The PapG-Adhesin at the Tip of P-Fimbriae Provides *Escherichia coli* with a Competitive Edge in Experimental Bladder Infections of Cynomolgus Monkeys

By Jan Winberg,* Roland Möllby,‡ Jörgen Bergström,§ Karl-Anders Karlsson,§ Iréne Leonardsson,§ Maan A. Milh,§ Susann Teneberg,§ Dave Haslam,‖ Britt-Inger Marklund,¶ and Staffan Normark‡

*From the *Department of Woman and Child Health, Karolinska Hospital and Karolinska Institute, 171 76 Stockholm, Sweden; ‡Microbiology and Tumorbiology Center, Karolinska Institute, 171 77 Stockholm, Sweden; §Department of Medical Biochemistry, Göteborg University, 413 90 Göteborg, Sweden; ‖Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110; and ¶Department of Microbiology, Umeå University, 901 87 Umeå, Sweden*

Summary

Human urinary tract infection is an infectious disease that depends on a series of host–microbial interactions. The bacteria first colonize the colon and then the periurethral/vaginal areas; they ascend to and infect first the bladder and then the kidneys. Expression of *Escherichia coli* P-fimbriae constitutes the strongest correlation to renal pathogenicity, but is also related to first-time cystitis in children. The role of P-fimbriae in the preceding steps in the infectious process is unknown. To examine this, we constructed, from a P-fimbriated *E. coli* strain with a class II G-adhesin preferentially binding to globoside, one isogenic mutant lacking the G-adhesin and another isogenic mutant in which we replaced the *papG* class II allele with a class III adhesin preferentially binding to the Forssman antigen. We report here the comparison of the adhesin knockout mutant (DS17-8) and the class-switch mutant (DS17-1) with the wild-type (DS17) for in vivo colonization of the gut, vagina, and bladder of cynomolgus monkeys. It was recently shown that the class II tip G-adhesin is a prerequisite for acute pyelonephritis to occur in the monkey model in the absence of other kidney-specific adhesins or obstruction of the urinary flow. Here we show that it is not required for bladder infection but gives a competitive advantage in mixed infections. In the vagina and colon, the G-adhesin gives no competitive advantage.

The process that results in urinary tract infection (UTI)[1] is initiated by colonization in the colon of the infecting *Escherichia coli* strain (1, 2). This strain next colonizes the periurethral area and the vaginal introitus (3, 4). From there, the organism may ascend to the bladder and eventually to the kidney. The infecting *E. coli* strain generally expresses a series of virulence factors. The strongest correlation to uropathogenicity is the expression of P-fimbriae that are found on ~90% of urinary isolates from anatomically healthy children with their first acute pyelonephritis as compared with 5–10% among the commensal fecal *E. coli* flora (5, 6). In children suffering from their first bladder infection, ~50% of the urinary isolates are P-fimbriated (7). The role of P-fimbriae in colonization of the gut and the periurethral/vaginal areas is unknown.

P-fimbriae are heteropolymeric fibers extending out from the bacterial surface that consist of a rigid shaft and a flexible tip fibrillum (8). The receptor binding PapG-adhesin protein is a very minor constituent of the pilus located at the distal end of the linear tip fibrillum (9–11). *E. coli* may express P-fimbriae with three distinctly different G-adhesins recognizing different glycosphingolipid isoreceptors in the globoseries family (12, 13). The class I G-adhesin is rare in *E. coli* and binds preferentially to globotriaosylceramide (14), whereas *E. coli* with a class II adhesin preferentially bind globoside and dominate in human UTI. P-fimbriae with a class III adhesin bind to the Forssman antigen and to globoA but significantly less to globoside, the dominating isoreceptor in the human kidney (12, 13, 15). The class III G-adhesin is common among canine UTI isolates, which correlates with the abundance of the Forssman antigen in the dog kidney (12), but *E. coli* with class III G-adhesin have also

[1] *Abbreviation used in this paper:* UTI, urinary tract infection.

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been found in humans with cystitis (16). It has been suggested that individuals who are blood group A and secretor positive would be susceptible to P-fimbriated *E. coli* with a class III adhesin because of the content of globoA on their uroepithelial cells (17).

