Rare Emergence of Symptoms during Long-Term Asymptomatic Escherichia coli 83972 Carriage without an Altered Virulence Factor Repertoire

Béla Köves,* Ellaine Salvador,* Jenny Grönberg-Hernández, Jaroslaw Zdziarski, Björn Wullt,† Catharina Svanborg and Ulrich Dobrindt‡

From the Department of Microbiology, Immunology and Glycobiology, Institute of Laboratory Medicine, Lund University (BK, JG-H, BW, CS), Lund, Sweden, and Institute for Molecular Biology of Infectious Diseases, University of Würzburg (ES, JZ, UD), Würzburg and Institute for Hygiene, University of Münster, Münster, Germany (UD)

Purpose: Asymptomatic bacteriuria established by intravesical inoculation of Escherichia coli 83972 is protective in patients with recurrent urinary tract infections. In this randomized, controlled crossover study a total of 3 symptomatic urinary tract infection episodes developed in 2 patients while they carried E. coli 83972. We examined whether virulence reacquisition by symptom isolates may account for the switch from asymptomatic bacteriuria to symptomatic urinary tract infection.

Materials and Methods: We used E. coli 83972 re-isolates from 2 patients in a prospective study and from another 2 in whom symptoms developed after study completion. We phylogenetically classified the re-isolates, and identified the genomic restriction patterns and gene expression profiles as well as virulence gene structure and phenotypes. In vivo virulence was examined in the murine urinary tract infection model.

Results: The fim, pap, foc, hlyA, fyuA, iuc, iroN, kpsMT K5 and malX genotypes of the symptomatic re-isolates remained unchanged. Bacterial gene expression profiles of flagellated symptomatic re-isolates were unique to each host, providing no evidence of common deregulation. Symptomatic isolates did not differ in virulence from the wild-type strain, as defined in the murine urinary tract infection model by persistence, symptoms or innate immune activation.

Conclusions: The switch from asymptomatic E. coli 83972 carriage to symptomatic urinary tract infection was not explained by reversion to a functional virulence gene repertoire.

Accepted for publication July 22, 2013.

Abbreviations and Acronyms

ABU = asymptomatic bacteriuria
cnf1 = cytotoxic-necrotizing factor1
fyuA = yersiniabactin receptor
hlyA = α-hemolysin
IFNγ = interferon-γ
IL = interleukin
iroN = salmochelin receptor
iuc = aerobactin
kpsMT K5 = K5 capsule
LPS = lipopolysaccharide
PMN = polymorphonuclear leukocyte
UTI = urinary tract infection
wt = wild type
Bacteria invading the urinary tract may cause symptomatic disease or give rise to ABU, a symptom-free carrier state resembling commensalism. ABU is even more common than symptomatic UTI. Epidemiological studies show that asymptomatic carriage protects the patient against symptomatic superinfections compared to patients in whom bacteriuria is eradicated by antibiotic therapy. This protective effect has been used as a rationale to deliberately establish ABU in patients prone to UTI. The therapeutic efficacy of this approach was demonstrated in randomized clinical trials. Observational studies established that therapeutic inoculation is safe and decreases UTI morbidity.

The prototype ABU Escherichia coli strain 83972 is extensively used for human inoculation since it produces no adverse effects, fails to express virulence factors associated with symptomatic UTI and lacks conjugative plasmids. E. coli 83972 and other ABU strains have a smaller genome size than uropathogenic strains. This is due in part to virulence gene deletions that abolish fimbrial expression and adherence, suggesting that ABU strains adapt to the human urinary tract by undergoing reductive evolution.

After therapeutic E. coli 83972 inoculation in a series the number of symptomatic episodes decreased during E. coli 83972 bacteriuria and patients experienced a longer infection-free interval than a placebo group. While most symptomatic UTI episodes were caused by superinfection with other E. coli or non-E. coli strains, we identified a few patients in whom symptoms developed during E. coli 83972 bacteriuria, suggesting a transition from ABU to symptomatic UTI.

In the current study we examined whether E. coli 83972 evolves toward virulence during asymptomatic carriage in the urinary tract. We compared phenotypic and genotypic traits of E. coli 83972 to those of re-isolates from patients with symptomatic episodes. We found no evidence of increased expression of traditional virulence factors by E. coli 83972 in hosts with symptomatic UTI during asymptomatic carriage.

