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The Gal(α1-4)Gal-specific tip adhesin of *Escherichia coli* P-fimbriae is needed for pyelonephritis to occur in the normal urinary tract

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ABSTRACT **Nonobstructive acute pyelonephritis in humans is most often caused by P-fimbriated *Escherichia coli*. P-fimbriae are heteropolymeric fibers carrying a Gal(α1-4)Gal-specific PapG adhesin at its distal end. The pyelonephritic strain DS17 expresses P-fimbriae from a single gene cluster. A mutant strain, DS17-8, which expresses P-fimbriae lacking the PapG adhesin, was constructed by allelic replacement introducing a 1-bp deletion early in the *papG* gene. In cynomolgus monkeys, DS17 and DS17-8 were equally able to cause bladder infection, whereas only the wild-type strain DS17 could cause pyelonephritis as monitored by bacteriological, functional, and histopathological criteria. Since DS17, but not DS17-8, adheres to renal tissue, these data underscore the critical role of microbial adherence to host tissues in infectious disease and strongly suggest that the PapG tip adhesin of P-fimbriae is essential in the pathogenesis of human kidney infection.**

Escherichia coli is the most common bacterial species causing urinary tract infections in humans. Such infections may manifest themselves as asymptomatic bacteriuria, acute cystitis, or most severely as acute pyelonephritis. Strains associated with acute pyelonephritis in patients with an anatomically normal urinary tract often express proposed virulence factors such as P-fimbriae, hemolysin, type 1 fimbriae, and iron-chelating aerobactin and are usually resistant to serum bacteriocidal activity (1, 2). A clonal theory has been advanced to explain the concomitant presence of these properties (3, 4). P-fimbriation is probably not the only virulence factor important in the etiology of acute pyelonephritis. However, it is the property that is epidemiologically best correlated with the disease in that it is reported in over 95% of children and 50–90% of adults (5–10). P-fimbriae are so named because they act as lectins recognizing the globoseries of glycosphingolipids, which constitute the P-blood group antigens (11, 12). Even though the Gal(α1-4)Gal disaccharide constitutes the minimal binding epitope for P-fimbriae (13), the location of this epitope within the carbohydrate chain affects the receptor activity of the glycosphingolipid (14). Globoside [GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer], which is abundant in human kidney, represents the preferred isoreceptor for those P-fimbriae that are expressed by pyelonephritic *E. coli*. (15).

In both BALB/c mice and nonhuman primates, immunization with homologous (and in the monkey, heterologous) purified P-fimbriae protected against pyelonephritis after either a bladder (mouse) or renal inoculation (monkey) of P-fimbriated *E. coli* (16–19). In addition, studies by Svanborg-Edén *et al.* (20) showed that a receptor analogue *in vitro* would prevent adhesion of P-fimbriated *E. coli* to human urothelial cells. Additionally, experimental ascending urinary tract infection in the BALB/c mouse was inhibited by treatment of P-fimbriated *E. coli* with the specific receptor molecule globotetraose prior to bladder inoculation. In the monkey, similar treatment of the bacteria with the soluble minimal receptor Gal(α1-4)Gal-O-methyl significantly delayed the onset of pyelonephritis after ureteral inoculation (21).

P-fimbriae are heteropolymeric structures composed of a rigid stalk containing the major subunit protein linked end-to-end with a flexible tip fibrillum consisting of four proteins of which PapG, the receptor-binding adhesin, is located at its distal end (22–24). A *papG* mutant is still able to express morphologically normal pili that, however, cannot mediate Gal(α1-4)Gal-specific attachment *in vitro* (22–24). Three classes of P-fimbriae-associated G-adhesins—GI, GII, and GIII—have been identified among different *E. coli* isolates mediating binding to different subsets of Gal(α1-4)Gal-containing isoreceptors (14, 15). The GII adhesin, which preferably binds to globoside, dominates in human cases with acute pyelonephritis (15, 25).

In this paper, our objective was to establish whether or not the GII adhesin is required for P-fimbriated *E. coli* to cause cystitis and/or acute pyelonephritis. For this purpose, we constructed an isogenic *papG* mutant of a known pyelonephritic clinical isolate and tested wild type and mutant for ability to cause cystitis or acute pyelonephritis *in vivo* in cynomolgus monkeys.