In a recent set of experiments using cynomolgus monkeys as an infectious model, we could demonstrate that a P-fimbriated *E. coli* isolate with a class II G-adhesin was able to cause pyelonephritis, whereas its isogenic mutant producing fimbriae lacking the tip adhesin was not. Both wild type and mutant, however, were able to cause cystitis when inoculated separately into the bladder (18).

Aims. We wanted to investigate whether there is a competitive advantage in the bladder for bacteria expressing the PapG-adhesin, and to examine a possible role for both the class II and class III G-adhesin in the preceding steps of an ascending UTI, i.e., the colonization of the gut and the vagina. To accomplish these goals, we constructed a second isogenic mutant from the wild-type strain in which we replaced the *papG* class II allele with an allele expressing a class III adhesin, resulting in an altered receptor binding specificity. Next, we compared the adhesin knockout mutant and the class-switch mutant with the wild type for in vivo colonization of the gut, vagina, and bladder of cynomolgus monkeys.

Materials and Methods

Bacterial Strains

The wild-type *E. coli* strain DS17, originally isolated from a child with acute pyelonephritis, is of serotype 06:K5:H$^-$, expresses P-fimbriae from a single *pap* gene cluster, and expresses type I fimbriae but lacks S-fimbriae and Afa-1 adhesin. The strain produces hemolysin and is resistant to ampicillin and trimethoprim-sulphonamide but sensitive to ciprofloxacin (18, 19). The mutant derivatives DS17-8 and DS17-1 (see below) were of the same serotype and exhibited the same resistance pattern as the parent strain DS17. *E. coli* HB101 (20) was used for molecular cloning and plasmid propagation, and *E. coli* TG1 was used for M13 cloning and phage propagation.

Construction of the Class-Switch Mutant DS17-1

Strain DS17 carries one *pap* gene cluster encoding P-fimbriae with a class II G-adhesin (21). The procedure to generate mutant strain DS17-8 carrying a 1-bp deletion in the *papG* adhesin gene has been described (18).

To create a class-switch mutant of DS17, we made use of the *prs*G allele previously cloned from strain J96 that encodes a class III G-adhesin (21). To substitute the *pap*G_{DS17} allele with the *prs*G_{J96} allele, a 3.4-kb KpnI-BamHI DNA fragment from plasmid pPAP601 (22) that carries the *prs*E-*prs*G_{J96} genes as well as a 1.2-kb region downstream from the adhesin gene was introduced into the cloning cassette of plasmid pPAP655. This plasmid is a kanamycin-resistant derivative of pMAK705 with a temperature-sensitive replicon permissive at 30°C but nonfunctional at 42.5°C (23). The resulting plasmid pPAP672 was introduced into DS17 by electroporation, and kanamycin-resistant transformants were selected at 30°C. By restreaking transformants on kanamycin-containing plates at 42.5°C, selection for the integration into the DS17 chromosome was achieved. Some of the clones had lost the ability to agglutinate human erythrocytes (22). One such clone was subjected to continuous growth at 42.5°C without antibiotic selection, and kanamycin-sensitive offspring were tested for their ability to agglutinate human and sheep erythrocytes: Unlike class II, the class III G-adhesin mediates binding to sheep rather than human erythrocytes (22). One strain, designated DS17-1, efficiently agglutinated sheep erythrocytes but was negative for hemagglutination of human erythrocytes.

DNA and Other In Vitro Techniques

PCR were performed using the oligonucleotides 5′-CTGAATTCACGGAAGTGATTTCTG-3′ and 5′-CTGGATCCGGCTCCGGATAAACCAT-3′, which are complementary (except for three and two mismatches, respectively, introduced to obtain an EcoRI and a BamHI site) to sequences immediately upstream and downstream from the *prs*G_{J96} gene. PCR amplification with these two oligonucleotides resulted in a 1,060-bp-large fragment. Amplifications were performed with Ampli taq polymerase and the reaction buffers and protocol included in the Gene Amp reagent kit (Perkin-Elmer Corp., Norwalk, CT). The polymerase reactions were carried out on a PHC-2 apparatus (Techne, Cambridge, UK) with a program of 35 cycles: denaturing step, 94°C for 30 s; annealing step, 55°C for 1 min; elongation step, 72°C for 3 min.

The DNA sequence of *pap*G_{DS17} has been reported (21). To confirm the DS17-1 mutant, the 1,060-bp fragment obtained by PCR amplification was separated on a 5% PAGE gel and electroeluted. The ends were trimmed with T4 DNA polymerase and ligated into SmaI-digested M13 mp18. Sequencing was performed using the dideoxy chain termination method (24) and a T7 sequenase kit (Pharmacia AB, Uppsala, Sweden).

