URINARY FACTORS AFFECTING ADHERENCE OF *ESCHERICHIA COLI* TO UROEPITHELIAL CELLS

by

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ABSTRACT

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Escherichia coli is the most frequent cause of urinary tract infections. A common interaction between *E. coli* and uroepithelial cells is one that is mannose sensitive. This study has demonstrated that a mannose containing glycoprotein (Tamm-Horsfall protein - THP) of urine has the ability to reduce adherence of *E. coli* to uroepithelial cells. In adherence assays, bacteria treated with THP displayed an approximate 48% decrease in adherence ($p < 0.05$). The observed antiadherence activity of the THP could be reversed by preincubating the THP in 5.0 mM calcium, prior to its use in an assay. Adherence in these cases was not reduced and was statistically equivalent to control adherence ($p < 0.05$). The effect of calcium on THP was modulated using cranberry juice cocktail (CJC). Studies both *in vitro* and *in vivo* have shown that CJC can protect the antiadherence activity of THP which had been preincubated with calcium. It is suggested that any condition

which results in an increase in calcium in the urine may act to reduce the antiadherence activity of THP and thus increase the potential for a urinary tract infection. CJC and other dietary supplements like CJC might be helpful in reducing the effect of increased calcium in the urine.

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CHAPTER I

Introduction

Bacteria have the ability to adhere to almost any surface and that adherence is an important ecological step in the colonization of a specific site by the bacteria. Adherence is also an important step in the development of bacterial infections that may arise on plant or animal tissues (Ofek and Beachey, 1978). In particular, adherence has been implicated as an important and sometimes essential step in the pathogenesis of bacterial infections that arise on mucosal surfaces (Editorial, 1981).

Because mucosal surfaces function to promote the flow of fluids and nutrients across them, they are thin, moist, and rich in nutrients and thus provide a suitable environment for microbial colonization and invasion. This functional design of mucosal surfaces increases their susceptibility to bacterial infection. Intestinal and respiratory mucosa have other structural requirements which further compromise their vulnerability. These surfaces have evolved convoluted structures to maximize exposure to ingested and inspired contents. This however, also maximizes exposure to bacteria. The urinary tract has an increased susceptibility to infection because of its close

proximity to heavily colonized mucosa of the intestinal tract (Christensen and Beachey, 1984).

Adherence of bacteria to a mucosal surface leads to colonization of that surface. The bacteria anchor to the surface, multiply and grow. Adherence becomes significant when the bacteria invade the tissue causing infection. The ability to adhere is one of several virulence factors involved in determining whether a population of bacteria will cause infection (Editorial, 1981).

Bacterial adherence is mediated through specific molecular surface structures of the bacteria. It is these structures, termed adhesins, that recognize and interact with specific complementary surface structures of the host. Lipoteichoic acid of Streptococcus pyogenes is one of the best described adhesins. Immunologically it has been identified in the fibrillae structures that coat the Streptococcal surface. Electron microscopy studies have shown that this fibrillae coat is responsible for making contact with the eukaryotic host cell. Another type of adhesin, a lectin, refers to any carbohydrate-binding protein on the bacterial surface. Fimbriae are specialized organelles on the bacteria that act as lectins. They are proteinaceous filaments that radiate outward from the bacterial surface where their tips initiate contact with surfaces. They contain carbohydrate-binding proteins for

recognition of host cells. Expression of fimbriae varies with environmental conditions and growth phase of a bacterial population. Other functions of fimbriae include mediating sexual conjugation (Christensen and Beachey, 1984).

The receptors on the host cell surface can be of three general types. Viral neoreceptors are new bacterial receptors for adherence that have been induced by an antecedent viral infection. Fibronectin is a second type of host cell receptor. Fibronectin is an ubiquitous adhesive glycoprotein that exists in an insoluble form on cell surfaces. It is responsible for mediating adherence of group A streptococci and a variety of other gram positive cocci to cells. Fibronectin has been implicated in promoting adherence of gram positive cocci to oral tissues since it exists in an exposed form on the surface of buccal cells. The third type of host cell receptor is a cell surface sugar residue of the intrinsic glycocalyx. It is these receptors that are most frequently involved in bacterial adherence to mucosal surfaces. Bacteria exploit the glycocalyx sugar residues by evolving complementary lectin adhesins which allow them to recognize and attach to the cell thereby increasing their specificity for that tissue. The precise orientation and size of the sugar moiety along with neighboring submolecular structures of

the active binding site add to the specificity of the bacteria-host reaction (Christensen and Beachey, 1984).

Bacterial adherence mediated through fimbrial lectins has been implicated as a virulence factor in the development of urinary tract infections (Svanborg-Eden, 1986). Urinary tract infections are common bacterial infections of humans of all ages. Urine is normally sterile, however, if it is not too acidic, it serves as a fine growth medium for bacteria (Mims, 1982). It is thought that the bacteria are introduced to the urinary tract from the intestinal flora. The intestinal mucosa is a reservoir of gram-negative bacteria that can cause extra-intestinal infections. Bacteria from the intestine colonize vaginal and periurethral areas. From here they gain entrance to the urinary tract via the urethra where they usually cause infection (Svanborg-Eden, 1986). Gram-negative bacteria that cause urinary tract infections are commonly from the Enterobacteriaceae family. *Escherichia coli* accounts for approximately 80% of all urinary tract infections with *Klebsiella*, *Proteus* and *Enterobacter* species accounting for approximately 10-15%. *Pseudomonas*, staphylococci and group D streptococci account for about 5-10% of the remaining infections (Kunin, 1975). Bacterial growth in the bladder is easily sustained as the growth medium (i.e. urine) is continuously renewed.

Consequently, growth is thought to be continually in the log phase. In the case where residual urine is maintained or reflux occurs, it might be possible to obtain post-log cultures. Although both populations possess adhesive ability, it is greater in post-log phase bacteria. Generally, 10^5-10^9 bacteria per ml of urine is achieved in an infection (Svanborg-Eden *et al.*, 1977).

