Prevention of Urinary Tract Infection in Rats with an Indigenous *Lactobacillus casei* Strain

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Our previous studies have shown that indigenous bacteria are able to block the in vitro attachment of uropathogenic bacteria to human uroepithelial cells. In the present study, we applied the concept of competitive exclusion to an animal model. A chronic urinary tract infection was established in female rats with bacteria incorporated into agar beads injected periurethrally into the urinary bladder via a no. 3 French ureteral catheter. Five strains of uropathogenic organisms were used in the first set of experiments, and their colonization of the bladder and kidneys of the animals was confirmed up to 2 months after injection. The uropathogens stimulated an immune response, detected by serum antibodies against the uropathogens, and an inflammatory response noted in sections of the kidneys stained with hematoxylin and eosin. Using this animal model, we established the persistent adherence of bacteria in the urinary tract without the need for creation of obstruction or implantation of a foreign body. In a second set of experiments, an isolate of *Lactobacillus casei* GR1 taken from the urethra of a healthy woman was incorporated into agar beads, instilled within the rat bladders on day 1, and then swabbed twice weekly for 21 days onto the introitus before challenge with uropathogens instilled into the urinary bladder. In 21 of 25 animals, no uropathogenic bacteria were recovered from the bladder and kidney tissues up to 60 days after challenge, and no immune response was detected. Our results show that *L. casei* prevented onset of urinary tract infection in 84% of the animals tested. The lactobacilli appeared to exclude the uropathogens from colonizing the urinary tract, within the first 48 hours after challenge, and the net effect was a complete eradication of bacteria from the uroepithelium. It is hoped that the demonstration of a protective role for indigenous bacteria in preventing urinary tract infection in an animal model will lead to the application of this technology to prevent recurrent urinary tract infection in female patients.

A number of studies have demonstrated the importance of bacterial adherence to epithelial surfaces in the initiation and maintenance of urinary tract infection (UTI) in humans (2, 15, 20, 25, 29). Our recent investigations have shown that indigenous bacteria adhere to human uroepithelial cells in vitro and that they are able to competitively exclude the attachment of uropathogens (5). The adherence of an indigenous *Lactobacillus* strain to uroepithelial cells was mediated by lipoteichoic acid, and the competitive exclusion phenomenon appeared to be caused by steric hindrance (6). Furthermore, *Lactobacillus* cell wall fragments were also capable of inhibiting uropathogenic adherence to uroepithelial cells (6). The aim of the present study was to devise an animal model to test the blockage effect of viable whole cells of *Lactobacillus* sp. in preventing uropathogenic colonization of the urinary tract.

Several animal models have been described to investigate the role of mucopolysaccharides (18), the importance of immune responses (30), and the role of type 1 fimbriae (8, 14) and P-fimbriae (11, 12, 22) in the pathogenesis of UTIs. We now describe the establishment of a nonobstructive, chronic UTI in female rats with bacteria incorporated into agar beads. In addition, we describe the prevention of infection in these animals by using an indigenous strain of *Lactobacillus* sp. instilled within the bladder and onto the vaginal introitus and urethra before challenge with the uropathogens.

MATERIALS AND METHODS

Bacteria. Five strains of uropathogenic bacteria were isolated from the urine of patients with chronic cystitis (4) and stored at −70°C on brain heart infusion agar slants. These comprised one *Escherichia coli* strain possessing a mannose-sensitive adhesin (MS), one *E. coli* strain possessing a mannose-resistant adhesin (MR), one encapsulated *Klebsiella pneumoniae* strain, one *Pseudomonas aeruginosa* mucoid (M) strain, and one *Pseudomonas aeruginosa* nonmucoid (NM) strain. An indigenous strain of *Lactobacillus* sp. was isolated from the urethra of a healthy adult female, and stored on MRS *Lactobacillus* growth media (Bacto; Difco Laboratories, Detroit, Mich.) at −70°C (5, 6). This strain was identified in our laboratory as *Lactobacillus casei* var. *rhamnosus* GR1, using methods described in the VPI (Virginia Polytechnic Institute, Blacksburg, Va.) manual. All strains of bacteria were cultured at 37°C in filter-sterilized urine, and this was supplemented with 0.5% glucose and 0.5% lactose for *Lactobacillus* growth. The organisms were harvested by centrifugation and suspended to a concentration of 10^{11} bacteria per ml in phosphate-buffered saline (PBS; pH 7.1).

