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Mannose Inhibition of *Escherichia coli* Adherence to Urinary Bladder Epithelium: Comparison with Yeast Agglutination

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Abstract. The adherence of piliated strains of *Escherichia coli* (*E. coli*) to mammalian epithelial cells has been reported by several investigators to be specifically inhibited by D(+)-mannose or its derivatives. Much of this work utilized mannose type compounds to inhibit agglutination of mannan containing yeast cells by *E. coli* to demonstrate mannose sensitivity. This report investigates the ability of the neotype strain of *E. coli* (which is sensitive to mannose inhibition of yeast cell agglutination) to bind and metabolize radiolabeled D(+)-mannose. In addition the relative efficacy of D(+)-mannose and heparin to inhibit the adherence of *E. coli* to rabbit bladder mucosa was compared. Results showed that although D(+)-mannose did block *E. coli* – yeast cell agglutination in a reversible manner, radiolabeled D(+)-mannose binding by *E. coli* could not be displaced by 1,000 fold excess unlabeled D(+)-mannose. This suggests uptake of the sugar as opposed to a surface binding phenomenon which was confirmed by the demonstration of significant metabolism of mannose by *E. coli*. The same concentration of D(+)-mannose which prevented *E. coli* – yeast cell agglutination was not particularly effective in preventing *E. coli* adherence to the acid denuded rabbit bladder. Heparin treatment of the acid denuded bladder was very effective in preventing *E. coli* adherence but was ineffective in preventing *E. coli* – yeast cell agglutination. This indicates that *E. coli* – yeast cell agglutination should not be correlated with *E. coli* adherence to mammalian epithelial tissue.

Key words: *E. coli* adherence, Mannose binding, Yeast agglutination, Urinary bladder, Urinary tract infection.

Introduction

Many workers have suggested that the adherence *Escherichia coli* (*E. coli*) and other strains of gram negative organisms with type 1 pili to mammalian epithelial cells is specifically inhibited by D(+)-mannose or its derivatives [1, 2, 5, 10, 11, 14]. The inference has been made that mannose residues on the surface of epithelial cells serve as receptors for the binding of these organism. Indeed, a mannose binding lectin on the surface of *E. coli* has been demonstrated and its amino acid composition has been determined [4].

In most instances the definition of "mannose binding" by *E. coli* has been equated with the ability of D(+)-mannose, mannan, or alpha methyl mannopyranoside to inhibit the agglutination of *E. coli* with yeast cells (which contain mannan on their cell surface [9, 11, 12, 15]). If a competitive mannose binding lectin on the surface of *E. coli* is responsible for its adherence to mammalian epithelial tissues then excess cold mannose should not only inhibit the agglutination of *E. coli* with yeast cells but should also prevent adherence of *E. coli* to epithelial cells.

In the present investigation the pharmacological characteristics of D(+)-mannose binding by *E. coli* was determined using the neotype strain of *E. coli* which is clearly mannose sensitive in the yeast cell agglutination model. D(+)-mannose metabolism by this *E. coli* strain was also determined. In addition the relative efficacy of D(+)-mannose and heparin to inhibit the adherence of *E. coli* to rabbit bladder mucosa was compared.

Methodology

Preparation of Bacteria

Lyophylized Neotype *E. coli* 11775 purchased from the American Type Culture Collection (ATCC) were rehydrated in sterile 0.9% NaCl and serially passed in static brainheart infusion broth (BHI; Difco Laboratories, Detroit, Michigan) at 37 °C. For adherence

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assays the organisms were labeled with ^3H. A 0.4% inoculum (v/v) was added to 4 ml of fresh BHI containing 5 μCi ^3H-Adenine (New England Nuclear, Boston, Massachusetts) per ml and incubated statically at 37 °C for 18–24 h. The bacteria were sedimented at 3,000 x g for 20 min and resuspended in their original volume of 0.9% NaCl. Colony forming units (CFU) were determined by making serial 10 fold dilutions in 0.05% Tween 80. A 0.025 ml of each dilution was placed on tripticase soy agar supplemented with 5% (v/v) yeast extract and incubated at 37 °C overnight. After incubation, the numbers of colonies were counted on plates containing 10 to 30 colonies per dilution and the number of colony forming units per ml of the original suspension was calculated.