The urogenital tract by design possesses inherent defenses against uropathogenic organisms. The bladder mucosa secretes a mucin layer which has anti-bacterial activity (although the exact mechanism is not known). A large soluble glycoprotein, Tamm-Horsfall glycoprotein (THP), which exists in the urine traps bacteria which are then removed with the urine. By the flushing of the urinary tract with urine every couple of hours a mechanical disturbance is created for any adhering bacteria. In males, where the urethra is 20 cm long this flushing is especially effective. The proximal two-thirds of the urethra remains sterile. In females, the urethra is much shorter, approximately 5 cm and it is more readily traversed by microorganisms. Consequently, urinary tract infections are 14 times more common in women than men. When there exist structural abnormalities or interferences with the bladder or urethra, these defenses are compromised and a urinary tract infection usually results. An enlarged

prostate or any stone that may prevent complete emptying of the bladder can increase chances of an infection occurring. Another example occurs in pregnant women whose bladder muscles may be sluggish and result in incomplete emptying (Mims, 1982).

A significant correlation has been demonstrated between the presence of fimbriae on _E. coli_ and the ability to adhere to human urinary tract epithelial cells. It was found that bacteria with very few or no fimbriae were non-adherent. When bacteria were treated in various ways to remove the fimbriae, adherence decreased parallel to the loss of fimbriae. (Fimbriae were removed by heating or washing of the bacteria.) These findings implicate fimbriae as the mediators of adherence for bacteria to uroepithelial cells in urinary tract infections (Svanborg-Eden and Hanson, 1978). Works by Selander and Levin, (1980) and Svanborg-Eden _et al_., (1977) demonstrated a correlation between bacterial adherence to human uroepithelial cells and to the severity of the urinary tract infection. Individuals prone to urinary tract infections have an increased carriage of gram-negative bacteria in the vaginal and periurethral areas. These cells seem to have an increased receptivity for attaching bacteria and perhaps this is the result of an increased density and/or availability of receptors (Svanborg-Eden,

1986). This also implicates fimbrial mediated adherence of bacteria in recurrent urinary tract infections.

Several types of fimbriae have been identified on uropathogenic bacteria. The first type recognized was classified as mannose-sensitive (or alternatively type 1) bacause it was found that D-mannose inhibited bacterial hemagglutination. This inhibition was interpreted to mean that mannose was part of the host receptor. Although no mannose-containing glycoconjugate with receptor activity could be found on the cell surface, mannose is part of the high mannan glycoproteins found in epithelial cell glycocalyces (Svanborg-Eden, 1986). Mannose-sensitive *E. coli* were also shown to cause aggregation of mannan-containing yeast cells. Type 1 fimbriae were found to be abundant on gram-negative bacteria. In particular, *E. coli* that were mannose-sensitive were shown to adhere to uroepithelial cells and this adherence could be inhibited by D-mannose. Based on these findings it was concluded that mannose-binding activity among human urinary bacterial isolates accounts for the adherence of the organisms to mannose residues on epithelial cells (Ofek and Beachey, 1978). Nearly all *E. coli* which cause urinary tract infections in humans express type 1 fimbriae, although it is probable that multiple ligands exist. Populations of bacteria may differ in types and number of types of fimbriae that are present (Hagberg *et al.*, 1981).

Another class of antigenically distinct *E. coli* adhesins were demonstrated on urinary isolates causing infection. P-type fimbriae were discovered when mannose resistant strains of *E. coli* were found to attach in high numbers to epithelial cells. These fimbriae were found to be morphologically similar to type 1 by electron microscopy analysis (Hagberg *et al*., 1981). When a fraction of glycolipids, isolated from human urinary tract epithelial cells, inhibited attachment of *E. coli* to cells from the same donor it was postulated that the glycolipids had a role at receptor sites (Leffler and Svanborg-Eden, 1980). These fimbriae were also characterized by their ability to agglutinate human red blood cells carrying the ubiquitous P blood group antigen, which is a member of the globoseries glycolipids. This agglutination could be inhibited by isolated and purified P blood group antigen or by a synthetic Galp-(β 1-4)-Galp-ceramide (Christensen and Beachey, 1984). This observation demonstrated that the host receptor was a glycolipid. Recently, the minimal structure recognized by the bacteria on the uroepithelial cells and on human erythrocytes has been shown to be the alpha-D-Galp-(β 1-4)-D-Galp disaccharide entity (Korhonen *et al*., 1982). The P antigen has also been identified on the kidney tissue of men and mice. In a study with mice, it was found that organisms with primarily P-type fimbriae

localized in the upper urinary tract while organisms with a combination of P-type and mannose-sensitive type localized the organisms to the bladder (Christensen and Beachey, 1984).

A third type of fimbriae, only recently characterized, recognize sialyloligosaccharides and are referred to as S-type. These fimbriae were found to be morphologically similar to the other types. Hemagglutination of organisms possessing S-type fimbriae could be inhibited by oligosaccharides containing sialyl galactoside sequences (Korhonen *et al*., 1984). Five major sialyloligosaccharides have been isolated and characterized from urine and are thought to be structurally similar to the host cell surface receptor. In the urine these may block adherence of the bacteria to the epithelial cells and possibly reflect some of the inhibitory factors of urine (Parkinnen and Finne, 1983).

Although fimbriae play a predominant role in adherence, other surface properties contribute. A study by Ljungh *et al*. (1985) showed that freshly isolated strains of *Staphylococcus aureus* from clinical infections including septicemia, wounds and urinary tract infections often possess high surface hydrophobicity. Protein A, a fibronectin-binding protein and other yet undefined surface proteins are thought to contribute to this hydrophobicity.

A hydrophobic cell surface may provide an advantage for the bacteria *in vivo* by increasing their resistance to phagocytosis or by contributing to the colonization of mucosal surfaces (Ljungh *et al*., 1985). Ohman *et al*. (1981), have shown that a negative surface charge and liability to hydrophobic interaction found on *E. coli* correlate with enhanced association with cells.