MATERIALS AND METHODS

***In Situ* Adherence Assay.** Formalin-fixed monkey kidney tissue was deparaffinized and processed as described by Falk *et al.* (26). Bacterial strains DS17 and DS17-8 were labeled with fluorescein isothiocyanate as described (26). Tissue sections were overlaid with 150 μl of fluorescent-labeled bacteria diluted in PBS/0.01% Tween and incubated at 4°C for 1 hr. Slides were then washed extensively in PBS and then visualized under fluorescent microscopy.

Anti-DS17 PapG Monoclonal Antibody and Electron Microscopy. One hybridoma (MC22) was raised against the amino-terminal 205 amino acids of DS17 PapG, and supernatant was used for electron microscopy (27). Bacterial colonies were scraped from agar plates and washed once with PBS. Grids were floated on a bacterial suspension for 5 min, blotted dry, and then blocked with 1% (wt/vol) bovine serum albumin in PBS for 15 min. After washing with PBS, grids were floated on a drop of MC22 or rabbit anti-pilus antiserum diluted 1:10 in PBS for 1 hr. After washing with PBS/0.1% Tween 20 and then with distilled water five times, grids were floated on gold-labeled antibodies to mouse IgG (MC22) or rabbit IgG (anti-pilus antiserum) diluted 1:10 in PBS for 30 min. Grids were washed in distilled water five times, and then bacteria were negatively stained with 1% uranyl acetate.

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Animals. Adult female outbred cynomolgus monkeys (*Macaca fascicularis*) were used for both bladder challenge and ureteral challenge of the wild-type and mutant bacteria. For the bladder studies, seven females were inoculated with wild-type DS17 and six with the mutant DS17-8. For the ureteral inoculations, five females were inoculated with the wild-type strain and six received the mutant strain. All experiments were done under ketamine anesthesia. They resumed water intake within 1 hr. They were housed separately and given free access to water and food. Before anesthesia, food but not water was withheld overnight. History as to sibling relationships or secretor status was not known.

Bacterial Inoculation. In the case of bladder infections, 1 ml of bacteria (1×10^7 organisms) was inoculated into the bladder by means of a urethral catheter. In the case of ureteral inoculations, 0.4 ml of a solution containing 1×10^9 bacteria per ml plus 0.2 ml of contrast material and ^{131}I-labeled hippuran were prepared for a total of 0.6 ml. Of this inoculum, 0.35 ml was injected via a cystoscopically inserted ureteral catheter into one ureter, leaving the other kidney as a noninfected control. Pyelotubular backflow (retrograde flow of inoculum from renal pelvis into renal tubules) was not seen by fluoroscopy in any monkey. Blood was obtained at 1, 10, and 60 min after inoculation for culture and isotope counting. Both showed that pyelovenous backflow had not occurred in any monkey.

Studies After Infection. Suprapubic bladder aspiration was used to obtain urine for culture at days 2, 7, 9, 14, and 16 after bladder inoculation. The monkeys that were subjected to ureteral inoculation had urine obtained by suprapubic aspiration at 24 and 48 hr and then weekly for 1 month. In these animals, blood was also taken for white blood cell count and complement at 24 and 48 hr and then weekly.

Radionuclide scans were done after the injection of ^{131}I-labeled hippuran 7 days prior to infection and 21 days after infection to determine renal function change after infection. These measures of renal function were done to allow longitudinal studies without the effect of obstructing ureteral catheters, which might well complicate infection. The monkeys were positioned over the sodium iodide crystal of a General Electric scintillation camera and 50 μCi (1 Ci = 37 GBq) of ^{131}I-labeled hippuran was given intravenously. A CAMII computer (Adac Laboratories, Sunnyvale, CA) program provided information about isotope uptake at designated areas after subtraction of background, which was determined at the 1- to 2-min time interval. At that time, radionuclide would still be within the renal parenchyma and not in the collecting system. Repeated measurement of percent function of each kidney in four control monkeys, who had the study repeated five times in as many weeks with this technique, showed a mean value of 50% ± 3.8% function per kidney. A one-factor repeated measure analysis of these normal kidneys ($n = 40$) showed that the percentage function over time did not change ($P = 0.99$).

