PapG-Dependent Adherence Breaks Mucosal Inertia and Triggers the Innate Host Response

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Mucosal pathogens differ from normal flora constituents in that they provoke a host response that upsets mucosal integrity. We investigated whether the elaboration of discrete adherence factors is sufficient to break the inertia of the mucosal barrier. PapG-mediated adherence was selected as an example, because P fimbrial expression characterizes uropathogenic Escherichia coli and because adherence starts the attack on the mucosal barrier. Patients were inoculated intravesically with transformed nonvirulent E. coli strains expressing functional P fimbriae (E. coli pap+) or mutant fimbriae lacking the adhesin (E. coli ΔpapG). E. coli pap+ was shown to activate the innate host response, and adherent gfp+ bacteria were observed on excreted uroepithelial cells. E. coli ΔpapG failed to trigger a response and was nonadhesive. We conclude that PapG-mediated adherence breaks mucosal inertia in the human urinary tract by triggering innate immunity and propose that this activation step differentiates asymptomatic carriage from infection.

The innate host response reflects the reactivity of mucosal sites to microbial attack. Commensal organisms often fail to evoke this response; thus, the mucosal barrier remains inert to these microbes and to their secreted products, and a state of asymptomatic carriage is established. The pathogens, in contrast, are equipped to break the inertia of the mucosal barrier by triggering a host response [1]. This dichotomy puts renewed emphasis on the molecular interactions that determine whether the mucosal response will be activated. Studies from several laboratories have shown that pathogens use highly sophisticated mechanisms to gain access to the host tissues and to corrupt the host response [2–4], but the molecular interactions that actually trigger the innate host response are not well understood.

The urinary tract provides an excellent model to study how pathogens break mucosal inertia [1]. Bacteria frequently enter this normally sterile site and may establish populations of >10⁵ cfu/mL of urine, but the presence of bacteria does not necessarily lead to disease. The most frequent outcome is asymptomatic bacteriuria (ABU), which occurs in 1%–2% of young female patients and in ~20% of elderly patients [5, 6]. Strains that cause ABU fail to express a number of the virulence factors that characterize uropathogen Escherichia coli; in particular, bacterial adherence is an essential first step in the pathogenesis of symptomatic urinary tract infection (UTI) [7, 8]. Fimbriae with different receptor specificities have been examined, and P fimbriae invariably show the strongest association to acute disease severity, with ≥90% of acute pyelonephritis–causing strains, but <20% of asymptomatic carrier strains, expressing this phenotype [8, 9].

P fimbriae recognize cell-surface glycosphingolipid (GSL) receptors [9], and the binding specificity is determined by the PapG adhesin located at the tip of the heteropolymeric fimbrial rod structure [10]. Coupling of P fimbriae to the GSL receptors triggers an epithelial cytokine response in vitro, through the recruitment of Toll-like receptor 4 (TLR4) for signal transduction [11, 12]. The in vivo consequences of P fimbriae–mediated adherence have been studied in murine and primate models of UTI, with contradictory results [13–15]. In the human inoculation model, P fimbriae were shown to facilitate the establishment of bacteriuria and the in-
duction of mucosal inflammation [16, 17], but other studies have failed to reveal this association [18]. Further human studies are thus needed to resolve the question of whether and how P fimbriae–mediated adherence perturbs the mucosa and to define the molecular interactions needed to trigger a host response.

The present study tested the hypothesis that PapG-mediated adherence breaks mucosal inertia by triggering the innate host response. We selected a strain that normally causes ABU and transformed it with a fully functional \( \text{pap} \) operon or a mutated, \( \text{papG} \)-deficient sequence. The results demonstrate that PapG-mediated adherence activates a mucosal host response.

**PATIENTS, MATERIALS, AND METHODS**

**Patients.** Patients with a history of recurrent, symptomatic lower UTI (5 women and 1 man) were enrolled in the present study. They had experienced recurrent infections with highly resistant strains and had experienced gastrointestinal adverse effects or allergic reactions to antibiotics. Conservative treatment measures, such as antibiotic prophylaxis and frequent clean intermittent catheterization (CIC), had been tested and failed. The 5 women had lower motor neuron lesions that caused postvoid residual urine, and the man had neurogenic bladder dysfunctions due to suprasacral spinal cord injury. Five of the 6 patients were receiving CIC. Lower urinary-tract function was assessed by use of cystoscopy and medium-fill cystometry, and residual urine measurements were performed for all patients. All had normal upper urinary tracts, as determined by use of intravenous pyelography, and intact renal function, as determined by serum creatinine and \(^{51}\)Cr-EDTA clearance (table 1). The Medical Faculty Ethics Committee of Lund University approved the study, and informed consent was obtained from all patients.