Several natural chemical defenses against adhering bacteria are intrinsic properties of the bladder. The glycocalyx of the transitional cells that line the bladder possesses excretions which may serve as receptor analogues and block adherence. Also, genetic variation within this layer may deny bacteria a receptor. Secretory IgA can bind to the bacterial surface and prevent adherence (Christensen and Beachey, 1984). The transitional cells secrete a mucin layer which serves as a barrier and blocks the epithelial cell receptors. Experiments by Parsons *et al*. (1978) have demonstrated the antiadherent activity of this mucosal lining. When the bladder was stripped of the mucin layer with 0.3 N HCl, bacteria freely adhered to the cells. In efforts to restore the anti-adherent activity of the bladder, IgA was added but the stripped bladder remained unprotected against the bacteria. Sodium citrate was added to the bladder to bind available divalent cations. Since increased doses of sodium citrate did not decrease adherence it was concluded that divalent cations do not have

a role in adherence. Changing the pH of the bladder to 7.0 restores the electrochemical surface charge and thus restores the mucin layer. Consequently, this restores the antiadherent nature of the transitional cells. It was postulated that the layer of mucin provides a poor electrochemical substrate for bacteria and blocks adherence sites for the bacteria (Parsons et al., 1978). Finally, Tamm-Horsfall glycoprotein produced in the kidney and secreted into the urine in a soluble form acts to trap bacteria and consequently wash them out of the urinary tract (Christensen and Beachy, 1984).

Tamm and Horsfall in 1950 discovered in urine a potent inhibitor of hemagglutination by influenza viruses. They isolated this substance by precipitation with 0.58 M NaCl. The substance was a large glycoprotein consisting of approximately 68% polypeptide, 28% carbohydrate and 1% lipid. It exists over a wide range of molecular weights averaging approximately 90,000 daltons. The average adult excretes 45 mg/24 hr or 25 mg/L of urine. It is generally agreed that Tamm-Horsfall glycoprotein originates in the kidney and, although the precise site is not certain, it has been suggested that it is the glycocalyx of the lumenal cells of the proximal convoluted tubule (Fletcher, 1972).

Tamm-Horsfall glycoprotein is soluble in the urine and can coat mannose-sensitive bacteria. THP contains

mannose moieties which bind to the mannose-sensitive fimbriae and provide a coat or partial coat on the bacteria. Receptors now blocked, the bacteria cannot bind to the mannose residues on the bladder epithelial cells. Kuriyama and Silverblatt (1986) have demonstrated this THP-fimbriae interaction. They showed that purified mannose-sensitive fimbriae were retained on a column containing purified THP. The fimbriae could then be eluted with methylα-D-mannoside. These findings implicate the mannose recognizing receptor of the bacteria as being involved in the THP coat (Kuriyama and Silverblatt, 1986).

THP exhibits changes in its physical nature in different ionic environments. The most striking of these is the increase in viscosity leading to gel formation with addition of 5.0-7.0 mM calcium ions. Other divalent and monovalent cations such as Mg^{2+}, Ba^{2+} and Na^{+} also affect viscosity but to a lesser degree. The effects of these cations are additive (Fletcher, 1972). A decrease in pH also increases viscosity but only for concentrations of THP well above those ever likely to be attained *in vivo* (McQueen and Engel, 1966). Stevenson *et al*. (1971) have suggested that THP, as the calyx of lumenal cells in the proximal tubule, may help regulate the absorptive process thereby responding physicochemically as it does to the calcium (Stevenson *et al*., 1971). The THP response to

calcium in the urine, however relates to the potential pathophysiology of this glycoprotein. The THP-Ca^{2+} complex is a possible initiator and/or promoter of renal calculi formation. THP has been identified immunologically in the matrix of various kinds of kidney stones (Fletcher, 1972).

Calcium excretion in urine is usually under several hormonal control processes. In some cases, however, there exists an excess of calcium in the urine. Idiopathic hypercalciuria is an often undiagnosed condition where there is an increased urinary excretion of calcium. This is due to a poorly characterized defect in calcium metabolism where there is an inappropriately high dietary absorption of calcium or abnormal renal handling of filtered calcium loads. Excess calcium may also exist in the urine transiently due to a high calcium load or in other disease states (Bondy and Rosenberg, 1974).

The purpose of this study is to demonstrate the antiadherent activity of THP on mannose-sensitive clinical isolates of *E. coli* and to examine the effect of selected cations and other parameters on this activity.

CHAPTER II

Materials and Methods

Bacteria

A total of 27 clinical isolates of *E. coli* were obtained from the microbiology laboratory of Alliance City Hospital. These urinary isolates were cultured from hospital patients with diagnosed urinary tract infections, whose urine had greater than 10^5 bacteria per mL. Upon receipt, the organisms were streaked on MacConkey agar plates and incubated for 24 hours at 37°C. Individual colonies were picked, cultured in tryptic soy broth overnight and the identity of each *E. coli* isolate was then verified using API 20E strips which identify gram negative organisms on the basis of their specific biochemical reactions. To obtain cultures for long-term storage, the organisms were transferred to Brain Heart Infusion (BHI, Difco) agar slants, grown at 37°C for 48 hours and then stored at 2-6°C. When bacteria were needed for testing, they were transferred to BHI or tryptic soy broth and grown at 37°C for 24 hours.

Epithelial Cells

Epithelial cells used in this study were obtained from freshly voided urine. Urine specimens were obtained

from one healthy individual with no prior history of urinary tract infection. The cells were harvested by centrifugation of 10 ml samples at 1000 Xg for 10 minutes in conical glass tubes. The supernatant was discarded and the pellet was washed once with 0.010 M phosphate buffered saline (PBS) pH 7.2. Epithelial cells were collected on the day they were to be used (Sobota, 1984).