Bacterial incorporation into agar beads. Preliminary experiments had shown that the animals were able to clear bacterial suspensions of uropathogens in PBS from the bladder after periurethral instillation. Accordingly, the bacteria were incorporated into agar beads in an attempt to obtain persistent bacterial colonization of the urothelium. The agar beads were prepared by using a method similar to that described by Cash et al. (3) and Klinger et al. (16). A 1-ml bacterial solution was added to a 1-ml solution of 2%

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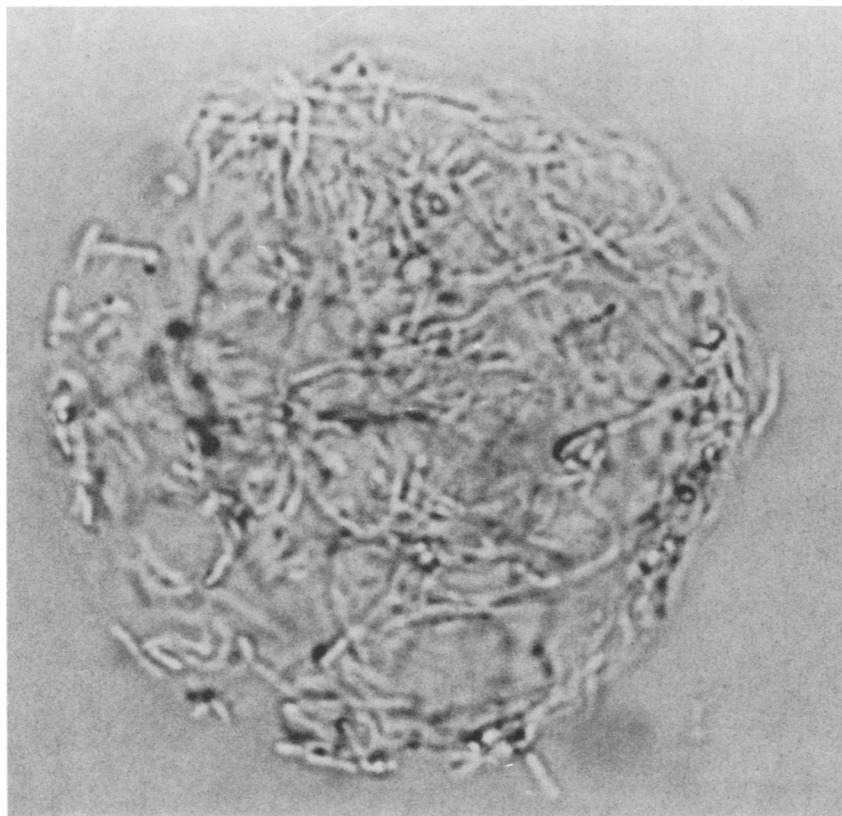
FIG. 1. *L. casei* incorporated into an agar-coated bead. The beads were uniform, although varying in size, and the organisms were seen to protrude from the thin capsule (×2,000).

agar and vortexed; 3 ml of peanut oil was then added. The mixture was vortexed, and the tubes were placed in an ice bath for several minutes to allow the beads to form, after which PBS was added and the samples were centrifuged four times at $1,200 \times g$ to remove the excess oil. The beads were examined by light microscopy and were found to be well formed and to contain large numbers of bacteria, many of which were seen to extend outside the agar coat (Fig. 1). They ranged in size from 20 to 150 μm in diameter (mean, 48 μm). There were approximately 5×10^3 agar beads (mean of 10 samples counted in a hemacytometer) in a 0.05-ml inoculum sample containing 5×10^9 bacteria. Allowing for the presence of free bacteria not incorporated into beads and for the differences in bead sizes, this represents up to 10^5 to 10^6 organisms per bead.