Electroporation of DS17 was achieved with a GenePulser apparatus (Bio-Rad Laboratories, Richmond, CA) according to established protocols, with the exception that the preincubation with plasmids on ice was excluded, resulting in an increased transformation frequency.

Immunoelectron microscopy using one hybridoma raised against the class II PapG_{DS17} adhesin (MC 22) and another against the class III (*prs*G_{J96}) adhesin was performed as described (18).

For the binding assays, *E. coli* DS17 and its mutants, DS17-8 and DS17-1, were cultured on colonization factor agar with the addition of 10 μl [^{35}S]methionine (400 μCi; Amersham International, Little Chalfont, UK) at 37°C for 24 h. The bacteria were harvested by scraping, and after two washes with PBS, pH 7.3, the cells were resuspended to 1×10^9 CFU/ml in PBS. The specific activities of the suspensions were ~1 cpm per 100 bacterial organisms.

The microplate binding assay has been described in detail elsewhere (25). Briefly, serial dilutions of selected pure glycosphingolipids in methanol were applied in 96-well microplates (Cooks M24; Nutacon, Holland). When the solvent had evaporated, the wells were blocked with 200 μl of solvent A (2% BSA and 0.1% NaN_3) for 2 h. Thereafter, 50 μl of ^{35}S-labeled bacteria were added per well. After 12 h of incubation at room temperature, the wells were washed six times with solvent A and cut out, and the radioactivity was counted.

In Vivo Colonization Experiments in Monkeys

Healthy, adult, female outbred cynomolgus monkeys (*Macaca fascicularis*) were used. They were fed and housed as described earlier (18).

Intestinal Colonization. Fecal colonization occurred spontaneously after active vaginal colonization (see below). Fecal speci-

mens were obtained by a cotton-tipped swab (26) and were identified as described below for vaginal samples.

Vaginal Colonization. The *E. coli* strains were grown overnight at 37°C on blood agar plates and were then suspended in PBS to a concentration of $\sim 10^9$ CFU/ml. Using a catheter, the vagina was flushed with 3 ml of this suspension containing either DS17, DS17-8, or DS17-1. In 10 experiments, equal numbers of bacteria of two strains were given as a mixture (for details see 27, 28).

Sampling of vaginal specimens was performed daily (weekends excluded) with a sterile cotton swab. The sample was suspended in 1 ml of PBS and vortexed for 20 s, giving a suspension referred to as vaginal fluid. A 0.1-ml sample was then spread on a Cled agar plate (Oxoid Ltd., Hampshire, UK) and incubated for 18 h in 37°C. Colonies with *E. coli* appearance were semiquantitated and identified as DS-17 or its derivatives by antibiotic susceptibility (ampicillin [resistant], trimethoprim-sulphonamide [resistant], ciprofloxacin [sensitive]) and by biochemical fingerprinting method (29). Strain DS17 and its mutant derivatives DS17-8 and DS17-1 were distinguished by human red cell agglutination; P-specific particle agglutination, identifying P-fimbriae with a class II tip adhesin (DS17 only) (30); and sheep red cell agglutination, identifying Prs fimbriae with a class III adhesin (Prs-adhesin) preferentially binding to the Forssman antigen (DS17-1 only). DS17-8, because of the lack of the P-fimbriae tip adhesin, did not agglutinate either of the red cells and was negative in the P-specific particle agglutination test. When two strains had been administered simultaneously by an intravaginal flush, 10 or 20 colonies were randomly chosen from the Cled agar plate and distinguished by their agglutination properties. A successful colonization was defined as persistence of $>10^4$ CFU/ml vaginal fluid for at least six consecutive days.

Bladder Inoculation. Bladder colonization/infection experiments were performed on eight animals (four with DS17-1 and four with a mixture of DS17 and DS17-8), which had never been in contact with any of the three strains used in this study. We have previously reported on bladder inoculations with either DS17 or DS17-8 (18). 1 ml of *E. coli* DS17-1 containing 10^7 bacteria or 1 ml of a mixture of DS17 and DS17-8 (5×10^6 bacteria each) was inoculated into the bladder by means of a urethral catheter. Suprapubic bladder aspiration was used to obtain urine for culture at days 2, 7, 9, and 14 after inoculation or until two negative cultures had been obtained. Bladder inflammatory reaction was measured by a leukocyte esterase test (Ecur[4]-Test; Boehringer Mannheim GmbH, Mannheim, Germany). Blood was taken for white cell count. For anesthesia, ketamine was used.