<u>Preparation of Tamm-Horsfall Glycoprotein</u>

The method used was that described by Hoyer and Seiler (1979) which is a modification of the original by Tamm and Horsfall (1952). THP can be collected from fresh human urine by precipitation with NaCl concentrations greater than 0.58 M. Urine was collected and brought to a final concentration of 0.01% sodium azide with a 0.10% sodium azide stock solution. A 3.5 M NaCl stock solution was added to the urine to achieve a final concentration of 0.58 M. After a minimum of 4 hours at 4°C, a bulky gelatinous precipitate forms. The precipitate was collected by centrifugation at 1000 Xg for ten minutes and the supernatant was discarded. The pellet was washed three times in 0.58 M NaCl, then solubilized in deionized water and centrifuged to remove any water insoluble impurities that would pellet. The supernatant was then brought to 0.58 M NaCl by addition of the NaCl stock to reprecipitate the sample. The THP preparation was dialyzed against

deionized water in dialysis tubing with a cutoff of 30,000 daltons for at least 18 hours at 4'C.

Analysis of THP

The concentration of THP was determined by quantifying the protein in a sample using the modified Lowry Technique (kit from Sigma). A 0.2 mL sample of each THP preparation to be tested was added to a test tube. To generate a blank, 0.2 mL of 0.85% NaCl was added to a tube. Then, to each tube, 2.2 mL of biuret reagent was added, the tubes were mixed and allowed to stand at room temperature (18-26'C) for 10 minutes. Next, 0.1 mL of Folin-Ciocalteu's Phenol Reagent was added to each sample, after which the tubes were immediately mixed and allowed to stand at room temperature (18-26'C) for 30 minutes. After this time, the samples were transferred to cuvets and absorbances were measured against the blank at 725 nm on a Bausch and Laumb Spectronic 20. Using the measured absorbance value, the concentration was then determined from an absorbance versus concentration of protein calibration curve. This calibration curve was generated by diluting a 100 mg/dL solution of bovine serum albumin with 0.85% NaCl to yield 0, 25, 50, 75 and 100 mg/dL protein solutions. Then, using the modified Lowry Technique absorbance values were obtained and plotted versus the concentration of bovine serum albumin to create a calibration curve.

The THP was analyzed by SDS-polyacrylamide gel electrophoresis. The method used is a modification of the methods of Weber and Osborn (1969) and Davies and Stark (1970). A 7.5% acrylamide solution was prepared by dissolving 16.65 g acrylamide and 0.45 g N,N'-Methylenebisacrylamide in 100 mL distilled water. The gels were prepared by mixing 13.5 mL of acrylamide solution with 15 mL of gel buffer (7.82 g $NaH_2PO_4.H_2O$, 20.45 g Na_2HPO_4, 2.00 g sodium dodecyl sulfate in 1000 mL distilled water, pH 7.0), degassing the solution by aspiration and then adding 1.50 mL of fresh ammonium persulfate solution (100 mg in 15 mL distilled water). Next, 0.05 mL TMEDA (N,N,N',N'-Tetramethylethylenediamine) was added to the gel solution. Slab gels of 3 1/4" X 4" dimensions were cast 0.8 mm in thickness using a glass plate sandwich apparatus. The smaller glass plate was treated with silane prior to casting the gels to aid in the polymerization of the gel onto the plate. The slab was placed upright in an EPHORTEC Mini Vertical Slab apparatus (Haake Buchler Instruments, Inc.). The chambers were filled with a diluted gel buffer, pH 7.0. (The gel buffer was diluted 1 part buffer to 2 parts deionized water). The gel was then loaded with a 10 uL sample of protein per lane. Prior to electrophoresis the protein samples were prepared in a sample buffer (0.39 g $NaH_2PO_4.H_2O$,

1.02 g Na_2HPO_4, 1.00 g sodium dodecyl sulfate, 1.00 mL 2-mercaptoethanol, 0.015 g bromphenol blue, 36.00 g urea in 100 mL distilled water, pH 7.0), saturated with sucrose and incubated for 2 hours at 37°C. Molecular weight marker proteins were run along with the THP sample and included pepsin (34,700 daltons), ovalbumin (45,000 daltons) and bovine albumin (66,000 daltons). The concentration of the THP sample was 1.0 mg/mL, pepsin: 5.0 mg/ml, ovalbumin: 1.5 mg/mL and bovine albumin: 1.5 mg/mL, which are optimum concentrations for electrophoresis. The electrophoresis was run at 10-15 mA for 4-5 hours or until the bromphenol blue tracking dye was about 1 cm from the anodic end of the slab. The slab was then immersed in a fixative solution (400 mL methanol, 70 mL glacial acetic acid and 530 mL distilled water) for a minimum of 10 hours with several changes of fixative solution. The slab was stained in Coomassie Blue G-250 (Stenesh, 1984) overnight and then immersed in the fixative solution for a minimum of 15 hours (not exceeding 25 hours) for destaining. Destaining was continued with a destaining reagent (50 mL methanol and 75 mL glacial acetic acid diluted to 1000 mL with distilled water). The gel was dried and photographed.

Bacterial Adherence Assay

The method used in these experiments was that of Parsons *et al*. (1980) as modified by Sobota (1984). For

all adherence assays a mannose-sensitive clinical isolate of E. coli (1401) was used. Mannose-sensitivity was demonstrated by determining adherence of the bacteria after incubation with 2.5% D-mannose. A 24 hour culture of E. coli (10^8 organisms per ml) grown in BHI at 37°C was used. For each experimental condition, a 2.0 mL sample of E. coli was centrifuged at 1000 Xg for 10 minutes to harvest a bacterial pellet. The pellets were then washed twice in 5.0 mL of 0.010 M phosphate buffered saline (PBS), pH 7.2. Bacterial pellets to be treated were layered with 0.25 mL of the treated THP solution, gently mixed and incubated for 10 minutes in a 37°C water bath. (Treatments to the THP were incubated with a THP sample for ten minutes in a 37°C water bath). One pellet was resuspended in 2.0 mL of PBS without undergoing treatment and was incubated for 10 minutes along with the treated pellets. This will serve as the control for adherence. After incubation, the treated samples were pelleted by centrifugation, the supernatant was discarded, and the pellets were resuspended in 2.0 mL of PBS pH 7.2. One mL of each suspension was mixed with an epithelial cell pellet containing approximately 10^5 cells per mL. This epithelial cell-bacteria mixture was gently mixed and incubated for 30 minutes in a 37°C water bath with mixing every 5 minutes by inversion. After incubation, 1 mL of the cell-bacteria mixture was removed using a tuberculin syringe. The tuberculin syringe was then attached to a filter apparatus