Yeast Cell Agglutination

Lyophylized *Candida albicans* purchased from the ATCC was rehydrated in sterile 0.9% NaCl and serially passed in Sabouraud Broth. A 48-h culture in 1 l of broth was washed three times in 0.9% NaCl and resuspended in 10% of its original volume of broth with 1.0% gluteraldehyde at room temperature for 1 h. After one saline wash the yeast cells were resuspended in 0.1 M glycine for 30 min at room temperature, washed twice with saline and resuspended to a final 3% pellet wet weight/volume of saline containing 0.2% Sodium Azide. On a standard microscope slide 0.2 ml of the yeast cell suspension was mixed with 0.2 ml of the *E. coli* suspension and 0.2 ml of saline or 50 mM D(+)-mannosed and gently rocked for 1 min after which agglutination was assessed visually.

Mannose Binding

E. coli was prepared as described above without the addition of ^3H-Adenine. 0.1 ml of the saline suspension of *E. coli* containing approximately 10^8 C.F.U. per ml was incubated in a Dubnoff shaking water bath with 0.1 ml of either ^3H-mannose or ^{14}C-mannose (New England Nuclear, Boston, Mass.) at various concentrations for 2 h at 0 °C. 0 °C was utilized to prevent bacterial growth and metabolism during the period of incubation. Preliminary experiments demonstrated that maximal "binding" occurred at 2 h of incubation. Unbound radioligand was separated from that associated with the bacteria by the addition of 5 ml of ice cold saline and rapid filtration through a 0.45 μM cellulose filter (Gelman Sciences, Ann Arbor, Mich.) with varying volumes of saline rinse. In competition experiments, after the 2 h incubation in the presence of the labeled D(+)-mannose a 200 fold dilution was made into ice cold saline or into saline containing 1,000 fold higher concentration of unlabeled D(+)-mannose. Samples were taken at various intervals, filtered and rinsed with 2, 10 ml aliquots of ice cold saline. Filters were assayed for radioactivity by suspension in 7 ml of Aquasol (National Diagnostics Laboratories, Sommerville, NJ) and counting in a Serle Isocap liquid scintillation spectrophotometer.

Mannose Metabolism

5 ml of *E. coli* (10^8 C.F.U.) was incubated in the presence of varying concentrations of ^{14}C–D(+)-mannose at and 37 °C in a closed flask. Within the flask was a well containing 1 ml of phenylethylamine to absorb the released ^{14}C–CO_2. The incubation was stopped at 30 min by the addition of 1 ml of 0.625 M perchloric acid. The flask was maintained in the shaking bath for an additional 1 h to allow for the complete absorption of the ^{14}C-CO_2 released by the acid. The concentration of D(+)-mannose metabolized was calculated from the specific activity of the D(+)-mannose and the bacterial concentration.

Adherence Model

Male New Zealand white rabbits weighing 2–2.5 kg were sedated with 0.7 ml/kg of a Ketamine/Zylazine mixture (29.2 mg/ml ketamine, 8.3 mg/ml zylazine) I.M. Surgical anesthesia was induced with 1 ml sodium pentobarbital (50 mg/ml) given I.V. over the course of surgery. The urinary bladders were catheterized with a #8 catheter and were emptied of urine. Prior to introduction of bacteria, the bladder was flushed with 3, 10 ml aliquots of 0.9% NaCl introduced through the catheter. Approximately 8×10^8 CFU ^3H labeled bacteria suspended in 1.5 ml of 0.9% NaCl were introduced into the bladder and flushed in with 5 ml of 0.9% NaCl. After a 20 min exposure to the labelled bacteria the bladder was emptied and flushed with 3, 10 ml aliquots of 0.9% NaCl. The animal was then sacrificed, the bladder removed and the mucosa was dissected free from the underlying muscle layer and assayed for ^3H activity.

Acid Treatment of Bladder

Prior to introduction of bacteria into the bladder 10 ml of 0.4 N HCl was infused through the catheter, left in the bladder for 1 min and removed. The bladder was then neutralized by flushing with 3, 10 ml aliquots if 0.5 M dibasic potassium phosphate followed by 3, 10 ml aliquots of 0.9% NaCl. Labeled bacteria were then introduced.