Results

In Vitro Binding Characteristics of E. coli Strain DS17 and its Mutant Derivatives, DS17-8 and DS17-1

Strain DS17 and its two mutant derivatives, DS17-8 and DS17-1, all express fimbriae. Whereas the tip of DS17 fimbriae reacts with a class II–specific mAb, those of DS17-8 and DS17-1 do not. The DS17-1 fimbriae uniquely react with a class III–specific mAb, whereas neither mAb can interact with the tip of DS17-8 fimbriae (Fig. 1). The in vitro binding characteristics of the three strains were compared using ^{35}S-labeled bacteria and a set of well-defined glycosphingolipids (Fig. 2) coated on microtiter wells. Whereas the parent strain DS17 preferentially binds globoside, the class-switch mutant DS17-1 binds best to the Forssman antigen, and no binding to Galα(1–4)Gal–containing isoreceptors was seen with DS17-8.

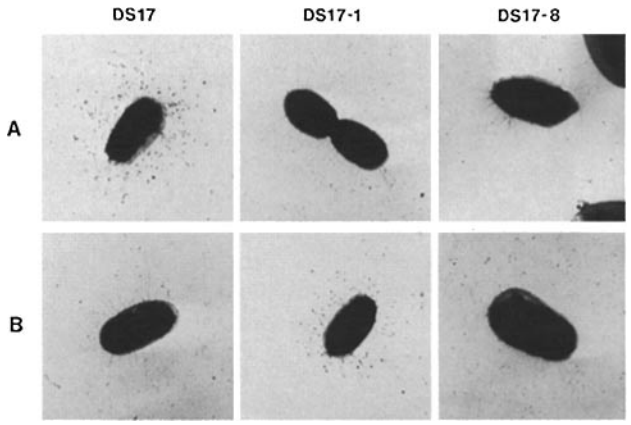

Figure 1. Immunoelectron microscopy of negatively stained DS17, DS17-1, and DS17-8 using the class II–specific, $PapG_{DS17}$-specific mAb MC22 (14) and the class III–specific $PrsG_{J96}$ mAb (*A* and *B*, respectively).

In Vivo Colonization and Infection Studies in Cynomolgus Monkeys Using the E. coli Strain DS17 and its Two Mutant Derivatives

Fecal Colonization. Earlier data have shown that strain DS17 readily colonizes and persists in the human and monkey gut (19, 26). In the monkey, this strain colonizes the gut also after vaginal exposure. To see whether or not gut colonization and persistence was dependent upon the class II adhesin, the fecal flora was monitored in eight animals after vaginal inoculation of the two mutant strains DS17-8 and DS17-1. Fecal colonization occurred for each of the two mutants. Once established in the gut, the mutants tended to persist (Table 1). (This was shown earlier for the wild strain DS17 [26]).

In four experiments, the vagina was exposed to an equal mixture of DS17 and DS17-1. Both these strains were found simultaneously in the stool of three monkeys. In one case, the mutant took over the wild type (data not shown).

Taken together, these data indicate that the presence of the tip G-adhesin does not provide the organism with a competitive advantage in the intestinal tract.

Vaginal Colonization. In pilot studies on four additional monkeys, *E. coli* DS17 and DS17-8 were found to colonize the vagina with equal efficiency (data not shown). Since these initial experiments did not indicate any difference between the two strains with respect to vaginal colonization, a set of competition experiments was performed. Mixtures of equal numbers of DS17 and DS17-8 were administered into the vagina in three experiments. No selective advantage for either strain was noted over a 9–14-d observation period, except for one trial in which DS17 was rapidly eliminated whereas DS17-8 persisted. In one separate experiment, DS17-8 was given to a monkey already persistently colonized with DS17. Both wild type and mutant persisted in the vagina over an observation period of 25 d