**Urinary tract colonization.** The human inoculation protocol has been described elsewhere [17, 19]. In brief, preexisting bacteriuria was eliminated by treatment with norfloxacin and/or ampicillin. At 72 h after the last dose of antibiotics, the patient underwent catheterization by use of a Low-Fric Fr. 14 (Astra). The bladder was emptied, the bacterial inoculum (30 mL, \( 10^5 \) cfu/mL) was introduced, and the catheter was removed. Urine samples were obtained before and 1, 3, 5, and 7 h after inoculation and then at regular intervals for 48 h.

**Bacteria.** *E. coli* 83972 was originally isolated from a girl with ABU who had carried it for 3 years without symptoms [19]. This strain was previously used for urinary tract–colonization studies and was shown to cause persistent ABU [17,
Like many ABU-causing strains, E. coli 83972 carries adhesin gene clusters homologous to pap, pap and IA2 as template and the forward primer upstream of the BamH1 site near papG (lane 2), whereas E. coli DpapG lacked papG (lane 2), as verified by PCR.

The reporter plasmid pGFP1 was constructed to monitor the papB promoter activity. A 654-bp DNA fragment containing the 3′ end of papB in the papB promoter was amplified by use of PCR (Taq Gold; Applied Biosystems), was cut with BamH1 (Fermentas), and was ligated into the BamH1 site of the vector pST1, which carries gfp [22] (figure 1A). The primers 5′-CTGTGGGATCCCTGTGAATTGATG-3′ (forward) and 5′-TTGCCACTACCATCTTTTCT-3′ (reverse) amplified a 448-bp internal papA fragment in both the pap+ and the ΔpapG strain. The primers 5′-TGATTCTCAGAAATGGTTCCAGCT-3′ (forward) and 5′-GATAAACGTTTAGGCAATGATGACA-3′ (reverse) amplified 1034 bp of papG that was absent in the ΔpapG mutant.

The reporter plasmid pGFP1 was transferred to E. coli 83972 by use of electroporation. The recombinant strains were stored at −80°C in 15% sterile glycerol and were passaged on tryptic soy agar (TSA; Difco) with appropriate selection.

For intravesical inoculation, bacteria were taken from TSA plates, cultured overnight in Luria broth, harvested by centrifugation, and resuspended in PBS (pH 7.2) to a concentration of 10⁶ cfu/mL. The order of inoculation of the pap+/ΔpapG isogens for each patient is shown in table 1.

P fimbrial integrity. The shape of surface-expressed fimbriae was examined by use of transmission electron microscopy. P fimbrial function was investigated by 2 receptor-specific assays. Direct binding to receptor GSLs was examined by use of the thin-layer chromatogram (TLC) overlay assay [23]. Receptor-specific cell adhesion was analyzed by use of hemagglutination, using human erythrocytes differing in receptor expression. Receptor-positive A, P, or receptor-negative A, P erythrocytes (4%) were mixed with bacteria in PBS with 2.5% α-methyl-o-
Figure 2. Properties of Escherichia coli pap+ and E. coli ΔpapG. A, E. coli pap+ and E. coli ΔpapG both expressed fimbriae, as shown by use of electron microscopy. Fewer copies of the fimbriae were observed on the ΔpapG mutant. B, E. coli pap+ adhered to the human kidney epithelial A498 cell line in vitro (left panels), but E. coli ΔpapG failed to adhere (right panels, bright-field microscopy; middle panel, fluorescence microscopy of gfp+ bacteria [lower panel]). C, Loss of receptor-specific binding. Thin-layer chromatograms of glycosphingolipid extracts from A498 cells were overlaid with labeled P-fimbriated bacteria. Gb3, globotriaosylceramide; Gb4, globotetraosylceramide; Gb5-7, Forssmann, globohexaosylceramide [9]. Deletion of papG caused the loss of receptor-specific hemagglutination. Human erythrocytes of blood group A 1P1 (receptor positive) or A1p (receptor negative) were used.

mannose on a glass slide, and agglutination was recorded as positive or negative.

P fimbrial expression. The papB promoter activity was quantified by use of flow cytometry (FACSCalibur; Becton Dickinson), using the green fluorescent protein (GFP) reporter. Bacteria were harvested by centrifugation of 50 mL of urine for 5 min at 3645 g, and the pellet was resuspended in 250 μL of PBS with 1% formaldehyde; 20,000 bacteria were examined in each sample.