Heparin Treatment of Bladder

Immediately after the acid treatment and neutralization, 0.25 ml of sodium heparin (Pan Heparin 20,000 U.I./ml, Abbott Laboratories, North Chicago, I11.) suspended in 5 ml of 0.9% NaCl was infused through the catheter and left in the bladder for 15 min. The bladder was emptied and labeled bacteria were introduced.

Mannose Treatment of the Bladder and Bacteria

Radiolabeled bacteria were suspended in 0.9% NaCl containing 50 mM unlabeled D(+)-mannose. Immediately after the acid treatment and neutralization the bladder was rinsed with 3, 10 ml aliquots of 0.9% NaCl containing 100 mM (1.8 mg/ml) D(+)-mannose. After introduction of radiolabeled bacteria into the bladder they were flushed in with 5 ml of 0.9% NaCl containing 100 mM unlabeled D(+)-mannose.

Recording of Radioactivity

Bladder mucosae were dissolved by overnight incubation in 1 ml of 1.0 N NaOH at 37 °C. The volume was then brought to 5 ml with 0.9% NaCl and 1.5 ml triplicates were placed in 20 ml glass scintillation vials and acidified with 0.1 ml glacial acetic acid. The remaining 0.5 ml of mucosal homogenate was used for determination of protein content by the method of Lowry [8]. Eighteen ml of Hydroflour was added and the vials were vortexted. 0.1 ml triplicates of the bacterial suspensions were also suspended in Hydroflour. The radioactivities of the tissue and the bacterial viable count (CFU) to ^3H uptake per ml of bacterial suspension were used to convert counts per minute to the actual number of bacteria (in colony forming units) attached to the mucosa per milligram of mucosal protein.

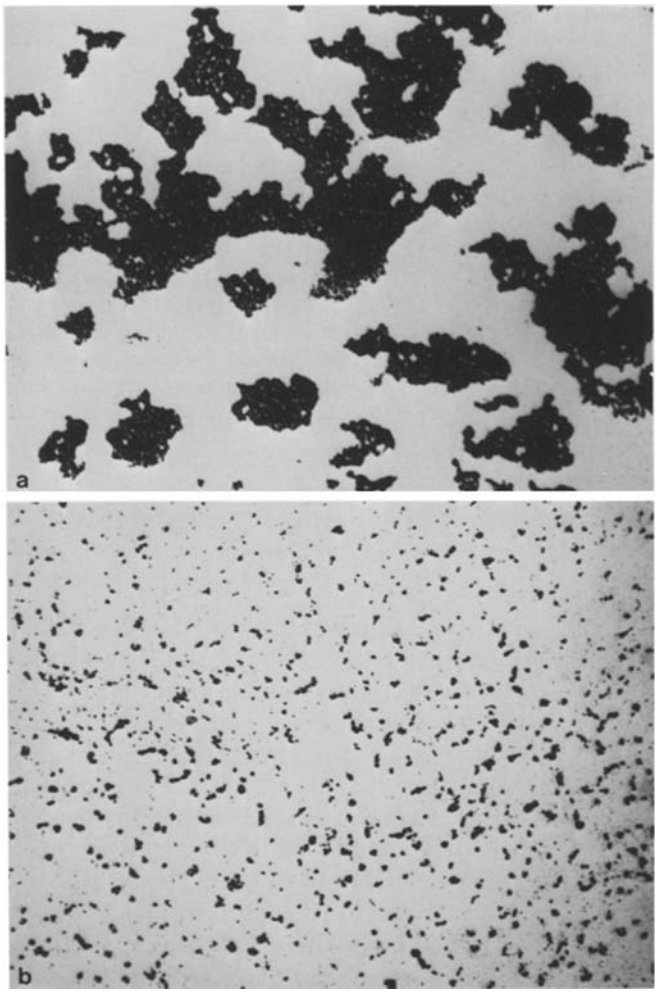

Fig. 1a, b. Effect of mannose on *E. coli*-yeast cell agglutination: a Photomicrograph of agglutinated *E. coli*-yeast (*Candida albicans*) suspension. b similar suspension in the presence of 50 mM D(+) mannose

Results

Growth curves for this strain of *E. coli* in brain heart infusion broth showed that the stationary phase was reached well within 18 h. 24 h broth cultures were used since this has previously been reported to induce maximal pilus formation [3, 12].