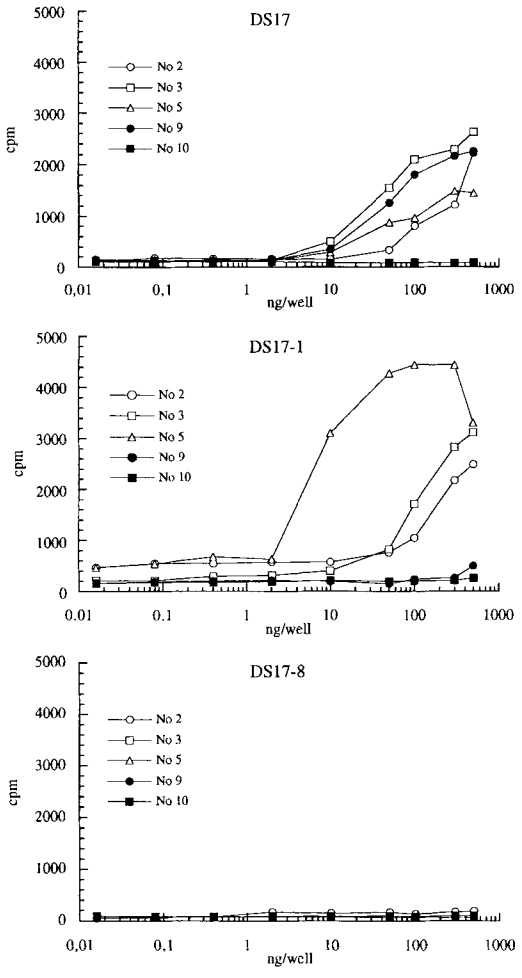

Figure 2. Binding of ^{35}S-labeled wild-type *E. coli* DS17 and its two isogenic mutant derivatives, DS17-8 and DS17-1, to a set of well-defined glycosphingolipids coated in microtiter wells. The PapG knockout mutant was unable to bind to Galα(1–4)Gal–containing compounds. The class-switch mutant DS17-1 bound according to a class III–expressing strain, i.e., preferentially to the Forssman antigen rather than to globoside, which is the preferred isoreceptor for DS17 with a class II adhesin. Open circles, Galα(1-4)Galβ4GlcβCer triaosylceramide; open squares, GalNAcβ3Galα-(1–4)Galβ4GlcβCer globoside; open triangles, GalNAcα3GalNAcβ3Galα-(1–4)Galβ4GlcβCer Forssman antigen; solid circles, Galα(1–4)Galβ4Glc-NAcβ3Galβ4GlcβCer PI glycolipid; solid squares, Galβ3GalNAcβ4Gal-β4GlcβCer.

in this experiment. Similar competition experiments were also performed with the combinations DS17/DS17-1 (three experiments) and DS17-1/DS17-8 (four experiments). No advantage for any of the two competing strains was found in these sets of experiments. A representative competition experiment for each of these pairs is shown in Fig. 3.

Bladder Infections. We have recently demonstrated that both the wild-type strain DS17 and its PapG-negative mutant derivative DS17-8 are able to generate bladder infection after intravesical inoculation of bacteria (18). The present study was extended to include also the class-switch mutant DS17-1. Infection was established in four out of four monkeys inoculated. The median duration for infection with DS17-1 was 9 d, similar to that previously found for DS17 and DS17-8. The infection was self limited in three monkeys, and leukocyturia was observed in all instances. Thus, there were no demonstrable differences in the ability to generate bladder infection between the three strains (data not shown).

Table 1. *Fecal Persistence after Intestinal Colonization by DS17 and Its Two Mutant Derivatives*

Strain	Longest observed persistence*
	>d
DS17	>17 mo in all four‡
DS17-8§	77, 79, 54, 131
DS17-1§	7, 21, 52, 50

Each strain was tested on four monkeys.
*The figures indicate minimum duration of persistence.
‡Taken from reference 26.
§In these experiments, the intestine was colonized secondary to vaginal challenge.

Competition experiments were performed in which a mixture of DS17 and DS17-8 was inoculated intravesically in equal numbers. Four monkeys that had no prior exposure to either of the two strains were used. Infection was induced in all four animals and was spontaneously eliminated in three monkeys after 23, 16, and 14 d, respectively. The fourth monkey became persistently infected for a period of >56 d. As shown in Fig. 4, the wild-type strain DS17 outcompeted the PapG-negative mutant DS17-8 in all four monkeys. 7 d after inoculation, only the wild type could be recovered from the urine. When a mixture of the two strains was cultured in monkey and human urine for a period of 1 wk, both strains were recovered in roughly equal numbers during the entire period (data not shown). Thus, the inability of the mutant to compete with the wild type in the bladder was not due to a different growth and survival in urine.