Bacterial adherence to uroepithelial cells in vivo. Cells were harvested from each urine sample (50 mL) by centrifugation for 5 min at 1230 g, washed in 2 mL of PBS, and resuspended. GFP-expressing bacteria were analyzed by use of light and fluorescence microscopy.

Urine cultures. Urine samples were refrigerated immediately after collection and were transported to the laboratory for quantitative culture. Total counts of E. coli pap+ and E. coli ΔpapG were determined on TSA plates. Selective counts were determined on TSA plates with ampicillin (100 μg/mL) and chloramphenicol (20 μL/mL).

Host response parameters. The concentration of neutrophils in uncentrifuged fresh urine was determined by use of microscopy, using a Bürker chamber. The cytokines interleukin (IL)–6 and IL-8 were quantified by use of IMMULITE IL-6 and IMMULITE IL-8 (both from DPC). Samples with undetectable cytokine concentrations were assigned a value corresponding to the lower limit of detection of the assay (5 pg/mL for both IL-8 and IL-6).

Statistical analyses. Samples from pap+ and ΔpapG inoculations of individual patients were ranked and compared by use of a paired, nonparametric test (Wilcoxon). P values are 2-tailed.

RESULTS

E. coli pap+ and the papG adhesin–deficient mutant E. coli ΔpapG. P fimbriae are rodlike heteropolymers of the PapA subunit and of minor proteins (PapE, PapF, and PapG) that form the flexible tip adhesin complex [10]. The PapG tip adhesin recognizes glycolipid receptors that are present along the urinary tract mucosa and in the kidneys.

The asymptomatic carrier strain E. coli 83972 has been shown elsewhere to establish bacteriuria in patients with neurologic bladder disorders [17, 19]. The patients did not develop symptoms, and the mucosal host response was low or absent. In the present study, E. coli 83972 was used as a host for recombinant plasmids encoding functional or adhesin-deficient P fimbriae (figure 1 and table 2). E. coli pap+ carried the papA2 sequences in pRHu1280 and the gfp reporter in-frame of the papBA promoter in pEGF1. E. coli ΔpapG carried a papG-deficient papA2 copy in pEG3 and pGFP1. The strategy used to construct the PapG adhesin–deficient mutant is outlined in figure 1A. The papG deletion was confirmed by use of PCR (figure 1B).

By electron microscopy, E. coli pap+ and the ΔpapG mutant were shown to express morphologically intact P fimbriae (figure 2A). E. coli ΔpapG had lost receptor-specific binding, as demonstrated by TLC overlay of GSLs purified from human uroepithelial cells (figure 2). Loss of receptor-specific cell adhesion
Figure 3. In vivo adherence to exfoliated uroepithelial cells. A, Fimbrial expression by Escherichia coli pap+ (green line, left panel) and E. coli ΔpapG (green line, right panel), as detected by the gfp reporter. Bacterial cells were analyzed by use of flow cytometry (FACSCalibur; Becton Dickinson). Green line, gfp+ cells; black line, E. coli 83972. B, Bright-field and fluorescence microscopy of exfoliated uroepithelial cells with adhering E. coli pap+ bacteria (left panels). Adherence was not observed in patients inoculated with E. coli ΔpapG (right panels). C, More than 50% of the samples from patients inoculated with E. coli pap+ contained uroepithelial cells with adhering bacteria (mean of pooled patient samples; error bar represents SD). This result was not seen after inoculation of the same patients with E. coli ΔpapG (left panel). Adherence of the pap+ strain varied over time, as exemplified by data from 3 patients (right panel).

was confirmed by the failure of the ΔpapG mutant to agglutinate human erythrocytes of the A, P, and OP, blood groups, expressing the globo-series of GSLs. E. coli pap+ and the mutant failed to agglutinate both the receptor-positive and the receptor-negative A, P erythrocytes (figure 2). The PapG-deficient mutant strain had also lost the ability to adhere to the human kidney epithelial cell line A498 in vitro (figure 2). E. coli 83972 carries a chromosomal copy of the pap gene cluster and may bind weakly to short receptor GSLs in vitro, but E. coli 83972 does not adhere to epithelial cells. The results confirmed the importance of PapG as the adhesin necessary for receptor-specific binding and cell adhesion of P-fimbriated E. coli.

PapG mediates in vivo adherence to uroepithelial cells in the human urinary tract. For intravesical inoculation, E. coli pap+ or the ΔpapG mutant were cultured in liquid medium with appropriate selection. Fimbrial expression was monitored by use of flow cytometry using the gfp reporter plasmid. The majority of the bacteria in both inocula were GFP positive, and no difference in pap transcription was detected between the pap+ and the ΔpapG mutant (figure 3A).