Yeast Cell Agglutination

Each *E. coli* suspension was tested each day for yeast cell agglutination prior to performing the binding or in-vivo studies. The results were the same every day in that *E. coli* did agglutinate the yeast cells and 50 mM D(+)-mannose did prevent this agglutination (Fig. 1a, b). Adding the mannose after the agglutination had taken place reversed the agglutination. When the mannose was removed from the *E. coli* – yeast cell suspension by centrifugation, resuspension in the same volume of saline resulted in agglutination identical to that which occurred before the mannose was added. The addition of heparin in concentrations up to 20,000 I.U./ml had no inhibitory effect on yeast cell agglutination. Higher concentrations of heparin were not possible due to lack of sufficient solubility.

Experiments at 37 °C demonstrated that *E. coli* is capable of significant metabolism of mannose (Fig. 2). In order to prevent both the quantitative metabolism of mannose and bacterial growth during the incubation period, the experiments were generally performed at 0 °C. Even at this temperature some degree of D(+)-mannose metabolism was observed.

Mannose Binding

Preliminary experiments using a maximal concentration of 30 nM ^{14}C D(+)-mannose did not show any appreciable "binding" of the sugar to the bacteria. Increasing the con-

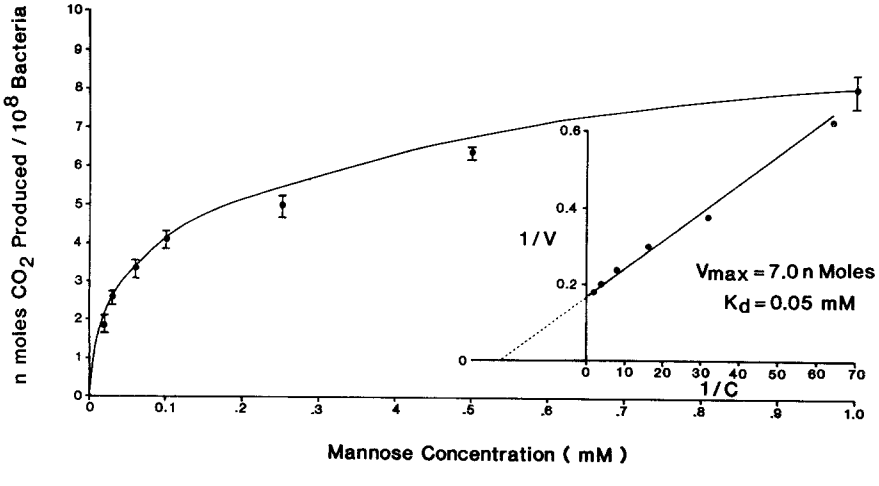

Fig. 2. Mannose metabolism by *E. coli*. ^{14}C-CO_2 produced from ^{14}C-mannose was determined as described in the methods. Each point represents the mean and standard error of 4 separate triplicate determinations

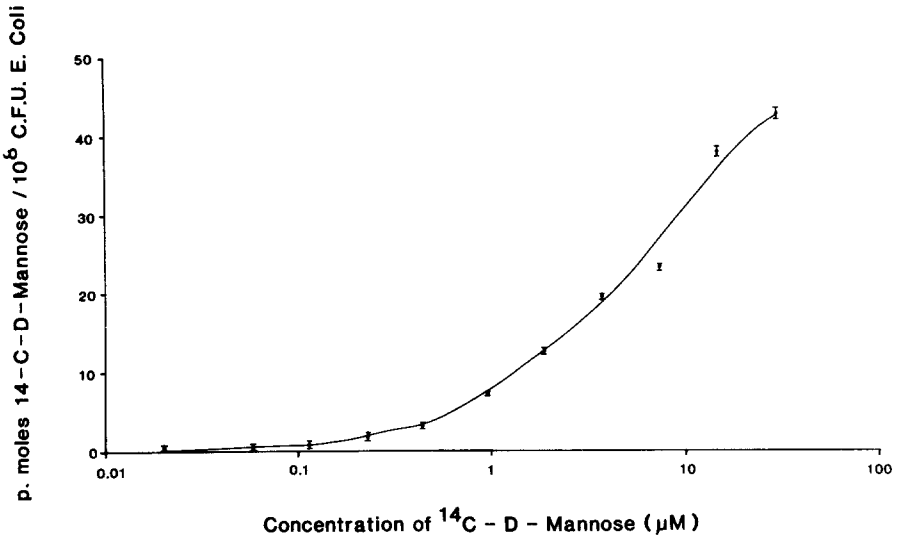

Fig. 3. Effect of increasing mannose concentration on mannose "binding" by *E. coli*. The binding assay was performed as described in the methods. Each point represents the mean and standard error for a representative triplicate determination