**METHODS**

**Patients and Study Design**

Patients with incomplete bladder emptying due to spinal or lower motor neuron lesions who had recurrent lower UTIs were included in a placebo controlled study of intravesical inoculation with E. coli 83972. In all patients optimal treatment, including clean intermittent catheterization, had been tried but failed. Study exclusion criteria were upper urinary tract dilatation, febrile UTI episodes or pyelonephritis, corticosteroid treatment and significant comorbidity. The study was approved by the Lund University human ethics committee and patients provided informed consent. The study was designed to deliberately establish ABU in patients prone to UTI. The therapeutic efficacy of this approach was demonstrated in randomized clinical trials. Observational studies established that therapeutic inoculation is safe and decreases UTI morbidity.

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**DNA Techniques**

QIAGEN® products were used for genomic DNA isolation. Primers were obtained from Eurofins MWG/Operon, Ebersberg, Germany. Restriction enzymes were obtained from New England Biolabs®. Genomic DNA was analyzed by pulsed field gel electrophoresis. Phylogenetic
classification of re-isolates, and ExPEC virulence genes were determined as previously described.11

**Virulence Factor Expression**
Functional type 1, P, F1C fimbriae, hemolysis and motility as well as O antigen, aerobactin expression and biofilm formation were detected.11,12 Adhesion to the human urinary tract cell lines A498 and T24, and curli and cellulose expression were determined as described previously.13 Experiments were performed in triplicate. We determined growth rates at 600 nm optical density in triplicate experiments using different batches of pooled human urine.

**Gene Expression Profiling**
RNA preparation and microarray analysis were performed as previously reported.9 For statistical significance the 1-sample t-test was applied with the Bonferroni correction. A cutoff of 1.7 (ln2) was used at p ≤ 0.09.

**Experimental Infection**
Experiments were performed with the permission of the animal experimental ethics committee, Lund District Court, Sweden. Female C3H/HeN mice bred at the MIG animal facility were infected at age 6 to 12 weeks by intravesical inoculation with E. coli 83972 wt or re-isolates from each symptomatic episode.14 The mice were sacrificed at 6 or 24 hours, or 7 days, and the kidneys and bladders were removed. Infection was quantified by viable counts on kidney and bladder homogenates. Neutrophils were quantified in uncentrifuged urine using a hemocytometer chamber. For statistical analysis the groups were compared by the paired t-test or Mann-Whitney test.

**RESULTS**

**E. coli 83972 Bacteriuria Delayed UTI Recurrences and Decreased Number of Symptomatic UTI Episodes**
Re-isolates of E. coli 83972 were obtained from patients who participated in a placebo controlled crossover study of the protective effect of E. coli 83972 bacteriuria after deliberate inoculation of this strain into the urinary tract.6 Patients were protected from symptomatic UTI, as defined by the number of episodes, before study entry and while in the placebo arm of the study. The mean number of symptomatic episodes per patient-year was 1.2 during ABU and 4 before the study (paired t-test p = 0.000019, fig. 1, A). In the E. coli 83972 bacteriuria arm with 202 months of observation a total of 13 symptomatic UTI episodes developed in 9 patients (0.8 per patient-year). This was significantly lower than in the placebo arm with 168 months of observation time during which 4 of 20 patients had a total of 35 UTI episodes (2.5 per patient-year, p = 0.009). Median time to the first symptomatic episode was also significantly less in the placebo group than during ABU (11.3 vs 5.7 months, p < 0.013).

The 13 UTI episodes recorded during E. coli 83972 bacteriuria were further characterized. Ten episodes were superinfections. E. coli 83972 was replaced by a different E. coli strain in 7 episodes, and by Pseudomonas aeruginosa, Enterococcus faecalis and Proteus mirabilis in 1 each. In patients R4 and R15 only E. coli 83972 was recovered during 1 and 2 symptomatic episodes, respectively, suggesting that symptoms were caused by this strain (see table and fig. 1, B). Before symptoms developed these patients carried E. coli 83972 asymptotically without discomfort. The 3 symptomatic episodes were accompanied by increased urine polymorphonuclear leukocyte numbers and increased urine cytokine levels (see table, fig. 2, A and supplementary fig. 1, http://jurology.com/). In patient R4 an increase in RANTES, IP-10, sIL-2Ra, MCP-1, IL-1α, IL-1RA and IFNγ was observed and in patient R15 IL-6, sIL-2Ra and IL-1α were increased.
However, these isolates did not stimulate a higher cytokine response in human uroepithelial cells (fig. 2, B and supplementary fig. 1, http://jurology.com/). In all patients the peak mucosal response was several fold higher during the symptomatic episode compared to the preceding ABU period. In patient R10 the preceding ABU response was low or absent.