In vivo adherence was monitored after intravesical inoculation by counting GFP-expressing bacteria adherent to excreted uroepithelial cells. All patients who had been inoculated with E. coli pap+ excreted cells with adhering gfp+ bacteria on their surface (figure 3). In contrast, the ΔpapG mutant failed to adhere also in vivo (figure 3). We conclude that P-fimbriated E. coli adhere to uroepithelial cells in the human urinary tract and that in vivo adherence is PapG dependent.

PapG determines induction of host response. Each patient was inoculated with E. coli pap+ or the ΔpapG mutant, on separate occasions (table 1). The innate host response was quantified in consecutive urine samples. IL-6 was selected as an example of the cytokines involved in the acute-phase response, because IL-6 triggers C-reactive protein, which is a widely used parameter in the diagnosis of UTI [24]. IL-8 was selected as a relevant chemokine because it is secreted by epithelial cells in response to P-fimbriated E. coli in vitro and is essential for neutrophil migration across the epithelial barrier in experimental models of UTI [25, 26]. Finally, the urine neutrophil response was monitored, because pyuria is a classic diagnostic tool in UTI, and these effector cells are crucial for bacterial clearance and for the urinary-tract defense [27].

The kinetics of the IL-8, IL-6, and neutrophil responses are shown in figure 4, for inoculations with E. coli pap+ or E. coli ΔpapG. E. coli pap+ was shown to trigger the innate host response in each of the patients who was inoculated with this strain. In contrast to E. coli pap+, E. coli ΔpapG did not trigger a significant host response (figure 4). The statistical analysis of the intraindividual differences in host response to E. coli pap+ and E. coli ΔpapG is shown in table 3.

We conclude that the expression of adhesive P fimbriae enables E. coli to trigger the innate host response in the urinary tract. Activation was PapG dependent under these experimental conditions.

Host response in relation to bacteria counts. The magnitude of the host response depends, in part, on the bacteria
count. To properly interpret the effect of PapG-mediated adherence, it was essential to normalize the bacteria counts in the urine samples. In previous human inoculation studies, P fimbriae–mediated adherence was required for establishment of bacteria in the normal urinary tract, but not in patients with voiding disorders [16]. In the present study, patients with residual urine were selected to facilitate the establishment of the papG mutant strain.

The outcome of each inoculation was determined by quantitative bacteria counts on all urine samples obtained during the first 48 h. E. coli pap+ and E. coli ΔpapG were found to establish bacteriuria with similar efficiency in this group of patients.

The host response was further examined as a function of the bacteria counts in individual urine samples (figure 4C). Low numbers of E. coli pap+ were required to trigger the host response (∼10^3 cfu/mL), and a dose-dependent increase was observed. E. coli ΔpapG was a very poor inducer of the host response, and only bacteria counts >10^7 cfu/mL triggered any response at all. Thus, in each bacterial concentration interval, the PapG-positive strain was, by far, the most efficient inducer of the host response.

**DISCUSSION**

Mucosal surfaces are populated by a rich microbial flora, but, in most cases, they remain inert to the microbes or their products, and only the pathogens go on to trigger an innate host response in the mucosa. The implication is that discrete virulence factors are needed to activate the response and that the expression of such virulence factors differentiates the pathogen from the commensal. The present study has examined specific epithelial-cell adherence as a mechanism of induction of the host response by use of P-fimbriated uropathogenic E. coli, as an example, and intravesical inoculation of patients, as a model.
An ABU-causing strain, which failed to elicit a response in the patient’s mucosa, was transformed with pap sequences encoding functional or PapG adhesin--negative P fimbriae. The results demonstrate that PapG-mediated adherence breaks mucosal inertia in the human urinary tract by triggering the innate host response and suggest that this is an essential first step in the pathogenesis of UTI that differentiates asymptomatic carriage from symptomatic infection.

Early studies in the murine UTI model showed that P and type 1 fimbriae enhance bacterial persistence [13, 28]. The effect of P fimbriae on the establishment of bacteria in the human urinary tract was later confirmed in the human inoculation model. A P-fimbriated mutant of E. coli 83972 established bacteriuria faster and more efficiently than did the nonfimbriated strain [17]. Epidemiological studies have not related bacterial adherence mainly to long-term bacteriuria but to acute severity of disease in the urinary tract [7, 8]. After the identification of P fimbriae and their receptors, P fimbrial expression was shown to characterize the most-virulent strains, including 70%–90% of acute pyelonephritis–causing isolates and up to 100% of bacteremia–causing isolates [29, 30]. The present study helps to explain the strong link between P fimbriation and disease severity, in that P fimbriae enable the bacteria to directly activate the innate response in the human urinary tract.