Pathological Evaluation. Four weeks after infection, the monkeys were sacrificed after an overdose of barbiturate. The kidneys were removed under aseptic conditions and weighed; half was used for histology and the remainder was used for culture. Ureters and bladder were also obtained for histology. Blinded histologic grading was done by a veterinary pathologist (G.B.). The standard section of tissue was taken transversely through the midportion of the kidney to contain papilla, medulla, and cortex. Histologic sections were examined by the pathologist in a double-blind manner according to previously established parameters (28). Acute pyelonephritis is associated with a marked inflammatory exudate in areas of bacterial growth, tubular damage, and death with microabscess formation. The reparative response with fibrosis, scarring, and mononuclear cell infiltration, especially in subcapsular, pelvic, and periglomerular regions, was considered to be subacute to chronic pyelonephritis and is the typical finding following an untreated infection in our experimental model. Rating was on a 0 to 4 basis, with 4 being the most severe. The sections were rated for the following parameters: tubular and/or interstitial neutrophils, tubular and/or interstitial mononuclear cells, fibrosis, scarring, pelvic infiltration, glomerular change, subcapsular invasion, tubular dilatation, and tubular atrophy. In addition, the percent of kidney involved was estimated based on the amount of kidney involved on the hematoxylin/eosin slide of the standard section.

Statistics. Data were analyzed by a one-way ANOVA with repeated measures, and all data reported as the *P* value.

RESULTS

Construction of an Isogenic *papG* Mutant of the Pyelonephritic Clinical Isolate DS17. The *E. coli* strain DS17 (06:K5:H$^-$) was originally isolated from a child with acute pyelonephritis and was shown to spread epidemically to staff and babies in a neonatal unit; it expresses P-fimbriae, type 1 fimbriae, and hemolysin (29). Chromosomal DNA from strain DS17 was negative in PCR reactions with primers specific for the sialic acid-specific adhesin of S-fimbriae and the nonfimbrial adhesin AFA-1 (data not shown). Since some strains of *E. coli* carry more than one *pap* gene cluster encoding P-fimbriae (25), we analyzed restricted chromosomal DNA from DS17 by Southern blot DNA hybridizations utilizing as a probe a DNA fragment specific for the *papG* class II allele. Only a single *Sal* I and *Hin*dIII fragment hybridized with the probe, showing that strain DS17 carries only one *papG* adhesin gene (data not shown). PCR amplification of DS17 chromosomal DNA was performed using primers complementary to sequences immediately upstream and downstream from the previously sequenced $papG_{IA2}$ class II allele (25). The expected 1.1-kb PCR product was obtained and subsequently cloned into an M13 vector. Four separate clones were sequenced. One of these clones carried a 1-bp deletion relative to the wild-type sequence that would encode a truncated peptide 40 amino acids in length. The *papG* DNA fragment from this mutant clone was inserted into the cloning cassette of plasmid pPAP655, a derivative of the temperature-sensitive replicon pMAK705 (30). The resulting *papG*-bearing plasmid, pPAP661, was introduced into strain DS17 by electroporation. Transformants were selected on kanamycin and incubated at 42.5°C for selection of incorporation of the plasmid onto the DS17 chromosome. One kanamycin-resistant clone, unlike the parent DS17, was unable to hemagglutinate human erythrocytes. This clone was subjected to continuous growth at 42.5°C without antibiotic selection, and kanamycin-susceptible clones were scored for a hemagglutination-negative phenotype. One such mutant clone in which integrated plasmid DNA had been excised was designated DS17-8. The 1-bp deletion in the mutant clone was confirmed by PCR amplification, cloning, and sequencing of a 179-bp region from the 5′ end of *papG*.

Immunoelectron microscopy using P-fimbriae-specific antiserum reveals that both the parent DS17 and the nonhemagglutinating mutant express P-fimbriae (Fig. 1 *A* and *B*). A monoclonal antibody MC22 specific for the amino-terminal portion of PapG was also used in immunoelectron microscopy and found to react only with the tips of DS17 fimbriae but not with those expressed by the DS17-8 mutant (Fig. 1 *C* and *D*). Likewise, this antibody reacted only with DS17 and not DS17-8 in immunoblotting (data not shown). Finally, strain DS17 unlike DS17-8 binds *in vitro* to tissue sections from embedded cynomolgus monkey (*M. fascicularis*) kidney (Fig. 1 *E* and *F*).