Discussion

The ability of a particular microbe to colonize a particular host species, tissue, or cell lineage is dependent on a multitude of factors, most of which are unknown. It has been suggested, though, that microbial recognition of specific receptor structures in the host plays a crucial role in tropism (8). Many receptor architectures in humans may be more or less specific for humans. It may therefore be exceedingly difficult to show, in animal experiments, that a particular receptor interaction plays a role in human disease. Many host receptors for bacteria are carbohydrates exhibiting different degrees of avidity for a given adhesin. The distribution of isoreceptors, which is most likely also a factor in tropism, may be very different in humans and animals. Because of the relatively close genetic relationship, nonhuman primates may be more appropriate models than other animals when the role of adhesion in human disease is explored.

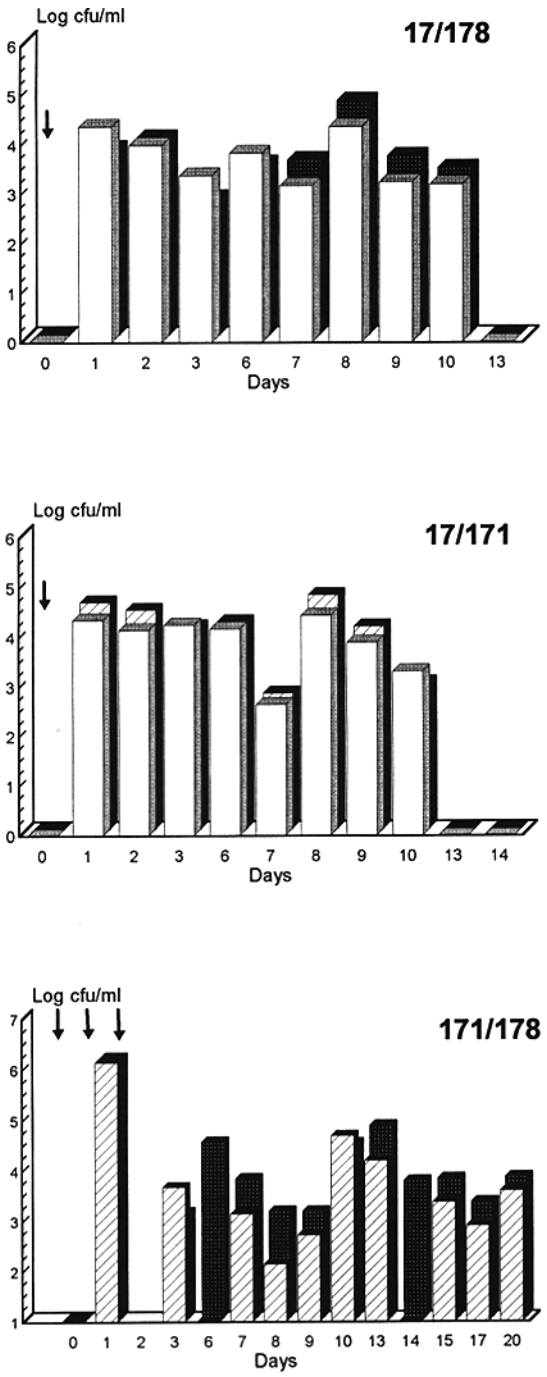

Figure 3. Vaginal colonization experiments, with simultaneous inoculation of two out of three strains in various combinations (1.5 ml of 10^9 CFU/ml of each strain). No advantage was demonstrated for the wild strain DS17 or its mutants. ↓, inoculation of strain mixtures; open bars, DS17; hatched bars, DS17-1; filled bars, DS17-8.

In natural human infections, the inoculum is usually much lower than in experimental infections, a factor that may lead to overinterpretation of animal data. Moreover, the initial natural inoculum may well contain many different organisms, sometimes creating a competitive situation and, in another situation, a beneficial microbial interaction.