Early studies in experimental-infection models confirmed the role of adherence as an essential early step in pathogenesis and identified P fimbriae as a virulence factor; however, the role of P fimbriae has been debated subsequently. Moleby et al. [15] showed that a pap deletion did not reduce the virulence of a clinical isolate in the mouse model. Similar results were obtained by Bahrani-Mougeot et al. [31], who used reporter constructs to monitor gene expression in vivo in the mouse model. In contrast, studies in a primate model of UTI showed enhanced colonization by a wild-type P-fimbriated pyelonephritogenic strain, compared with a papG deletion mutant [14]. Finally, Hull et al. have used the E. coli 83972 strain in patients with neurogenic bladders and have failed to see an effect of P fimbriae [18]. The present study has resolved this controversy by proving that the PapG adhesin mediates attachment in vivo in patients and that PapG-mediated adherence triggers the innate host response in the urinary tract. Importantly, the inoculated patients who responded to the PapG+ strain did not develop disease, emphasizing the need for additional virulence factors to fully activate or perturb the host response.

The mechanism of cell activation by P fimbriae has been studied in uroepithelial cell lines, human tissue biopsy specimens, and animal models. P fimbriae trigger the innate host response by activating distinct transmembrane signaling pathways in epithelial cells [11, 12]. The cell-surface GSLs are necessary as primary recognition receptors for the PapG adhesin, but then TLR4 is recruited for signal transduction, and both receptors are needed for a productive response [12]. Both the recognition receptors and TLR4 are needed for cell activation; thus, mucosal inflammation is suppressed by inhibition of GSL expression or by inactivation of TLR4. In mouse model studies [32] and in clinical studies, variant GSL receptor expression has been shown to influence the severity of disease and the adhesive phenotype of the infecting E. coli strain [33]. Different Tlr4 mutant mice failed to respond to challenge with P-fimbriated E. coli strains and actually developed long-term asymptomatic carriage [12, 34]. Thus, mucosal inertia is controlled both by the fimbrial adhesins and by host genes that regulate the signal-transduction pathways involved in the host response that follows bacterial adherence.

E. coli 83972 is an asymptomatic carrier isolate and, as such, is well adapted for survival in the urinary tract. We have used it as a model ABU-causing strain in several previous human inoculation studies [17]. The strain carries several adhesin gene clusters, but, like most ABU-causing strains, it does not express fimbriae in the urinary tract and evokes virtually no host response, despite high numbers in human urine. As a consequence, E. coli 83972 establishes bacteriuria only in patients with incomplete bladder voiding, where residual urine allows the bacteria to remain and multiply [35]. This is consistent with molecular analysis of ABU-causing strains showing that they carry fimbrial gene clusters but stop expressing the phenotype and persist in an apparently symbiotic state without breaking the inertia of the mucosal barrier [36]. We have proposed that the ABU-causing strains express virulence/colonization factors to facilitate intestinal carriage and early establishment in the urinary tract but not during long-term
persistence. These results have confirmed the relative inertia of the mucosa to the mucosa to persistence. These results have confirmed the relative inertia of bacteria, and have shown that the expression of functional P fimbriae in the E. coli 83972 background was sufficient to break inertia and trigger the host response.

The Koch-Henle postulates were originally formulated to prove the causal link between a microbial pathogen and the disease caused by infection with this organism. The postulates state that the microbe must be isolated from the site of infection in the patient, subcultured to homogeneity, and identified in vitro and that infection of a susceptible host with this isolate should reproduce disease. This line of reasoning may also be applied to individual bacterial virulence factors if they show clear disease associations in epidemiological studies [28, 37] and if discrete mutations attenuate the virulence in vivo. We have demonstrated elsewhere that type 1 fimbriae fulfill these criteria in the mouse model of UTI [28], but the role of type 1 fimbriae in humans remains unclear. Because bacterial virulence in the human urinary tract is multifactorial, we expected it to be quite difficult to discern the influence of a single gene or gene product. Yet, in the present study, we have detected a significant effect of papG in vivo in patients. On the basis of these results, we propose that PapG and P fimbriae fulfill the molecular Koch-Henle postulates as virulence factors of uropathogenic E. coli in the human urinary tract.

References