Infections in Cynomolgus Monkeys with DS17 and its Isogenic PapG Adhesin-Negative Mutant DS17-8. Strain DS17 and its *papG* mutant derivative DS17-8 were compared with

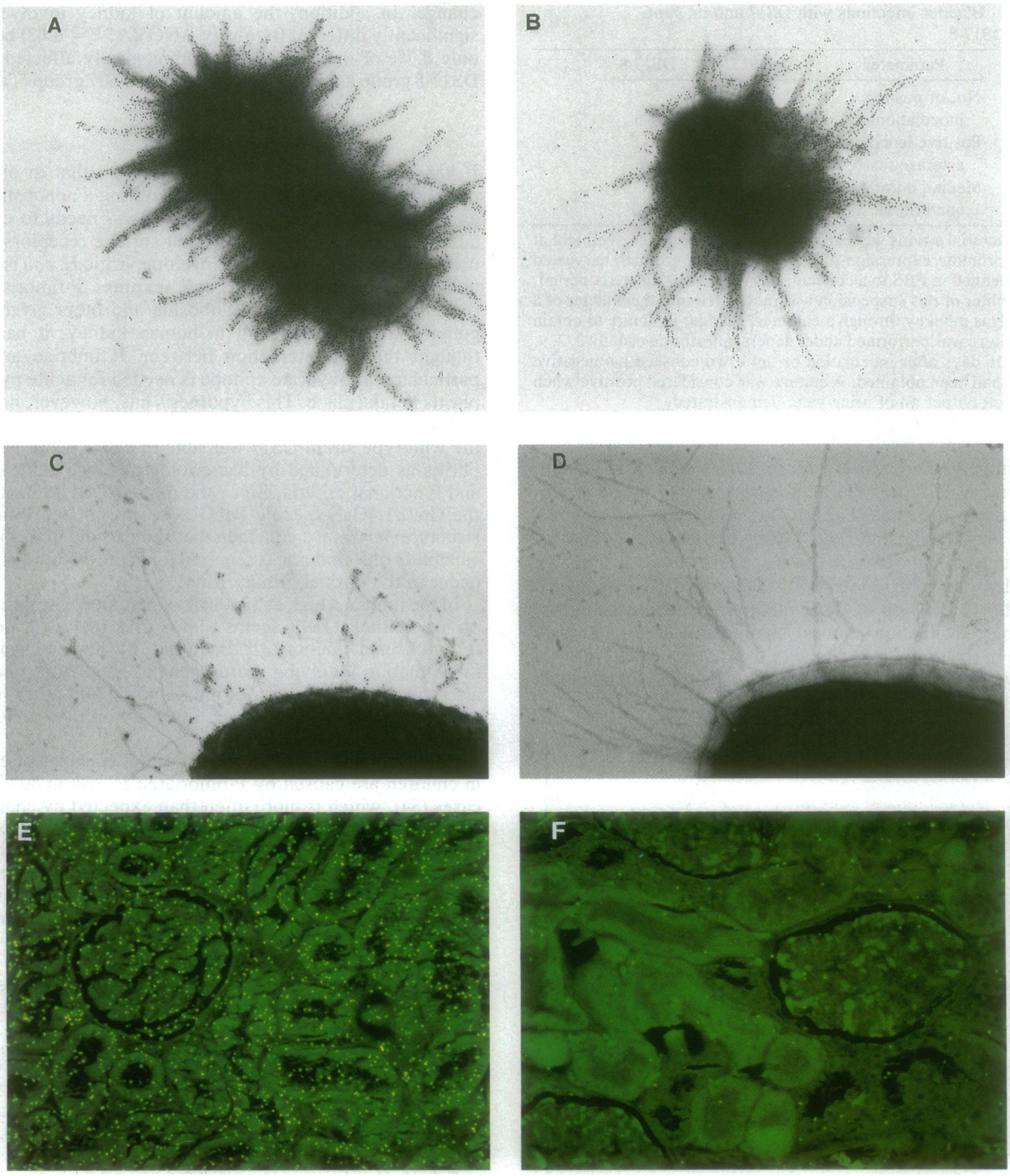

FIG. 1. Immunoelectron microscopy of negatively stained DS17 and DS17-8 using a polyclonal antiserum to purified P-fimbriae (*A* and *B*) as well as the $PapG_{DS17}$-specific monoclonal antibody MC22 (*C* and *D*), respectively. Note that both strains express P-fimbriae but only DS17 expresses the tip adhesin. (*E* and *F*) *In situ* binding of fluorescein isothiocyanate-labeled DS17 and DS17-8 cells to tissue sections from cynomolgus monkey kidney.

respect to their ability to cause bladder infection in cynomolgus monkeys. Both wild type and mutant induced bladder infections in all inoculated monkeys that was self-limited in five out of seven and three out of six instances, respectively (Table 1). The remaining five infections were eliminated by ciprofloxacin wash of the bladder between 10 and 21 days after infection. The infections with both wild-type and mutant were associated with a local inflammatory response.