containing a 8 um pore size polycarbonate membrane filter (Nucleopore). The sample was filtered and washed three times with 10 mL aliquots of deionized water to remove any non-adherent bacteria. The epithelial cells with adherent bacteria remain on the nucleopore filter. The wet filter was placed face down on a slide allowing the cells to adhere as it dried. The filter was then removed. The slide was stained using a Gram stain method. The method used involved 1 minute of Gentian Violet, followed by 1 minute of Grams Iodine, followed by a 30 second wash with acetone and finally 1 minute counterstain with Safranin. The number of gram-negative bacteria on each of fifty cells was counted using the oil-immersion objective of a bright field microscope and mean adherence was established. For each adherence assay the negative control consisted of urine-derived epithelial cells only, i.e. no bacteria were added. The positive control was the mean adherence of untreated _E. coli_ to the cells. The controls were run concurrently with the experimental samples. When the mean adherence in the negative control was greater than five for any bacteria, the run was discounted (Sobota, 1984).

Statistical Analysis

The mean adherence values were compared to the controls and to each other using the two tailed Student's T-test. The standard error of the mean was calculated for

each sample to determine dispersion around the mean (Zar, 1974).

CHAPTER III

Results

Mannose Sensitivity

E. coli isolate 1401 was found to be sensitive to 2.5% D-mannose. Sensitivity was demonstrated using the adherence assay. The *E. coli* was incubated with and without D-mannose for 10 min at 37°C prior to the assay and mean adherence values were obtained. There was an average 68% decrease in the adherence of the mannose-treated bacteria as compared to the control ($p < 0.05$). Thus, isolate 1401 was considered mannose sensitive and used in all adherence assays.

SDS-Polyacrylamide Gel Electrophoresis of THP

THP was analyzed by electrophoresis on 7.5% SDS-polyacrylamide gel slabs. Molecular weight markers were run along with the THP. These included bovine albumin (66,000 daltons), egg albumin (45,000 daltons) and pepsin (34,700 daltons). The glycoprotein migrated as a discrete band having an approximate molecular weight of 85,000.

Effect of Tamm-Horsfall Glycoprotein on Adherence

To determine the effect of THP on adherence, samples of *E. coli* were incubated with and without THP for ten minutes at 37°C. The bacteria were then mixed with uroepithelial cells and assayed for adherence. Uroepithelial cells without added bacteria served as a baseline control. The results are presented in Table 1. The epithelial cell preparation to which no bacteria were added contained an average of 0.72 bacteria per cell. When these cells were incubated with 10^8 *E. coli* per ml the average bacteria per cell increased significantly to 29.72. If the uroepithelial cells were incubated with the same amount of *E. coli* with the addition of approximately 80 mg/dL of THP the average number of bacteria per cell decreased significantly to a value of 15.34 bacteria per cell ($p < 0.05$).

Effect of Tamm-Horsfall Glycoprotein Preincubated with 5.0 mM Calcium on Adherence

Three different sources of calcium were used to treat the THP samples and thereby determine if calcium ions would have an effect on the antiadherence activity of the THP. (All THP samples were approximately 80 mg/dL). The sources were prepared to yield a final concentration of 25 mM calcium and included A) $CaCl_2$, B) normal urine supplemented with $CaCl_2$ and C) urine concentrated by

evaporation to reach the desired calcium concentration. *E. coli* was incubated with and without each THP sample plus or minus calcium to determine the effect on adherence. These data are presented in Table 2. Each column represents data from a different source of calcium. In column A the source of calcium was $CaCl_2$. It can be observed that baseline controls, to which no bacteria were added, had an average of 0.40 bacteria per cell. When the epithelial cell preparation was incubated with *E. coli*, the mean bacteria per cell increased to 43.52 ($p < 0.05$). As was observed in the previous section, when the *E. coli* was mixed with THP and then incubated with epithelial cells the adherence value dropped significantly to 27.54 bacteria per cell ($p < 0.05$). It can further be observed from the table that when THP was preincubated with 5.0 mM $CaCl_2$, the antiadherence effect of that THP was negated. The adherence value was comparable to and statistically equivalent to the control value.

The second source of calcium was a 25 mM calcium ion solution in urine. Urine was collected fresh and the concentration of total calcium was measured (kit from Sigma). $CaCl_2$ was added to achieve a final calcium ion concentration in the urine of 25 mM (stock). The calcium containing urine was incubated with a THP sample for 10 minutes in a 37°C water bath. *E. coli* (10^8 bacteria per

ml) was then incubated with and without this THP sample to determine its effect on adherence. This data appears in column B of Table 2. The baseline control had an average value of 0.16 bacteria per cell. And as observed in the previous sample the mean adherence on the control showed a significant increase in adhering bacteria as compared to the baseline ($p < 0.05$). Preincubation of the *E. coli* with THP significantly reduced adherence ($p < 0.05$). The mean adherence value of *E. coli* with THP preincubated in 5.0 mM calcium of urine was 30.38 bacteria per cell. This was comparable to control values and significantly greater than adherence values attained for THP samples without added calcium.

The third source of calcium was a concentrated urine sample containing 25 mM calcium (stock). Urine was collected fresh and the concentration of total calcium was measured (kit from Sigma) and followed during an evaporation period. Evaporation was achieved at 37°C on a large surface area. This calcium source was incubated with THP for 10 minutes in a 37°C water bath. *E. coli* was then incubated with and without this sample to determine its effect on adherence. This data appears in column C of Table 2. It can be observed from the table that these results parallel those found with the first two calcium sources. The mean adherence value of *E. coli* with THP

preincubated in calcium was 40.60 and was statistically greater than adherence of *E. coli* and THP without calcium (test B) ($p < 0.05$). The adherence of *E coli* and THP with calcium (test C) was not statistically different from the control (test A) ($p < 0.05$).