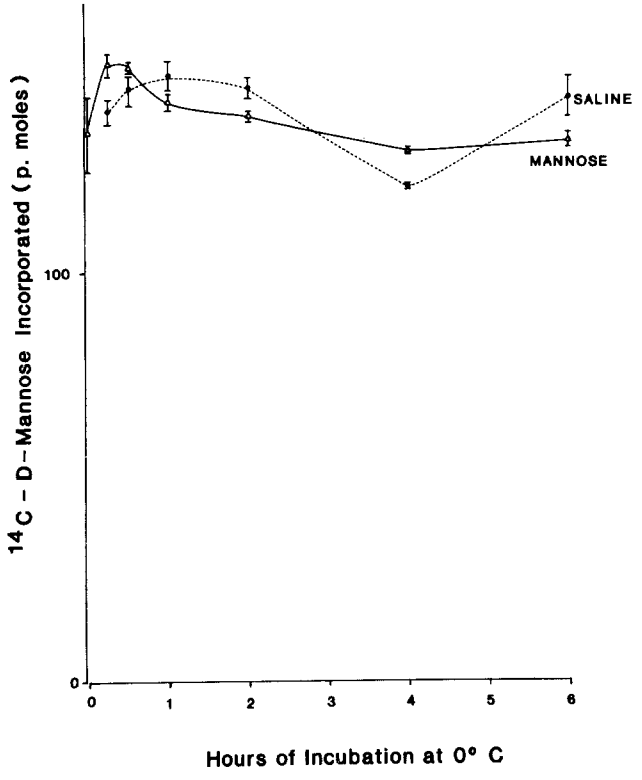

Fig. 4. Lack of dissociation of labeled mannose from *E. coli*. After 2 h incubation with 30 μM ^{14}C-mannose a 200 fold dilution was made into either 30 mM unlabeled mannose (*solid line*) or into 0.9% NaCl (*broken line*) and aliquots were sampled at the times indicated as described in the methods. Each point represents the mean and standard error for a representative triplicate determination

centration of the labeled D(+)-mannose to 30 μM resulted in detectable "binding" of D(+)-mannose by the bacteria. Wash volumes of 20 to 50 ml removed virtually all radioactivity from the filter but not from the bacteria; all subsequent experiments utilized 20 ml of saline wash. The effect of increasing concentrations of labeled D(+)-mannose on the association of the sugar with the bacteria is displayed in Fig. 3. As the concentration of the labeled D(+)-mannose increased the amount of sugar associated with the bacteria also increased. No saturation was observed up to 30 μM ^{3}H-D(+)-mannose so all subsequent experiments utilized this concentration.

The data displayed in Fig. 4 was obtained by first incubating the bacteria with 30 μM ^{14}C D(+)-mannose for 2 h at 0 °C then making a 200 fold dilution into 30 mM unlabeled D(+)-mannose (solid line) or saline (broken line). The hours of incubation on the X axis refers to the time after the 200 fold dilution. No dissociation of the labeled D(+)-mannose from the bacteria was seen even for the dilution into 1,000 fold excess unlabeled D(+)-mannose.

Bladder Adherence

This data is displayed in Fig. 5. The acid treatment increased the adherence of *E. coli* by about 20 fold. This increased adherence to the acid denuded bladder was prevented by heparin confirming earlier work [6, 7, 13], however, the D(+)-mannose treatment only prevented this increase by about 25%. This decrease was not statistically significant by analysis of variance.