Properties of E. coli 83972 Re-Isolates from Symptomatic Episodes
To examine whether a change in bacterial properties precipitated the symptomatic episodes we examined the 3 E. coli 83972 re-isolates. We also included E. coli 83972 re-isolates from symptomatic episodes in 2 patients. Patient R10 participated in the therapeutic study but symptoms developed after study completion. Patient Sp10, who received E. coli 83972 inoculation in a separate open study protocol, was excluded from analysis due to corticosteroid treatment (see table).

To identify E. coli 83972 re-isolates we screened 20 randomly chosen colonies for the presence of the cryptic 1.6 kb plasmid and the internal 4,253 bp fim deletion (fig. 3, A). Like the wt strain, re-isolates were phylogenetically classified into the B2 lineage and shared an identical genomic restriction pattern (supplementary fig. 2, http://jurology.com/).

E. coli 83972 carries the type 1 (fim), P (pap), F1C (foc) fimbrial genetic determinants and genes coding for hlyA or cnf1, fyuA, iroN, iuc, kpsMT K5 and the pathogenicity island marker malX.11 These genes were present in all re-isolates, suggesting that the overall pathogenicity island structure remained largely intact (fig. 3, B).

Re-isolates did not express functional P, F1C or type 1 fimbriae (fig. 3, C). To exclude other adhesins we monitored adherence to A498 kidney cells and T24 bladder cells but it was not detected (fig. 4, A). We observed no consistent change in biofilm, curli or cellulose formation (figs. 3, C and 4, B).

Re-isolates expressed an O antigen pattern identical to that of E. coli 83972 (supplementary fig. 2, http://jurology.com/). They had a growth rate in pooled human urine similar to that of E. coli 83972 except re-isolates R10 and R15-1 clone II, which grew more slowly (fig. 4, C).

Re-Isolate Population Heterogeneous Phenotypes
Although urine samples from symptomatic episodes were E. coli 83972 monocultures, re-isolates of samples R15-1 and R15-2 showed heterogeneous phenotypes and comprised colonies of different sizes or motility. For colonies from urine sample R15-1 about 75% the colony size and morphology resembled those of strain E. coli 83972 (R15-1 clone I). The remaining colonies (R15-1 clone II) were small and grew slowly (fig. 4, C). However, they had the same 1.6 kb cryptic plasmid, fim deletion, restriction pattern and virulence gene content as E. coli 83972. The slow growth and reduced colony size were reminiscent of small colony variants associated with persistent infection.15 Furthermore, individual colonies of urine sample R15-2 differed in motility and flagella expression.

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**Figure 1.** Symptomatic UTI episodes during E. coli 83972 bacteriuria. A, mean ± SEM frequency (n) of symptomatic UTI episodes during year before inoculation and during year with E. coli 83972 bacteriuria in 20 patients (paired t-test). B, innate immune response of patients to symptomatic E. coli 83972 episodes quantified as urine cytokine and PMN levels.
Re-Isolate Increased Motility
Since flagella were proposed to facilitate ascending UTI, we compared the motility of E. coli 83972 and the re-isolates. Increased motility was observed for re-isolates R15-2 and Sp10. R15-2 appeared as a phenotypically heterogeneous population with increased motility of individual cells (R15-2 clone I) and flagellar expression relative to E. coli 83972 (fig. 4, D and supplementary fig. 3, http://jurology.com). The remaining re-isolates (R15-2 clone II) were as motile as E. coli 83972.

Motile Re-Isolate Gene Expression Analysis
To analyze differences in the gene expression of re-isolates with phenotypes that markedly deviated from the wt we compared the transcriptome between E. coli 83972 and the motile re-isolates (R15-2 clone I and Sp10). Of 95 de-regulated genes in R15-2 clone I 80 were up-regulated and 15 were down-regulated while 81 and 17 of 98 genes in Sp10 were increased and decreased, respectively (fig. 5).