Reversal of the Effect of Calcium on the AntiAdherence Activity of Tamm-Horsfall Glycoprotein Achieved In Vitro

The data presented above demonstrates that calcium ions reduce the antiadherence activity of THP. It might then be expected that agents which remove calcium from solution would reverse this effect. It has been demonstrated in this laboratory that cranberry juice cocktail (CJC) will complex relatively high concentrations of calcium ions (Erme, MS thesis). Thus, CJC was used *in vitro* and *in vivo* to modulate the THP response to calcium.

In the *in vitro* experiments cranberry juice cocktail was added to urine preparations prior to extraction of the THP. Two concentrations of the juice were used at dilutions of 1:10 and 1:30. The juice was added to the urine and allowed to interact with the THP at 37°C for 10 minutes. THP was then extracted from the urine preparations in the normal manner. (All THP preparations used in these assays contained approximately 80 mg of protein per dL). Each of these THP samples (1:10 CJC and 1:30 CJC) was divided into two portions and incubated with 5.0 mM $CaCl_2$

for 10 minutes in a 37°C water bath. The resulting four THP samples were used in the adherence assay and this data appears in Table 3. Typical controls were run along with the test assays. Epithelial cells to which no *E. coli* were added served as a baseline control. The mean adherence on these cells was 1.12 bacteria per cell. When *E. coli* was added to epithelial cells, the adherence was significantly increased to 38.68 ($p < 0.05$). The adherence of *E. coli* with THP (test A) was significantly decreased to 14.40 as compared to the control ($p < 0.05$). Adherence of *E. coli* with THP and 5.0 mM $CaCl_2$ (test B) significantly increased to 29.42 as compared to adherence of *E. coli* with THP and no calcium (test A) ($p < 0.05$). These results are representative of previous data found in this text. They will serve as controls against which to compare the activity of THP that has been prepared with CJC. The adherence of *E. coli* with THP prepared in 3.3% CJC (test C) was significantly decreased to 16.52 as compared to the control ($p < 0.05$) and was comparable to the THP/*E. coli* preparations without added CJC. Adherence of *E. coli* with THP prepared in 3.3% CJC and then preincubated in 5.0 mM $CaCl_2$ (test D) was also significantly decreased to 12.94 as compared to the control ($p < 0.05$). The results for tests C and D are also statistically equivalent, i.e. the addition of CJC to the THP preparation negated the effect

of the calcium. The mean adherence of *E. coli* with THP prepared in 10% CJC (test E) was significantly decreased to 15.58 as compared to the control ($p < 0.05$). The mean adherence of *E. coli* with THP prepared in 10% CJC and then preincubated in 5.0 mM $CaCl_2$ (test F) was also significantly decreased to 15.74 ($p < 0.05$). There was no statistical difference between test E and test F.

Reversal of the Effect of Calcium on the AntiAdherence Activity of Tamm-Horsfall Glycoprotein Achieved In Vivo

In these assays, THP was extracted from urine collected 2-4 hours after ingesting 8 oz cranberry juice cocktail. The THP, collected after CJC, was prepared in the normal manner and divided into two portions and incubated with and without 5.0 mM $CaCl_2$ for 10 minutes in a 37°C water bath. The two samples were then tested in adherence assay to determine their effect on adherence. This data is presented in Table 4. Epithelial cells to which no *E. coli* had been added served as the baseline control. These cells had an average of 1.92 bacteria per cell. When *E. coli* was added to the epithelial cells, the adherence was increased significantly to 22.10 bacteria per uroepithelial cell ($p < 0.05$). The adherence of *E. coli* with THP (test A) was decreased significantly to 6.86 as compared to the control ($p < 0.05$) and the addition of calcium to the THP significantly reduced the antiadherence

activity of the THP (test B vs test A) ($p < 0.05$). These adherence values served as controls against which to compare the activity of THP that was collected after ingestion of CJC. The adherence of *E. coli* with THP after drinking CJC (test C) was found to be significantly decreased to 10.42 as compared to the control ($p < 0.05$). The antiadherence activity of THP collected after drinking eight ounces of CJC was not significantly different from THP obtained prior to drinking the cocktail (test A vs test C). However the antiadherence activity of THP, treated with calcium, before (test B) and after ingestion of the CJC (test D) differed significantly ($P < 0.05$). Drinking the CJC negated the effect of the calcium on the THP.

TABLE 1

EFFECT OF TAMM-HORSFALL GLYCOPROTEIN ON ADHERENCE OF *E.coli*.

Group	Epithelial Cell Treatment	Mean Bacteria per Cell ± S.E.[1,2]
Baseline Control	none	0.77 ± 0.27
Control	*E.coli*	*29.72 ± 4.56
Test	*E.coli* + THP	**15.34 ± 3.47

[1] Standard Error of the Mean.

[2] n=50

* Statistically Different from Baseline ($p < 0.05$).

** Statistically Different from Control ($p < 0.05$).

TABLE 2

EFFECT OF DIFFERENT SOURCES OF CALCIUM ON THE ANTIADHERENCE ACTIVITY OF TAMM-HORSFALL GLYCOPROTEIN.

Group	Epithelial Cell Treatment	Mean Bacteria per Cell $\pm$ S.E.[1,2]		
		A $CaCl_2$	B Urine + Ca^{2+}	C Conc. urine
Baseline Control	none	0.40 $\pm$ 0.16	0.16 $\pm$ 0.01	1.62 $\pm$ 0.44
Control	_E.coli_	*43.52 $\pm$ 3.77	*38.29 $\pm$ 5.78 (n=47)	*40.60 $\pm$ 6.41
Test A	_E.coli_ + THP	**27.54 $\pm$ 3.46	**18.08 $\pm$ 2.77	**12.66 $\pm$ 1.44
B	_E.coli_ + THP + Ca^{2+}	41.04 $\pm$ 4.51 (n=49)	30.38 $\pm$ 2.79	30.38 $\pm$ 5.49

[1]Standard Error of the Mean.