To study the role of PapG in pyelonephritis, five monkeys were inoculated with *E. coli* strain DS17, whereas six received mutant strain DS17-8 via a cystoscopically inserted ureteral catheter. Bacteriuria was significantly different between the two groups (two-way ANOVA, $P = 0.002$) as the DS17 group had a mean time of bacteriuria of 21 days compared to 6.8 days for the group receiving the mutant strain. There was, however, no significant difference in leukocytosis or complement levels after the infection in either group.

Renal clearance of the radionuclide during renal scans was also significantly different as shown in Fig. 2*A*. The percent function of the infected kidney was likewise significantly different in the two groups: the final percent function of the infected kidney in the DS17 group was 43% and that in the DS17-8 group was 50%; the normal expected amount of function for one kidney is 50% ± 3.8% (28) (Fig. 2*B*).

Table 1. Bladder infections with DS17 and its *papG* mutant DS17-8

Parameter	DS17	DS17-8
No. of positive inoculations	7/7	6/6
Positive leucocyte esterase test*	5/6	6/6
Median duration of bacteriuria, days	10	>10

The bacterial strains were grown on blood agar plates overnight at 37°C to promote expression of P-fimbriae and were then harvested and suspended in PBS to a concentration of 1×10^7 bacteria per ml. One milliliter of this suspension was inoculated into the bladder of a cynomolgus monkey through a catheter. Bladder puncture to obtain urine culture was performed under general anesthesia usually 2, 7, 9, 14, and 16 days after inoculation or until two consecutive negative cultures had been obtained. A culture was considered positive when >100 bacteria per ml of urine were demonstrated.
*Ecur[4]-Test (Boehringer Mannheim).

At autopsy, there was greater renal loss in the DS17 group, with a mean loss of 0.49 g, compared to the DS17-8 group, where mean loss was 0.02 g. This difference between the groups was significant at the 0.03 level (two-way ANOVA). The average renal weight in those animals was 7.0 g; thus, the 0.49-g loss from DS17 infection represents a 7% loss, which is similar to the loss of renal function in infected kidneys. Pathologic evaluation showed significantly less pathologic change in the group infected with the mutant strain, in 9 out of 13 values used in our standard evaluation of pathologic change. In addition, the amount of kidney involved was significantly different (two-way ANOVA, $P = 0.02$) because only 8.7% of the kidney was pathologically affected in the DS17-8 group as opposed to 26% in the DS17 group (Table 2).

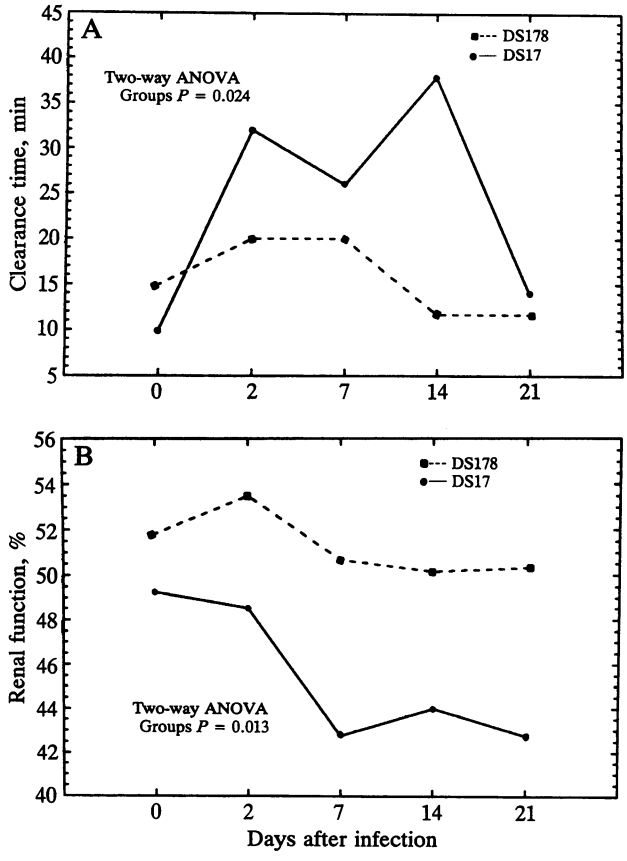

FIG. 2. (*A*) The clearance time of radionuclide shows that renal and ureteral function was much more affected in the animals inoculated with DS17 than DS17-8. (*B*) Renal function of infected kidney as percent of total. Infection with DS17 decreased renal function significantly, whereas DS17-8 did not impair the function of the infected kidney.

