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In vitro studies of epithelium-associated crystallization caused by uropathogens during urinary calculi development

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ABSTRACT

Infectious urinary stones account for about 10% of all urinary stones. In 50% of cases urolithiasis is a recurrent illness, which can lead to the loss of a kidney if not properly treated. One of the reasons for recurrence of the disease may be the ability of bacteria to invade urothelial cells, persist in the host cells and serve as potential reservoirs for infection. Various uropathogens are associated with the formation of bacteria-induced urinary stones but *Proteus mirabilis* is the most commonly isolated (70%). An *in vitro* model was used in this study to analyze intracellular growth and crystallization in the presence of *P. mirabilis*, *Klebsiella pneumoniae* and *Escherichia coli*. Human ureter (Hu 609) and bladder (HCV 29) epithelial cell lines were infected with bacteria and incubated (3–72 h) in the presence of synthetic urine and amikacin to prevent extracellular bacterial growth. During the incubation the number of bacteria (CFU/ml) inside epithelial cells and the intensity of crystallization were established. Crystallization was determined as an amount of a calcium radioisotope. The chosen strains of uropathogens were able to invade both types of epithelial cells but the Hu 609 cells were invaded to a higher extent. However, crystallization occurred only in the presence of *P. mirabilis* strains which were invasive and urease-positive. The highest intensity of cell-associated crystallization was observed when the number of bacteria within the urothelium remained stable during the time of incubation. These results show that *P. mirabilis* has an ability to form crystals inside the host cells. Under these conditions bacteria are protected from antibiotic killing, which leads to persistent and recurrent infections. We also suspect that this phenomenon may be an important stage of kidney stones formation.

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1. Introduction

Urolithiasis is one of the most common diseases, occurring in approximately 10% of the human population with an average recurrence rate of 25%. It is a multifactorial disease that can result from metabolic disorders, incorrect diet, obesity, concentration of crystallization inhibitors and promoters in urine and bacterial infection [1–3]. Urinary infectious stones develop as a result of urinary tract infection caused by urease-producing bacteria such as *Proteus*, *Klebsiella*, *Staphylococcus*, *Providencia* and others. In industrialized countries, these stones account for 10% of all urinary stones and pose a significant health problem due to their high rate of recurrence (in 50% of cases) and renal tissue damage [4]. Infectious stones are more common in women, male children and elderly people since these groups are more prone to urinary tract infection [2]. These stones are also characterized by very rapid growth (4–6 weeks are enough to form a stone) and chemical composition which is a mixture of struvite ($MgNH_4PO_4 \times 6H_2O$) and carbonate-apatite ($Ca_{10} (PO_4)_6 \times CO_3$). *Proteus* species are among the most common bacilli associated with the formation of bacteria-induced bladder and kidney stones [5]. *Proteus mirabilis* causes urinary tract infection (UTI) most frequently in patients with functional or anatomic abnormalities or with instrumentation such as prolonged catheterization [6,7]. These infections are often long-term and recurrent and lead to a renal stone disease. The course of infection depends on the host's ability to eliminate pathogens and specific virulence factors of *P. mirabilis* such as urease, haemolysins, fimbriae, proteases, lipopolysaccharide or invasiveness [6]. One of the reasons for the persistence of infection is probably the ability of *P. mirabilis* to invade epithelium and avoid the humoural immune

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Table 1
Description of the source of isolation and characteristics of tested bacterial strains.

Strains	Urease activity		Source of isolation	Patients/disease
	Medium detected[a]	Urease units[b]		
P. mirabilis				
C6	Positive	95.1 ± 7.8	Urinary catheter	Male
C7	Positive	209.9 ± 9.1	Urinary catheter	Catheterized
C11	Positive	149.9 ± 6.2	Urinary catheter	Due to benign
C31	Positive	34.8 ± 5.8	Urinary catheter	Prostatic hypertrophy
K5	Positive	77.9 ± 4.2	Urinary stones	
K8	Positive	65.0 ± 11.4	Urinary stones	Women, urolithiasis
K608	Positive	168.7 ± 9.6	Urinary stones	Urinary tract infection
KR37	Negative	1.0 ± 0.6	Swab of the wound	
KW28	Negative	2.4 ± 1.8	Swab of the wound	Women, leg ulcers
E. coli				
02	Negative	5.0 ± 2.8	Urine	
04	Negative	3.1 ± 1.9	Urine	Women, urinary tract
09	Negative	4.3 ± 2.9	Urine	Infection
C9	Negative	2.6 ± 1.9	Urinary catheter	
C11	Negative	2.1 ± 1.6	Urinary catheter	Male
K. pneumoniae				
C41	Positive	18.6 ± 7.2	Urinary catheter	Catheterized
C45	Positive	48.5 ± 5.8	Urinary catheter	Due to benign
C51	Positive	124.8 ± 10.1	Urinary catheter	Prostatic hypertrophy
C53	Positive	135.5 ± 12.1	Urinary catheter	