Animals. Female Sprague-Dawley rats (200 to 250 g; Charles River Breeding Laboratories, Quebec, Canada) were housed in The Best Institute, University of Toronto, with two animals per cage and food ad libitum.

Infection procedure. A total of 25 animals was anesthetized by intramuscular injection of 0.2 ml of sodium pentobarbitol (M.T.C. Pharmaceuticals, Mississauga, Canada), and a peanut oil-coated no. 3 French ureteral catheter was inserted into the bladder. A 0.05-ml quantity of the agar bead uropathogenic bacterial suspension (*E. coli* MS, *E. coli* MR, *K. pneumoniae*, *P. aeruginosa*, M, or *P. aeruginosa* NM) was injected into the bladder (approximately 5×10^9 bacteria) via the catheter, and the rats were placed in their cages, where they recovered to full activity within 2 to 4 h. The animals were sacrificed at intervals up to 60 days post-challenge (one rat from each group at 1 day, 1 week, 3 weeks, 1

month, and 2 months after injection) by intramuscular injection of 1 to 2 ml of sodium pentobarbitol. The bladder and kidneys were removed and homogenized in 1 ml of PBS and cultured for the presence of uropathogens by serial dilution plating on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). The bacterial count was expressed as the number of CFU per entire tissue. Tissue specimens were also collected, sectioned, and stained for histological examination. Blood samples were collected from the rats at time of sacrifice and serum was then separated from the clotted erythrocytes and stored at 4°C. Twofold dilutions of sera were tested for agglutination against the five strains of bacteria used in the animal experiments to further assess whether the organisms had infected the animals. Lactobacilli were incorporated into agar beads and instilled into the urinary tract of five animals, and the colonization of the urinary tract was monitored over 2 months, as described for the uropathogens. A total of 25 rats was used as controls, in groups of five, as follows: (i) instilled with PBS, (ii) instilled with saline, (iii) and (iv) instilled with sterile agar beads, and (v) left untreated. The controls were not challenged with bacteria.

Treatment with lactobacilli before challenge with uropathogens. Lactobacilli (5×10^9) incorporated into agar beads were instilled within the rat bladders (as described for the uropathogens) and swabbed over the introital area at this time and twice weekly for 3 weeks to enhance colonization of the uroepithelium. The animals were then challenged with periurethral injection of one of five strains of uropathogenic bacteria incorporated into agar beads. Animals were sacrificed at intervals for 60 days, and the numbers of bacteria

TABLE 1. Recovery of viable uropathogens from the urinary
bladder of female rats[a]

Bacterial strain	Log counts of viable bacteria recovered from bladder at time after transurethral injection of bacteria:				
	1 day	1 wk	3 wk	1 mo	2 mo
E. coli MS	9.3	9.3	Proteus sp.	6.9	7.3
E. coli MR	9.8	9.6	10.0	5.9	6.0
K. pneumoniae	7.0	4.9	10.3	10.3	6.9
P. aeruginosa NM	10.3	10.3	10.0	5.6	5.8
P. aeruginosa M	10.0	10.0	3.3	6.9	6.9

[a] 5×10^9 bacteria were instilled into the bladder on day 0. The counts represent total bacteria recovered from the bladder.

colonizing the bladder and kidneys was measured by growth of the homogenate on Trypticase soy agar. The lactobacilli were distinguished from the uropathogens by growth on MRS *Lactobacillus* medium and by colonial morphology and Gram stain.

Control rats consisted of a repeat of the previous controls, (i to v) in groups of five animals instilled with PBS, saline, sterile beads (×2), and untreated. Additional controls consisted of animals (vi) pretreated with sterile agar beads before challenge with uropathogens, (vii) 10 animals pretreated with lactobacilli and challenged with PBS, and (viii) 10 animals treated with lactobacilli and then left unchallenged. Specimens were examined histologically for inflammatory cells, and serum was collected and tested for the presence of antibodies against the uropathogens.

Histological examination of tissues. The urinary bladders and kidneys were fixed in Brazil fixative (1 g picric acid, 150 ml of 80% ethanol, 60 ml of 37% formaldehyde, 15 ml of glacial acetic acid) immediately and for 4 h after removal from the sacrificed animals. The tissues were then washed in 70% alcohol, processed through graded alcohols and xylene, infiltrated with tissue prep wax (Fisher Scientific Co., Pittsburgh, Pa.), embedded and sectioned to 4 μm, dried for 1 h at 60°C, added to water, and finally stained with hematoxylin and eosin by using standard techniques (7).