[2]n=50, unless otherwise stated.

*Statistically Different from Baseline ($p<0.05$).

**Statistically Different from Control ($p<0.05$).

TABLE 3

REVERSAL OF THE EFFECT OF CALCIUM ON THE ADHERENCE ACTIVITY OF TAMM-HORSFALL GLYCOPROTEIN ACHIEVED *IN VITRO*.

Group	Epithelial Cell Treatment	Mean Bacteria per Cell $\pm$ S.E.[1,2]
Baseline Control	none	1.12 $\pm$ 0.37
Control	*E.coli*	*38.68 $\pm$ 3.95
Test A	*E.coli* + THP	**14.40 $\pm$ 2.07
B	*E.coli* + THP + Ca^{2+}	29.42 $\pm$ 3.64
C	*E.coli* + $THP_{(3.3\%\ CJC)}$	**16.52 $\pm$ 2.07
D	*E.coli* + $THP_{(3.3\%\ CJC)}$ + Ca^{2+}	**12.94 $\pm$ 1.91
E	*E.coli* + $THP_{(10\%\ CJC)}$	**15.58 $\pm$ 2.32
F	*E.coli* + $THP_{(10\%\ CJC)}$ + Ca^{2+}	**15.74 $\pm$ 1.83

[1] Standard Error of the Mean.

[2] n=50

* Statistically Different from Baseline ($p<0.05$).

** Statistically Different from Control ($p<0.05$).

CHAPTER IV

Discussion

E. coli, the most frequent cause of urinary tract infections, accounts for approximately 80% of all infections (Kunin, 1975). Essential for initiating and sustaining an infection on a mucosal surface is the ability of the bacteria to adhere to that surface. It has been demonstrated with uropathogens that adhesive ability is linked to the virulence of the organism. *E. coli* isolated from symptomatic urinary tract infections adhere in larger numbers to uroepithelial cells than do *E. coli* isolated from urine of patients with asymptomatic urinary tract infections (Parsons and Schmidt, 1980). Although other factors contribute, adhesive ability has been linked to bacterial fimbriae. A significant correlation was found between the ability of the bacteria to adhere to human uroepithelial cells and the presence of fimbriae on *E. coli*. Bacteria with little or no fimbriae present did not adhere to the uroepithelial cells (Svanborg-Eden and Hansson, 1978). The type of fimbrial interaction most often found between uropathogenic *E. coli* and uroepithelial cells is one that is mannose sensitive. Mannose sensitive fimbriae are

classified as type 1 (Svanborg-Eden, 1986). Although the types and numbers of types are known to vary for a given bacterial population, most *E. coli* that cause urinary tract infections contain type 1 fimbriae (Hagberg *et al.*, 1981).

Kuriyama and Silverblatt (1986) demonstrated that purified THP could be retained on a column which contained purified type 1 fimbriae held in the stationary phase and could be eluted from the column using methylα-D-mannoside. The THP-fimbriae interaction was further supported by electron microscopy studies which revealed that non-fimbriated *E. coli* did not appear to bind THP and type 1 fimbriated *E. coli* did. Mannose residues of THP bind to fimbrial sites and through this interaction THP coats the bacterial surface. Since THP synthesized in the kidney is excreted daily into the urine (Fletcher, 1972), it would appear to have the potential to be a natural antiadherence agent in the bladder. This observation served as the basis for testing THP in adherence assays to determine if the protein could prevent the adherence of *E. coli* to uroepithelial cells.

For these adherence assays an isolate of *E. coli* bearing type 1 fimbriae was necessary. Clinical isolates of *E. coli* were screened for mannose sensitivity and an isolate found to be mannose sensitive was used in all assays. To maximize adherence, the bacteria were

cultured on brain heart infusion and tryptic soy media (Kuriyama and Silverblatt, 1986) and uroepithelial cells for the adherence assay were harvested only from fresh urine. Urine sediment contains cells of the squamous type thought to be derived from urethral and outer genital areas and transitional cells thought to be derived from ureters and bladder (Svanborg-Eden, 1986). Because bacteria reach the urinary tract after colonization of vaginal and periurethral areas (Stamey and Sexton, 1975), cells of these origins are relevant in studying the adherence phenomena.

Since bacteria are thought to adhere to uroepithelial cells through recognition of mannose residues on the bacterial surface (Svanborg-Eden, 1986), it would follow that if the sites on the fimbriae were blocked with THP, the bacteria would be less able to adhere to uroepithelial cells. This study demonstrates that THP has the ability to act as an antiadherence agent for mannose sensitive *E. coli*. In every case where THP was allowed to interact with the bacteria, prior to their use in an assay, those bacteria showed a reduced ability to adhere to uroepithelial cells. This observed decrease in adherence of THP treated *E. coli* was statistically significant ($p < 0.05$). Kuriyama and Silverblatt (1986) have demonstrated that a minimum of 2.5 g/mL of THP is required to coat the bacteria. For THP samples tested in this investigation,

the concentrations were consistently greater than 60 g/mL and therefore concentration was not adjusted for use in assays. Fletcher (1972) has shown that the concentration of THP in urine is approximately 25 g/mL. It therefore appears that there is sufficient THP in the urine to effectively neutralize the adherence of _E. coli_ to bladder epithelial cells. The results of this study support the observations of Orskov _et al_. (1980) who had shown that THP traps and washes the _E. coli_ out of the bladder.

Calcium ions are known to bind to THP and increase the viscosity of this glycoprotein (Fletcher, 1972). Work by Stevenson _et al_. (1971) demonstrated that 5-7 mM $CaCl_2$ caused marked increases in viscosity. These observations suggested that calcium might have the potential to affect the observed antiadherence activity of THP. Three different sources of calcium ions were tested to determine if they would affect this activity. These included $CaCl_2$, urine to which $CaCl_2$ was added and a concentrated urine sample containing calcium. The first source tested was a 5.0 mM $CaCl_2$ solution. It was observed that this concentration of $CaCl_2$ significantly decreased the antiadherence activity of the THP. To determine if this decrease in antiadherence activity might occur in the bladder, $CaCl_2$ was added to urine and incubated with THP. Again, a decrease in antiadherence activity was observed. Urine

concentrated to yield an effective calcium concentration of 5.0 mM had the same effect. Thus, calcium ions significantly decrease the ability of THP to prevent the adherence of *E. coli* to uroepithelial cells. It also appears that urine does not decrease the effect of calcium on the THP and therefore what we have demonstrated *in vitro* should potentially also occur *in vivo*.

