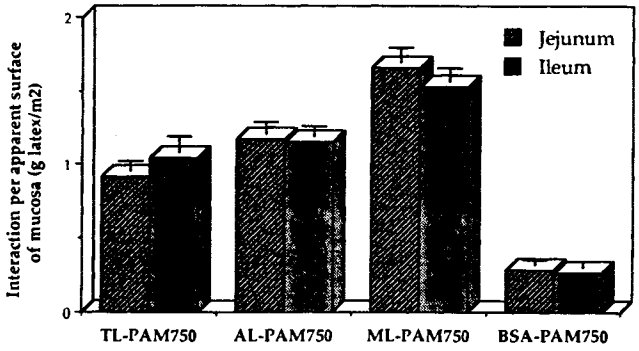

Fig. 3. Ligand-PAM750 conjugates interaction with intestinal segments with Peyer's patches. Control: BSA-PAM750 conjugates.

mucus. Intestinal mucus glycoproteins collected from proximal to distal intestine, i.e., duodenum to ileum, had the following sugar composition expressed in millimole per mole of mucin: 435 ± 10 for N-acetylglucosamine, 265 ± 64 for L-fucose, and 222 ± 26 for sialic acid (17).

For all lectin-latex conjugates, the extent of interaction decreased from duodenum to jejunum but no statistical differences were found between jejunum and ileum ($p < 0.05$). For the BSA-PAM-750 conjugates (used as control), no significant difference at all was observed ($p < 0.05$).

The decreasing amount of interaction of the three lectin-latex conjugates may be explained by the progressive diminution in the mucin concentration along the gastrointestinal tract (18). The greater amount of mucin glycoproteins in the upper regions of the small intestine provide a greater amount of receptors able to interact with the lectin-latex conjugates. This may also explain the presence of numerous particle aggregates which were found in duodenum by fluorescence microscopy (Figure 2) but not in jejunum and ileum (not shown).

Finally, the interaction of the BSA-PAM-750 conjugate (control) with the mucosa was always less than 30% of the interaction of the lectin-PAM-750 conjugates. As suggested by previous *in vitro* interaction studies with PGM (15), it was likely that bioadhesion provided by BSA conjugates was non-specific.

In conclusion, the most promising lectin to obtain specific bioadhesion to intestinal mucus glycoproteins appeared to be TL. It may be used for increasing the residence time of the drug delivery system at the mucous membrane, at least in the limits of the mucus turnover rate. There are two more reasons beside the specificity of TL that make it attractive for this purpose: non-toxicity and resistance to digestion in the gastrointestinal tract (3).

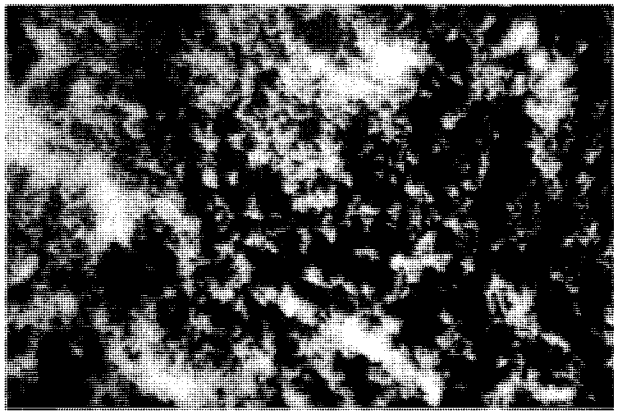

Fig. 2. Fluorescence photomicrograph of lectin-latex conjugates with intestinal segments without Peyer's patches: TL-FCM-1000 conjugates on mucus gel in duodenum. Original magnification: ×400.

Interactions Between Lectin-Latex Conjugates and Intestinal Mucosa with Peyer's Patches

Figure 3 shows the interactions of the ligand-PAM-750 conjugates with the mucosa samples having PP. The same as for mucosa without PP, no significant differences in the extent of interaction were found between jejunum and ileum ($p < 0.05$). When the interactions of lectin-PAM-750 conjugates with samples that have PP (Figure 3) were compared with samples without PP (Figure 1), a significant decrease of about 25% was observed for TL-latex conjugates, but an important increase of about 25% for AL-latex conjugates and 50% for ML-latex conjugates was found. No influence of the PP was found for BSA-PAM-750 conjugates (control). Therefore, specific binding of AL- and ML-latex conjugates to the PP region was calculated as the difference between the extent of interactions with and without PP. These results, which are listed in Table II, showed that the specific binding to the PP region was two times higher for the ML- than for the AL-latex conjugates.