Discussion

Results of the direct binding of radiolabeled D(+)-mannose to live *E. coli* at 0 °C indicates that this is probably an uptake site as opposed to a surface binding site. As stated previously if the bacteria bind the mannose with a surface lectin then this binding should be competitive and therefore displaceable with excess unlabeled mannose. These results clearly show that radiolabeled mannose association with *E. coli* cannot be displaced by excess unlabeled mannose. The possibility that an *E. coli* surface lectin binds mannose

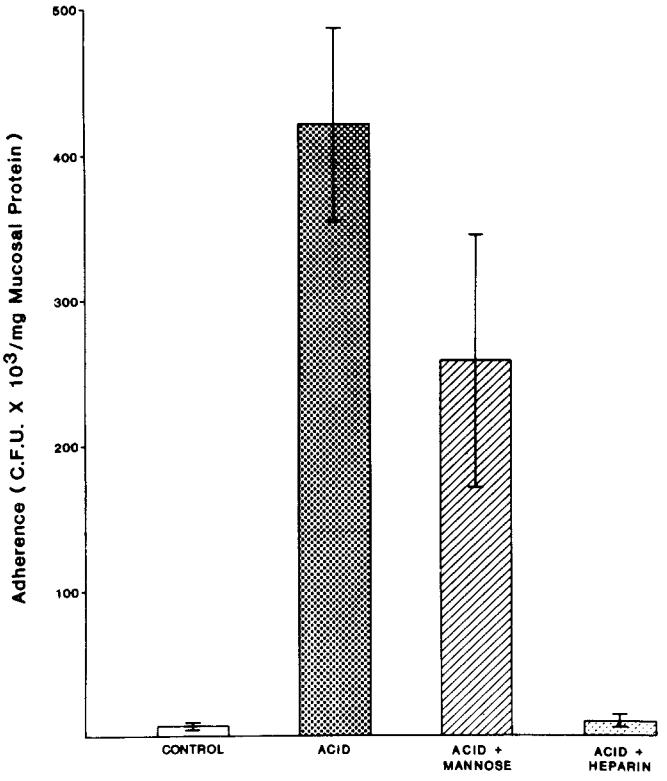

Fig. 5. Mannose and heparin effect on *E. coli* adherence to rabbit bladder mucosa. Adherence was assayed as described in the methods. Each bar represents the mean and standard error of 4 to 6 individual rabbits

irreversibly is not compatible with results from the yeast cell agglutination studies. If the mannose binds irreversibly with the *E. coli* surface then removing the unbound mannose by centrifugation would result in no agglutination upon resuspension (assuming that the mechanism of *E. coli* – yeast cell agglutination is mannose binding by *E. coli*). However, resuspension did result in agglutination which suggests a reversible binding phenomenon.

In some of the binding experiments using ^{14}C mannose a cotton pledget soaked with phenyl ethyl amine (PEA) was suspended in a cup above the enclosed atmosphere of each incubation tube to collect any $^{14}C\text{-}CO_2$. Appreciable radioactivity was found in the PEA pledget – about one tenth of the radioactivity associated with the bacteria. This did not occur in blank tubes without bacteria. It appears that the bacteria were metabolizing the mannose even though the temperature was maintained at 0 °C throughout the incubation period. This provides additional support that the bacteria were taking up the sugar since *E. coli* cannot metabolize a sugar unless it is first taken up into the bacterial cytoplasm.

The most likely explanation for the inability to find a competitive binding site for mannose on live *E. coli* is the masking of this site by the mannose uptake site. Experiments using metabolic inhibitors such as azide and flouride to prevent uptake and metabolism of the sugar by the bacteria may demonstrate such a binding site. Using a radiolabeled mannose analogue which is resistant to metabolism such as α-methyl mannopyranoside (presently not commercially available as a radioligand) or performing the binding on purified *E. coli* pili may be another useful approach.

Regardless of whether or not a competitive mannose binding site can be demonstrated on the surface of *E. coli* the results of the in-vivo rabbit bladder adherence studies indicates that 50 mM mannose is not of much use in prevention of *E. coli* adherence. Several investigators have shown that *E. coli* can produce more than one type of adhesion which may or may not display mannose sensitivity [2, 3, 10–12]. The results of the present investigation indicate that the strain of *E. coli* used possesses a mannose sensitive adhesion demonstrated by yeast cell agglutination, however this is only responsible for about 25% of the adherence of this strain to the rabbit bladder mucosa demonstrated by mannose inhibition. Heparin appears to hold much more promise in preventing adherence and thereby subsequent colonization of the bladder by *E. coli* since its antiadherence properties do not seem to depend on whether or not the bacteria possess a mannose binding lectin on their surface. The fact that heparin was ineffective in preventing yeast cell agglutination whereas mannose was effective places some doubt on the use of yeast cell agglutination as a model for bacterial adherence to mammalian epithelial tissues.

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