Since THP, in excess calcium, will increase in viscosity it would appear that this change is related to its inability to coat the bacteria. The inability of the calcium treated THP to coat could be due to a physical hindrance, i.e. the sites on the THP are still present but are prevented by steric hindrances from interacting with the fimbriae. As a consequence, the THP may form a partial or ineffective coat on the fimbriae (bacteria) and some fimbrial adhesins may still be accessible to the epithelial cells. At higher concentrations of calcium, where gel formation occurs, the mannose residues of the THP may be inaccessible. Or the calcium, in binding to the THP, may block the sites that would be recognized by the bacterial fimbriae.

The observed interaction of THP with calcium suggests that the concentration of calcium in urine at any given time becomes important when estimating the protective potential of THP. The normal concentration of calcium in

the urine of healthy individuals ranges from 0.5-6.0 mM (Bradley and Benson, 1974). The concentration of calcium that affects THP falls within the upper limits of this normal range. It is therefore conceivable that, on a daily basis, the integrity of the THP as an antiadherence agent varies due to changes in the calcium concentration. In the case of normal calcium excretion, where the concentration may vary daily within the normal range, THP may be able to maintain a net vigilance against any colonizing bacteria. In cases where the concentration of calcium is always elevated, the THP may not be able to block adherence and any adhering bacteria have a chance to colonize and possibly cause infection. The link between the adhesive ability and the virulence of uropathogens has been strongly supported in past works on adherence. *E. coli* that reach the urinary tract are selected out of the random *E. coli* fecal flora and represent a clonal population of bacteria. One of the traits that these uropathogenic *E. coli* possess is the ability to attach to human uroepithelial cells (Svanborg-Eden, 1986).

A defect in calcium metabolism may result in increased urinary calcium levels. This hypercalciuric state may reflect an abnormally high dietary absorption of calcium or abnormal renal handling of filtered loads (Bondy and Rosenberg, 1974). Dietary calcium supplementation may

also contribute to elevated calcium levels in the urine. Based on the observations presented here, it would appear that this increase in dietary calcium could lead to an accompanying increase in urinary calcium with a potential negative effect on the THP antiadherence activity. This has particular significance for females since they are most likely to use calcium supplements and are at the highest risk for urinary tract infections. The activity of the naturally occurring THP and the relationship of urinary calcium levels to that activity raises some interesting questions. Do individuals with chronic urinary tract infections have elevated urinary calcium excretion? Could management of urinary calcium help restore THP activity and prevent further or recurrent urinary tract infection?

One approach to managing urinary calcium levels would be to find a naturally occurring urine constituent that could bind calcium. Organic acids, for example, are found in the urine in varying concentrations (Oser, 1965) and have the potential to bind divalent cations including calcium (Vogel *et al.*, 1984). Citric acid has been shown to bind free calcium and remove it from solution (Erme, MS thesis). A good dietary source of citric acid is cranberry juice cocktail (CJC). This juice is known to contain high concentrations of citric acid and other organic acids.

Elevated levels of citric acid have been demonstrated in urine 2-3 hours after ingesting 8 oz CJC (Erme, MS thesis). In a previous study (Sobota, 1984), CJC and metabolites were shown to have a direct effect on the adherence of *E. coli* to uroepithelial cells presumably by interfering with some surface component of *E. coli*. Perhaps CJC also has an indirect effect on the adherence of *E. coli* by binding excess urinary calcium and allowing THP to have antiadherence activity.

To test the potential of CJC to protect THP from the effect of calcium the juice was added to urine preparations in 1:10 and 1:30 dilutions prior to extraction of THP. In control samples it was demonstrated that CJC had no effect on the antiadherence activity of THP. When calcium was added to the preparations, the expected decrease in the THP antiadherence activity did not occur. It thus appears that the juice has the ability to prevent the interaction of the calcium with THP. Based on previous observations it appears probable that the organic acids in CJC are complexing the excess calcium, thereby providing protection for the activity of the THP. Preparation of the THP does however include a dialysis step using dialysis tubing with a molecular weight cutoff of 30,000. During the dialysis process it might be expected that free organic acids having molecular weights of several hundred daltons would be lost

in the dialysate. The interaction of the CJC with THP, then, becomes unclear. If it is the organic acids, they may in some permanent association, have blocked the sites for calcium or competitively bound the calcium so as to protect the THP. It is also conceivable that some other factor in the CJC is responsible. All the experimental work described to this point was performed *in vitro*. The ultimate test of a suggested treatment is however *in vivo* performance. Positive results were achieved here also. Ingestion of 8 oz of CJC resulted in protection of the THP. THP collected 2 hours after drinking CJC retained its anti-adherence activity. In contrast, urine collected prior to ingestion of the juice showed the normal response to calcium. It appears that the CJC or some factor in the juice or some metabolic product accumulates in the urine and is able to protect the THP. The specific effect on the THP is unclear. It may involve a permanent structural change in the protein or perhaps more likely a transient interaction of the protein with some small molecule that protects the protein from the effects of the calcium.

This study has shown that a naturally occurring urinary glycoprotein, Tamm-Horsfall glycoprotein, has the ability to reduce the adherence of mannose sensitive *E. coli* to uroepithelial cells. When calcium in present with the THP, this antiadherence activity is negated. The

negative effect of calcium on the activity of THP suggests that urinary calcium levels are significant when estimating the antiadherence potential of THP. CJC has been shown to provide protection, both *in vitro* and *in vivo*, against the effect of calcium on THP activity.

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