Most up-regulated genes in R15-2 clone I encoded bacteriophage components. In addition, activated genes were involved in the SOS or stress response (recA, recN, lexA, ruvB, dinI, dinB, sulA, yebG, osmB and umuD), σ factor expression (rpoA, rpoE and rpoS) and acid resistance (gadA, gadB, hdeAB, cadB and slp). mglAB genes that code for a galactose transporter and some phage related genes were down-regulated. In E. coli R15-2, representing a heterogeneous group of motile and less motile colonies, increased flagellar gene expression was less pronounced.

In contrast, in re-isolate Sp10, in which all colonies showed increased motility, flagella biosynthesis and assembly genes (flgA, flgDEFG, flhA,

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**Figure 2.** Host response to ABU in vivo and in vitro. A, host response to symptomatic E. coli 83972 episodes. B, geometric mean ± SEM epithelial response to in vitro infection of A498 cells with E. coli 83972 wt, symptomatic E. coli 83972 re-isolates and uropathogenic E. coli strain CFT073 in 2 independent experiments. Fetal calf serum (FCS) (5%) was used to provide soluble CD14.
**fliA, fliG and fliO** were significantly up-regulated together with genes involved in heat shock response (*groEL* and *groES*), LPS biosynthesis (*rfagplijy, waaV, waaW* and *lpxAB*), amino sugar use (*glmUS*) and iron uptake (*chuATUWX* and *entCA*). Transport genes were significantly down-regulated, including *aga, srl* and *dgo*.

The *yjeE* and *yqiD* genes involved in biofilm formation were commonly up-regulated in R15-2 clone I and Sp10 compared to *E. coli* 83972, as were *gudD* and *gudP* involved in D-glucarate use, and genes coding for 30S and 50S ribosomal subunit components. Transcription of ribose transporter genes was repressed in each re-isolate relative to the wt.

Accordingly, transcriptional regulation was unique for bacteria recovered from each host. It provided no evidence of commonly deregulated genes in re-isolates from different symptomatic hosts.

**Bacterial Persistence and Host Response Activation In Vivo**

To investigate whether the re-isolates showed increased virulence we established in vivo infections in C3H/HeN mice. There was no significant difference in the bacterial number in kidneys and bladders 24 hours and 7 days after inoculation (fig. 6, A). Urine neutrophil counts reflected the low acute inflammatory response to *E. coli* 83972. Motility of the re-isolates did not influence bacterial numbers or urine neutrophil counts (fig. 6, A).

*E. coli* 83972 was compared to the strain 83972*Δ*fliC mutant, which does not express functional flagella. SN25, the most motile asymptomatic
re-isolate, served as the positive control (fig. 6, B). We observed a biphasic infection pattern. Five hours after infection E. coli 83972 and SN25 reached significant numbers while the kidneys of mice colonized with E. coli 83972ΔfliC remained sterile, consistent with a role for flagellation in the early phase of ascending infection.16 At 7 days persistent bacteriuria (greater than 10⁵ cfu/ml) developed in mice infected with E. coli 83972 and 83972ΔfliC. The highly motile isolate was eliminated more rapidly than the wt and no increase in the urine neutrophil number was related to flagellation (fig. 6, B).

**DISCUSSION**

After comparing the genome of E. coli 83972 re-isolates from different inoculated human hosts we previously suggested that evolution toward commensalism is favored during asymptomatic bladder colonization.9 The current study was designed to address whether evolution toward virulence may occur in parallel in specific hosts. To detect changes in bacterial properties associated with the rare development of symptoms during asymptomatic carriage we investigated phenotypic or genotypic changes in re-isolates that might have precipitated the symptomatic episodes.

After 202 patient-months of E. coli 83972 ABU only 3 symptomatic UTI episodes with E. coli 83972 were recorded. Analysis of these isolates and an additional 2 re-isolates from symptomatic episodes excluded regained expression of virulence factors as a cause of symptoms. LPS and capsule as well as biofilm formation and adherence properties remained unchanged. Deregulated genes were mainly involved in different stress responses, metabolic versatility and LPS biosynthesis but no common expression pattern was detected. Flagellation was perturbed but differences in virulence were not observed in the murine UTI model. Results suggest that the occasional symptomatic UTI episode does not reflect regained expression of established virulence factor in E. coli 83972 during long-term carriage.