RESULTS

Bacterial colonization of the urinary tract. Preliminary experiments established the optimal infection protocol procedure for the rats, namely, the use of bacteria incorporated in agar beads, the inoculum size (0.05 ml with 5×10^9 bacteria), the method of injection of the organisms, and the time intervals before sacrifice. The results from these preliminary experiments are not included. Electron microscopy examination confirmed that the beads contained large num-
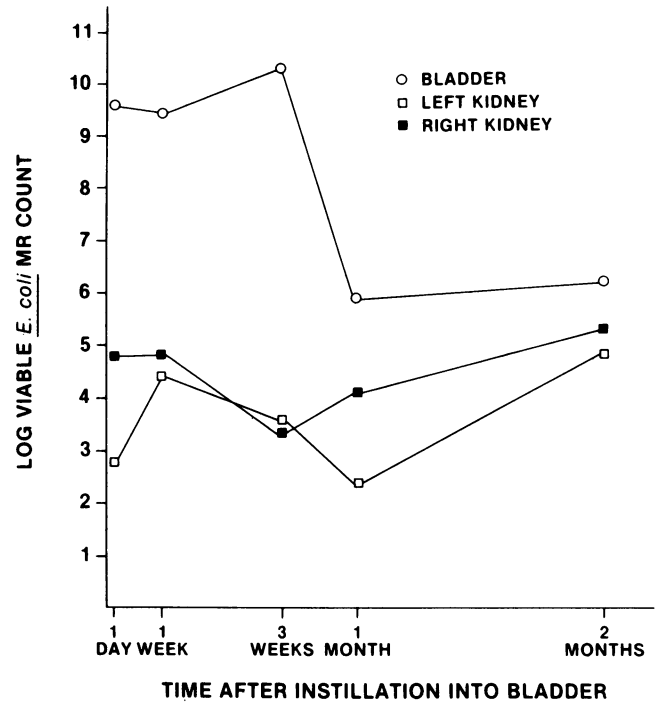

FIG. 2. Viable bacterial counts for *E. coli* MR strain from the bladder and kidneys of five female rats 1 day, 1 week, 3 weeks, 1 month, and 2 months after instillation of the uropathogens into the bladder.

bers of bacteria, and showed that the agar beads were attached to the mucosal tissues 1 day after instillation but that they were absent 7 and 21 days after instillation (K. Lam and J. W. Costerton, manuscript in preparation). By 7 and 21 days, the gram-negative uropathogens were present in microcolonies on the tissue surfaces, as seen by electron microscopy, showing that colonization had taken place and that the subsequent bacterial counts from the tissues of animals sacrificed at 1 week, 3 weeks, 1 month, and 2 months were not simply due to bacterial survival within the beads. The viable counts of each uropathogen colonizing the bladder and kidneys varied over 2 months, as seen from Tables 1 and 2 and as presented for an *E. coli* MR strain (Fig. 2). A chronic infection state was induced and maintained for up to 2 months after instillation of the uropathogens into the bladder. The infection appeared to be an ascending one and not simply due to forced ascension of the beads into the kidneys at the time of instillation as the uropathogens were recovered in high numbers from the kidneys (Table 2),

TABLE 2. Recovery of viable uropathogens from the kidneys of female rats[a]

Bacterial strain	Log counts of viable bacteria recovered from kidneys at time after transurethral injection of bacteria[b]:									
	1 day		1 wk		3 wk		1 mo		2 mo	
	L	R	L	R	L	R	L	R	L	R
E. coli MS	4.6	3.8	3.5	3.8	Proteus sp.	Proteus sp.	4.6	4.9	6.6	7.3
E. coli MR	2.9	4.9	4.6	4.9	3.6	3.3	2.5	4.0	4.9	5.3
K. pneumoniae	3.3	3.6	4.6	3.6	0	0	4.6	7.6	6.9	7.3
P. aeruginosa NM	4.9	3.3	3.3	4.0	3.6	4.9	2.6	6.6	7.3	8.9
P. aeruginosa M	4.6	3.8	4.6	7.9	2.9	8.6	7.3	2.9	6.9	7.3