The verification by fluorescence microscopy gave evidence of the specific binding of ML- and AL-latex conjugates to the PP region (Figures 4A and 4B, respectively), whereas TL-FCM-1000 conjugates hardly interacted with this intestinal portion (Figure 4C). On the contrary, the perimeter of the PP (without fluorescence) and the intestinal region (fluorescent) around the PP were clearly distinguished.

Table II. Extent of Specific Interaction of ML- and AL- Latex Conjugates with the Peyer's Patches of Jejunum and Ileum. Latex Per Apparent Surface of Mucosa (Calculated as the Difference Between Figure 1 and 3)

	Jejunum with PP (g/m^2)	Ileum with PP (g/m^2)
ML-PAM-750	0.537	0.505
AL-PAM-750	0.228	0.293

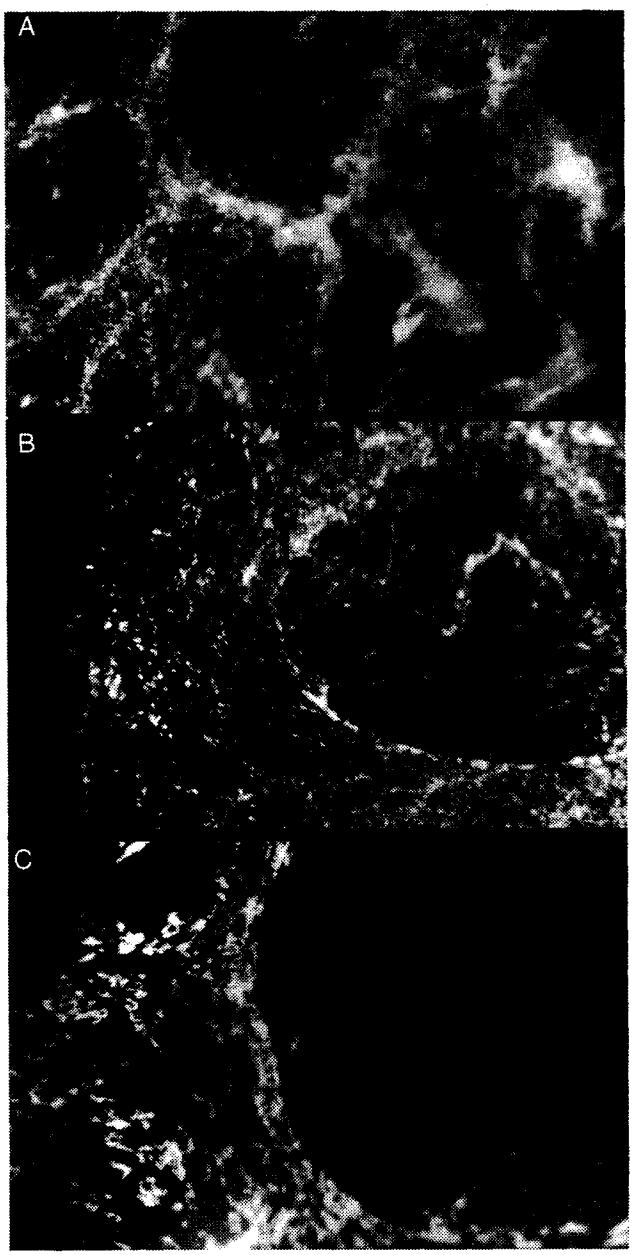

Fig. 4. Fluorescence photomicrographs of lectin-latex conjugates with intestinal segments with Peyer's patches: ML-FCM-1000 conjugates on Peyer's patch (PP) in jejunum (fig. 4A), AL-FCM-1000 conjugates on PP in ileum (fig. 4B) and TL-FCM-1000 conjugates uniformly arranged on mucus around PP in ileum (fig. 4C). Original magnification: ×400.

PP are aggregates of lymphoid follicles and they are mainly formed of absorptive cells termed M-cells but also some goblet cells, and occasionally tuft and enteroendocrine cells can be present (19). It was reported that PP and especially M-cells can be stained specifically by different lectins including peanut agglutinin (specific for sialic acid) (5) and *Ulex europaeus* agglutinin I (specific for L-fucose) (6). It is likely that the specific interactions observed with AL- and ML- conjugates are due to differences in glycoconjugate expressions either in local mucus glycoproteins or at the surface of M-cells. Taking into consideration the main function of the M-cells which consists in sampling macromolecules and antigens in order to activate the immunological response of the lymphatic system (20), the possibility of targeting the PP region by a drug delivery system becomes of interest.

CONCLUSIONS

On the one hand, this study demonstrated the specificity of the ML- and AL-latex conjugates for the PP regions. On the other hand, the TL-latex conjugates were much more specific for intestinal regions without PP. In drug therapy, the use of these lectins may increase the intestinal residence time of nanoparticulate drug delivery systems either at the site of absorption or action of a drug and thusly, improve the bioavailability and efficacy of the drug.

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