Interestingly, we observed phenotypic variation in the E. coli 83972 monoculture populating the bladder. This behavior mirrored adverse and stress conditions, and it may ensure the fitness and survival of a subset of cells in this niche. The presence of 2 phenotypes in a clonal population18 suggests bistable gene expression facilitating the exploitation of dynamic host environments and promoting gene

**Figure 4.** Phenotypic characterization of E. coli 83972 re-isolates from symptomatic episodes. A, adhesion to T-24 bladder epithelial cells and A498 kidney epithelial cells. B, biofilm formation in pooled human urine. C, growth kinetics in pooled human urine in vitro. D, motility on urine swarm agar plates.
expression changes, eg those favoring chronic infection. Examples of such heterogeneous phenotypes correlated with increased fitness of Vibrio cholerae and E. coli. Flagellin expression of Salmonella typhimurium also underlies bistable gene regulation but host environmental factors driving these changes remain poorly understood. Small colony variant formation in Staphylococcus aureus or P. aeruginosa has correlated with chronic infection and in Campylobacter jejuni it is considered a survival strategy relying on stress fit individuals in a heterogeneous population. Therefore, bacterial adaptation to long-term in vivo growth in the urinary tract could include phenotype switching. Alternatively, the occurrence of heterogeneous populations at symptomatic episodes may represent spontaneous stochastic events, including minor transient populations.

If symptom development were due to changes in bacterial virulence, the re-isolates should have shown increased fitness in the murine UTI model, as reflected by a higher count in the bladders and kidneys. In parallel, we would have expected these strains to trigger an inflammatory response that was not observed after infection with the wt strain. However, such changes were not observed. The highly flagellated asymptomatic strain SN25 attained a significant number in kidneys 5 hours after infection. This suggests that flagellar motility may be important for initial ascent of bacteria to the upper urinary tract but this was cleared earlier than the wt strain. In contrast, the 83972ΔfltC mutant established persistent bacteriuria without upper urinary tract involvement, similar to human ABU. This implies that increased flagellar expression may be counterproductive for long-term persistence.

Symptomatic episodes were accompanied by an innate immune response with increased cytokine and chemokine levels in urine as well as pyuria. IL-6 and 8 responses in symptomatic UTI have been extensively studied and the concentrations reflect disease severity. A recent experimental study suggested a potential role for noninflammatory host responses, showing distinct symptomatic responses to bacteriuria mediated by TLR4 that are independent of inflammation. However, in our study all symptomatic UTI episodes were accompanied by increased cytokine levels. Additional proinflammatory cytokines included IL-1α, which was counterbalanced by the IL-1 receptor antagonist IL-1RA, as well as RANTES, which was associated with eosinophil/mast cell activation. The increase in soluble IL-2Rα and IPNγ confirmed a response profile previously observed in patients with E. coli 83972 bacteriuria. These findings are consistent with local lymphocyte and dendritic cell activation, and infection dependent formation of lymphoid follicles in patients with long-term ABU. The follicles resolve after antibiotic eradication of bacteriuria, confirming that they are driven by infection. However, there was no evidence of follicle formation in patients who carried E. coli 83972.
Figure 6. Geometric mean ± SEM values of re-isolate virulence, as defined by in vivo infection (Mann-Whitney test). A, experimental infection of C3H/HeN mice by intravesical inoculation with $10^9$ cfu in 0.1 ml E. coli 83972 or re-isolates from symptomatic episodes. Bacterial number in urine, kidneys and bladders, and neutrophil response revealed no difference in virulence (Mann-Whitney test). B, role of increased flagellation and motility were compared for E. coli 83972 wt and highly motile re-isolate Sp10. Mutant strains E. coli 83972 $\Delta fliC::cat$ and SN25, highly motile re-isolate from asymptomatic carrier, served as negative and positive control, respectively. Bacterial numbers in urine, kidneys and bladders, and neutrophil influx revealed no long-term advantage for flagellated strains.
CONCLUSIONS
In the absence of functional virulence factors and shared molecular changes in bacteria the mechanism behind the emergence of symptoms remains unclear. A possibility is a host driven break in the tolerance of asymptomatic colonization, which triggers spurious pathogen recognition signaling. However, it is intriguing to speculate that the molecular events that precipitate cystitis symptoms have a different mechanistic nature than that of acute pyelonephritis. It is also intriguing that the few study patients in whom symptoms developed during ABU strain carriage may share what is to our knowledge an as yet undefined host response pattern that leads to immune overactivity and symptoms.

ACKNOWLEDGMENTS

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