[a] 5×10^9 bacteria were instilled into the bladder on day 0. The counts represent total bacteria recovered from each kidney.
[b] L, Left kidney; R, right kidney.

whereas the lactobacilli did not colonize the kidneys. In several animals, large numbers of pus cells were recovered from the bladder up to 1 month after inoculation of the uropathogens. No pus cells were found in animals inoculated with the *L. casei* despite colonization of the bladder with these indigenous bacteria 1 day (log viable count, 3.8), 1 week (log viable count, 8.9), 3 weeks (log viable count, 4.8), and for up to 1 month (log viable count, 5.3) after instillation. The bladders and kidneys of 23 of the 25 control animals were free of bacteria. The bladder of two animals and the right kidney of one animal were colonized by *Proteus* sp., which probably originated from the rat gastrointestinal tract.

Antibody was detected against the infecting uropathogens 1 month after challenge. The reciprocal titers obtained were 128 for *E. coli* MR and *K. pneumoniae* and 256 for *E. coli* MS and the two *P. aeruginosa* strains. The analysis of kidney specimens obtained 21 days after challenge with the uropathogens and stained with haematoxylin and eosin showed the presence of acute (polymorphonuclear leukocytes) and chronic (lymphocytes) inflammatory cells. There was no inflammatory response observed in the kidney sections prepared from control rats or from those injected with lactobacilli.

Prevention of UTI in animals pretreated with *L. casei* GR1 before challenge with uropathogens. Of the 25 animals which were pretreated with lactobacilli before challenge with uropathogens, only 1 animal was infected with *E. coli* MS (at day 3 or 48 h after challenge; bladder log viable count, 8.9; left kidney, 8.8; right kidney, 8.5) and 3 animals were infected with *Proteus* sp. (at day 3, 3, and 21 after challenge) which had apparently originated from the rat intestine. Uropathogens were not cultured from the bladders and kidneys of the other 21 animals, at 3, 10, 21, 35 and 60 days after challenge. Lactobacilli remained in the urinary tract of only three animals after uropathogenic bacterial challenge. One animal challenged and infected with *E. coli* MS on day 3 was colonized with lactobacilli (bladder log viable count, 8.0; left kidney, 8.3; right kidney, 8.7). One animal challenged with *E. coli* MR but infected with *Proteus* sp. on day 3 was colonized with lactobacilli (bladder log viable count, 4.7; left kidney, 4.6). The bladder of one animal challenged but not infected with *P. aeruginosa* mucoid strain was colonized with lactobacilli (log viable count, 5.3) on day 35. The protected animals did not have antibody against the uropathogens or inflammation of the urinary tract tissues. The results showed that 84% of the animals had been protected from onset of UTI. Animals instilled with sterile agar beads before challenge with the uropathogens developed UTI. Of the 25 repeat group of control animals, 21 were free of uropathogens; 4 were contaminated with *Proteus* sp. Lactobacilli were recovered from the kidneys and bladders of only two animals treated with lactobacilli and challenged with PBS at 1 and 3 weeks after challenge. Only lactobacilli were recovered from the urinary bladder of the 10 animals treated with lactobacilli and unchallenged for up to 1 month after instillation.

DISCUSSION

A chronic UTI was established in female rats by instilling five different gram-negative uropathogens into the bladder, using a technique which did not require the creation of an obstruction or implantation of a foreign body. The model is similar to that described for respiratory infections by Cash et al. (3) and Klinger et al. (16) and utilizes bacteria incorporated into agar beads. Our preliminary experiments with bacteria suspended in PBS were not effective in establishing bacterial colonization of the uroepithelial mucosa. It appeared that the agar coating enabled the organisms to resist the clearance mechanisms of the normal bladder (voiding of urine) and resulted in bacterial colonization of the urinary epithelium. The infection in each animal was an ascending one, and all five uropathogens were found to colonize the kidneys. The exact nature of the host cell receptivity for the five uropathogens was not determined. However, other studies have shown that type 1 fimbriae (MS adhesins) facilitate the development of UTI in mice (14) and mediate infection of the renal pelvis in rats (9) and that P-fimbriated *E. coli* attach in large numbers to specific receptors in mice and monkeys (11, 12, 21). In the present rat model, the initial bacterial interaction with the host cells may have been caused nonspecifically by agar coating. Alternatively, as the bacteria were protruding from the agar beads (Fig. 1), bacterial adherence factors (MS, MR adhesins, and capsule) expressed by the uropathogens may have mediated the colonization.