Human UTI is a good example of an infectious disease that depends on a series of host and microbial factors. In an otherwise healthy child that receives its first *E. coli* UTI, bacterial expression of P-fimbriae is the most frequently occurring potential virulence factor. Interestingly, P-fimbriae expression is almost a prerequisite in first-time acute pyelonephritis (5, 6) but also as much as ~50% of first-time cystitis isolates express P-fimbriae (7). This latter figure is high enough to suggest some form of advantage for a P-fimbriated *E. coli* a in the human bladder also, as only 5–10% of commensal fecal *E. coli* express P-fimbriae (5).

The P-fimbriated *E. coli* DS17 with a class II tip G-adhesin is an excellent colonizer of the gut in monkeys (26) as well as in healthy humans (19). Other evidence for a selective advantage for P-fimbriated *E. coli* in the gut comes from studies of children with acute pyelonephritis (5) and girls with asymptomatic bacteriuria (31). In the former group, P-fimbriated *E. coli* dominated in the fecal flora, whereas, in the latter, resident strains carried P-fimbriae more often than transient strains. In the present study, we found no advantage for the wild-type P-fimbriated strain DS17 as compared with the mutant strains DS17-8 (G deficient) or DS17-1 (class-switch mutant) for colonization of the monkey gut. This would suggest that the colonization of the gut is not dependent on the presence of the G-adhesin. This finding fits with the biochemical data suggesting a lack of receptor-active structures for class II and III adhesins in the mucosal layer in the monkey colon (Teneberg, S., J. Andziak, J. Bergström, K.-A. Karlsson, I. Leonardsson, M. A. Mihl, B.-I. Marklund, R. Möllby, J. Winberg, and S. Normark, manuscript in preparation). P-fimbriae are highly complex organelles consisting of at least six different polypeptides. The *pap* gene cluster that contains 11 genes is part of a large "pathogenicity island" of ~100 kb in size (32). Thus, expression of a particular binding property via the PapG adhesin on P-fimbriae may not be the only explanation for the occurrence of P-fimbriated *E. coli* in the human large intestine.

The next phase in UTI is a bacterial colonization around the external orifice of the urethra and the vaginal introitus. In humans, a mixture of bacteria may colonize this area, and it is not known if these preferentially express P-fimbriae. In our monkey model, there was no requirement for the class II adhesin for vaginal colonization by DS17. Even when given in mixtures, there was no advantage for the wild type over the mutants.

It is suggested that DS17 and its derivatives express another adhesin mediating binding to vaginal cells that is distinct from PapG and that is also mannose resistant (Teneberg, S., J. Andziak, J. Bergström, K.-A. Karlsson, I. Leonardsson, M. A. Mihl, B.-I. Marklund, R. Möllby, J. Winberg, and S. Normark, manuscript in preparation). Since this in vitro binding was not inhibitable by mannose, it is most likely not caused by the mannose-specific FimH protein on type I fimbriae. It is known that P-fimbriae may interact with fibronectin via a pilus protein that is different from the PapG tip adhesin (33). *E. coli* also has the genetic capability to express fibronectin-binding curli when growing to stationary phase at temperatures slightly below 37°C (34). Conse-