DISCUSSION

It has been a paradigm for several years that in order to establish itself in the human kidney during a nonobstructive ascending urinary tract infection, *E. coli* needs to express adhesins recognizing epithelial cell surface receptors in the ureter and kidney. Since most pyelonephritic *E. coli* isolates, unlike commensal fecal *E. coli*, express P-fimbriae that recognize Gal(α1-4)Gal in globoside and other glycosphingolipids that are abundant in human kidney, it has been thought that an interaction between P-fimbriae and this particular carbohydrate epitope is needed for acute pyelonephritis to take place. This hypothesis has, however, not been verified experimentally. In the primate model used here, only the wild-type strain DS17 was able to cause acute pyelonephritis as determined by bacteriological, histopathological, and functional criteria. Since the mutant DS17-8 lacks only the Gal(α1-4Gal)-specific PapG adhesin, not the P-fimbrial structure *per se*, we conclude that the carbohydrate-binding capability of P-fimbriae is required for acute pyelonephritis to occur in the normal urinary tract.

In the human a high availability of receptors for P-fimbriae on uro-epithelial cells seems to be a risk factor for deterioration of renal function (31). Bacterial attachment may induce inflammation involving recruitment of leucocytes and may also stimulate production of interleukin 6 and other inflammatory mediators as proposed by Svanborg and colleagues (32, 33).

Epidemiological data show that initial episodes of cystitis in children are caused by P-fimbriated *E. coli* in 40–50% of cases (34), which is more often than expected by chance. It was therefore surprising that DS17 and DS17-8 were equally able to cause bladder infection in cynomolgus monkeys. Relatively large inoculates were used in our experiments. Thus, even though our primate model does not show any need for PapG in bladder infection, this may still be the case with very small inoculates and an infection route via the intestine as in natural human infections.

In a recent paper by Mobley *et al.* (35), a double deletion *pap* mutant of a human pyelonephritic *E. coli* isolate re-

Table 2. Pathologic evaluation of kidney infected with either DS17 or its *papG* mutant DS17-8

Pathologic finding	DS17	DS17-8	*P*
Edema	0.6	0.2	0.06
Neutrophils			
Tubular	0.5	0.2	0.05*
Interstitial	0.5	0	0.001*
Monocytes			
Tubular	0	0	
Interstitial	2.1	1.2	0.02*
Scarring	2.2	0.5	0.0001*
Pelvic infiltration	1.8	0.2	0.008*
Epithelial destruction	0.3	0	0.02*
Glomerular involvement	1.2	1.2	0.2
Subcapsular involvement	2.6	1.0	0.003*
Tubular dilatation	0.9	0.2	0.003*
Tubular atrophy	2.5	1.1	0.001*
Vascular involvement	0.7	0.2	0.2
Kidney involvement, %	26	8.7	0.02*

Pathologic evaluation was done as described in *Materials and Methods*. The values given for DS17 and DS17-8 are ratings on a 0 to 4 basis, with 4 being the most severe.
*Statistically significant (by two-way ANOVA).

mained able to develop acute pyelitis or pyelonephritis in the CBA mouse model. Since the strain used in that study also carried the *sfa* determinant, the lack of P-fimbriae might be functionally compensated for by the expression of S-fimbriae. It may also be that the isoreceptor distribution and availability of globoseries of glycolipids may differ between humans and mice. The pattern of receptor active glycolipids in monkey and human kidney is similar and in both cases dominated by globoside (J.W., B.M., S. Teneberg, R.M., K. A. Karlsson, and S.N., unpublished results) arguing that cynomolgus monkey used here is relevant as an experimental animal model for human urinary tract infections.

Our findings finally suggest that a vaccine composed of PapG protein may well protect against pyelonephritis in humans. Since the sequence variation among PapG in different pyelonephritic *E. coli* strains is low (25), an immune response to one PapG variant may be protective against *E. coli* strains expressing P-fimbriae of different serotypes. Indeed, the monoclonal antibody described here, which was raised against DS17 PapG, reacts strongly with other class II adhesins (data not shown).

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