The chronic nature of the UTI was further detected by the presence of acute and chronic inflammatory cells only in the kidney tissues of unprotected animals challenged with uropathogens. In addition, an antibody response was detected against the gram-negative pathogens.

An isolate of *L. casei* GR1 taken from the distal urethra of a healthy woman was found to colonize the rat bladder for up to 1 month after instillation, but the organisms did not appear to colonize the kidneys. No inflammatory response was detected in the mucosal tissues of the animals. Our previous studies had shown that *Lactobacillus* whole cells (5) and cell wall fragments (6) attached to uroepithelial cells in vitro. In addition, these studies showed that the normal flora could competitively exclude uropathogens from adhering to uroepithelial cells. In the present study, this competitive exclusion concept was tested by pretreating the introitus, urethra, and bladder of female rats with an indigenous *Lactobacillus* strain before challenge with uropathogens. The result was an effective inhibition of uropathogenic colonization in 84% of the animals and the prevention of UTI. A number of the rats were infected with *Proteus* sp., which appeared to originate from the feces of the rats. As these pathogens were not recovered from the urinary tract of all animals pretreated with *L. casei*, it is possible that in some instances the lactobacilli were able to exclude the *Proteus* sp. Our earlier in vitro experiments had established that no single strain of indigenous bacteria was able to completely inhibit all strains of uropathogens from attaching to uroepithelial cells (5, 6). It is therefore not surprising that the distal urethral *Lactobacillus* strain used in the present study did not prevent the in vivo colonization by *Proteus* sp. in three of the animals. There is a need to test a large number of isolates of the normal flora, to select a few strains with an effective blockage capacity against uropathogens, including *Proteus* sp. In addition, it would be interesting to test the use of lactobacilli to prevent UTI in other animal models, such as mice and monkeys.

The inhibition of uropathogenic adherence in the rats may have been due to a competitive exclusion phenomenon, similar to that found in our in vitro studies. The mechanism for this inhibition in vitro appeared to have been a steric hindrance effect (6). Alternatively, in the in vivo situation other factors may have been involved, and we cannot be certain as to the exact mechanism responsible for the blockage effect in the rats. However, this was quite dramatic and appeared to take place within the first 48 h after challenge. The net effect was an eradication of the uropathogens and

the prevention of a UTI. The lactobacilli were also eradicated from the bladder, and therefore, to have withstood a second uropathogenic challenge, more lactobacilli would have to have been reinstilled into the rat bladder.

The competitive exclusion phenomenon has been investigated in relation to respiratory tract infection. In one study, streptococci isolated from the pharynx were found to prevent the growth of other bacteria in vitro, and this was thought to explain the maintenance of a bacterial status quo in vivo (28). Using this concept of competitive exclusion in the form of bacterioprophylaxis, Shinefield et al. (26) were able to stem an epidemic of virulent staphylococcus in infants.

Lactobacillus organisms have been used previously to treat diarrhea (1, 13), hepatic encephalopathy (19) and vaginitis (23). In addition, lactobacilli have been found to inhibit the adherence of *Candida albicans* to vaginal epithelial cells (27). It has been well established that lactobacilli are predominant indigenous bacteria in the cervix (10), vagina (24) and urethra (17) of healthy women. Our finding that lactobacilli were able to prevent uropathogenic colonization of the rat urinary tract suggests that the normal flora could be used in the form of viable whole cells or perhaps as cell wall fragments incorporated into a suitable suspension to colonize the urogenital tract and prevent the onset of UTI in females.

ACKNOWLEDGMENTS

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