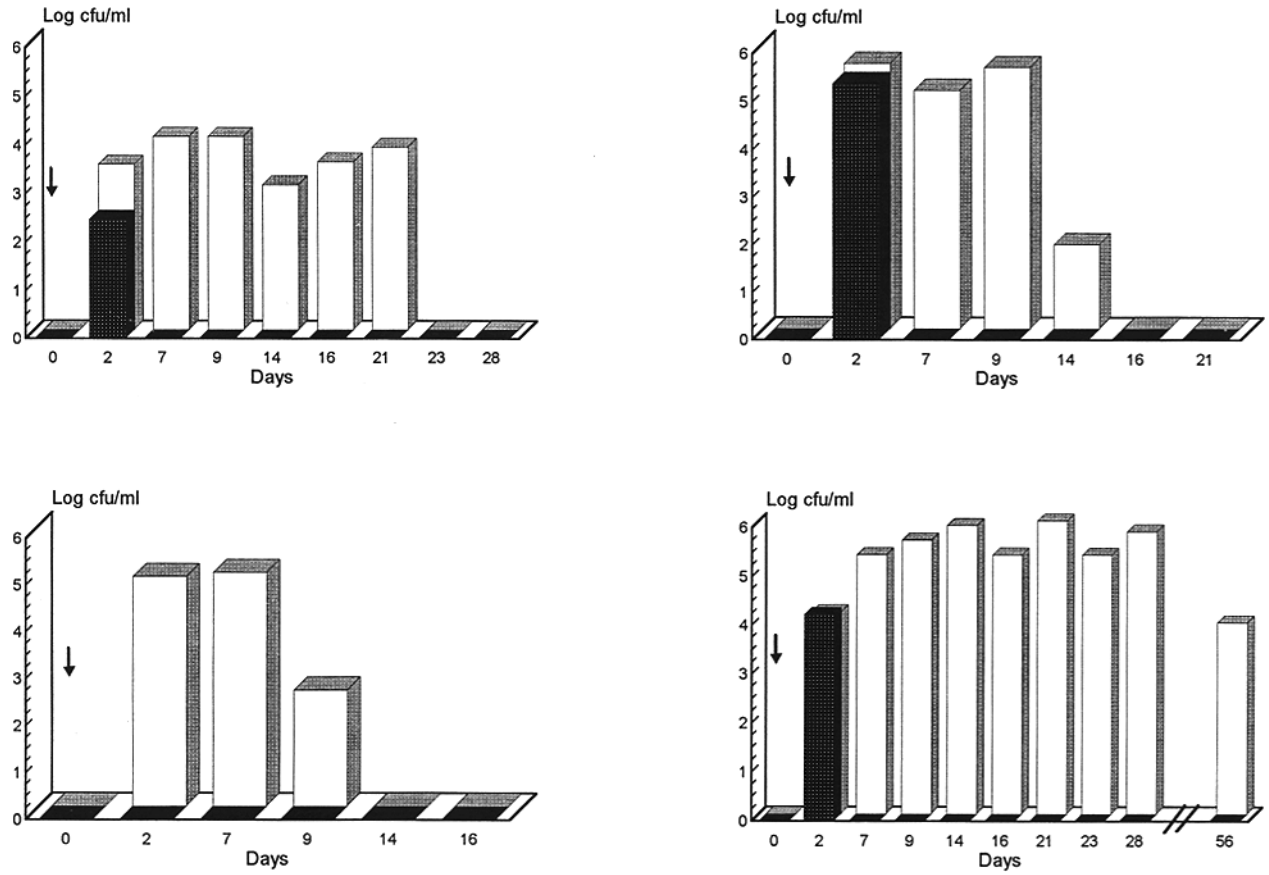

Figure 4. Inoculation of 1 ml of a mixture of strains DS17 and DS17-8 (5×10^6 CFU of each strain) into the bladder of four monkeys. The wild-type strain DS17 took over in all instances. All infections except one were self limited. ↓, inoculation of strain mixtures; open bars, DS17; filled bars, DS17-8.

quently, there are other adhesive properties that might explain binding to vaginal epithelial cells.

Clinical studies (4) and the experimental data shown here demonstrate that more than one *E. coli* strain may colonize the periurethral area/vagina simultaneously. It is therefore likely that the initial inoculum into the bladder frequently consists of a mixed culture. The experimental bladder infections in cynomolgus monkeys clearly show that *E. coli* carrying the class II adhesin on their P-fimbriae outcompete the mutant lacking the adhesive tip. However, when the PapG-negative mutant was inoculated alone, it was perfectly capable of generating bladder infection. It therefore seems that the presence of the G-adhesin is not necessary for pathogenicity in the bladder, but it provides *E. coli* with a competitive advantage over the mutant. A slightly improved epithelial binding to a small, specific region in the bladder such as the trigonum might select for the wild type during micturition.

Conclusions. The present study and a previous one (18) have clearly shown that the class II G-adhesin at the tip of the *E. coli* P-fimbriae is a prerequisite for the occurrence of acute pyelonephritis in the absence of other kidney-specific adhesins or obstruction to the urinary flow, but is not required for infection of the bladder. However, here we show that the tip adhesin provides *E. coli* with a competitive edge in mixed bladder infections. Clinical observations suggest that P-fimbriated *E. coli* strains have a selective advantage over non–P-fimbriated strains in the gut. However, in the monkey, such an advantage does not seem to be due to the G-adhesin. In the vagina, the G-adhesin is not required for colonization and gives no selective advantage. Whether other P-fimbrial proteins confer a selective advantage remains unknown.

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Address correspondence to Dr. Jan Winberg, Department of Pediatrics, Karolinska Hospital, S-171 76 Stockholm, Sweden.

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