[a] Urease activity was tested on Christensen's medium.
[b] Urease activity units [U] determined as μg NH_3 produced × 1 min^{-1} × (mg total bacterial protein)$^{-1}$.

system, which allows the bacteria to survive within the cells [7]. Bacterial urease is the most important virulence factor involved in urolithiasis. Ammonia, produced by the enzymatic hydrolysis of urea, elevates urine pH causing supersaturation and crystallization of magnesium and calcium ions as struvite and carbonate-apatite, respectively [8]. Additionally, bacteria have on their surface negatively charged polysaccharides such as a capsular polysaccharide (CPS) and a lipopolysaccharide (LPS). These polysaccharides can accelerate crystallization, serve as a crystallization nidus and bind crystals together into a stone [9,10]. The result of these processes is formation of aggregates of crystals, bacteria and various host components such as proteins or glycosaminoglycans, which are very difficult to eradicate. The stone can obstruct the urine flow and its surface is a nidus for *P. mirabilis* and other bacteria to establish urinary tract infection [5,11]. As mentioned above, different species of microorganisms including those that do not decompose urea were isolated from urinary stones. Therefore, the role of urease-negative uropathogens including *Escherichia coli* in the development of urinary stones cannot be excluded, although it has not been sufficiently examined. According to the current knowledge, development of infectious urolithiasis may be caused by several factors and in spite of long-term clinical and experimental investigations, some specific mechanisms responsible for urinary calculi formation still remain unclear. The aim of this study was to demonstrate the specificity and kinetics of crystal growth induced by intracellular bacteria. To achieve this goal *P. mirabilis* and *Klebsiella pneumoniae* strains with different urease activity and *E. coli* without this virulence factor were used.

2. Material and methods

2.1. Bacterial strains

Various strains of uropathogenic bacteria belonging to *P. mirabilis*, *E. coli* and *K. pneumoniae* species were used in the study. All strains were examined by standard diagnostic methods including identification of urease activity on Christensen medium. The strains of *P. mirabilis* (KW 28, KR 37) and *E. coli* (02, 04, 09) were obtained by courtesy of the Department of General Microbiology, University of Lodz. The other strains were part of the strains collection of the Department of Immunobiology of Bacteria. The list of the strains is presented in Table 1. Before the experiment bacteria were cultured on a tryptic soy broth (TSB, BTL, Poland) for 18 h at 37 °C and then suspended in synthetic urine with RPMI 1640 medium (mixed in a ratio of 1:4) (Lonza, Walkersville, MD, USA). The number of bacteria per ml was determined spectrophotometrically at 550 nm (Ultrospec 2000, Pharmacia Biotech, Vienna, Austria). As previously stated, the absorbance was directly proportional to the cell concentration, and the density of the suspension was adjusted to an A_{550} between 0.3 and 0.4, which corresponded to 1–5 × 10^5 bacteria per ml.

2.2. Cell lines

Hu 609 and HCV 29 cell lines were used to study the invasion of human urothelium by bacteria. These cell lines were derived from a histologically normal human ureter and the bladder epithelium, respectively [12,13]. HCV 29 and Hu 609 were obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland. They were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 2 mM ultraglutamine (Lonza, Walkersville, MD, USA) and antibiotics—100 μg/ml penicillin and 100 μg/ml streptomycin (Polfa Tarchomin, Poland) in a humidified incubator with 5% CO_2 at 37 °C.

2.3. Urease activity

Urease activity of the whole bacterial cells was analyzed using the phenol hypochlorite ammonia determination assay [14]. Urease activity was expressed as units (U). One unit was defined as μg NH_3 produced × 1 min^{-1} × (mg total bacterial protein)$^{-1}$. Overnight bacterial culture (TSB, 37 °C) was centrifuged (8000 g/5 min) and suspended in synthetic urine. After incubation (15 min, 37 °C) 10 μl of 1 ml of bacterial suspension in synthetic urine was mixed with 100 μl phenol sodium nitroprusside solution (1% w/v phenol, 0.05% w/v sodium nitroprusside) and 100 μl sodium hypochlorite solution (0.5% w/v sodium hydroxide, 0.5% w/v sodium hypochlorite) and incubated for 30 min at 37 °C. Absorbance was measured at 625 nm Multiskan Ex (Labsystems, Helsinki, Finland) and the intensity of the blue colour was compared to that given by standard solutions of ammonium sulphate. The amount of ammonium was recalculated to 1 ml. The suspension was centrifuged (8000 g/5 min), the pellet was mixed with 2M NaOH and incubated overnight at 37 °C to lyse bacterial cells. The amount of protein was measured by the Lowry method [15].

2.4. Synthetic urine

Synthetic urine was prepared using a modified version of the method previously described [16] and contained components present in normal urine, each at a concentration equivalent to the average concentration found during a 24 h period in the urine of a healthy person. It contained the following components (g/l): $CaCl_2 \times 2H_2O$, 0.651; $MgCl_2 \times 6H_2O$, 0.651; NaCl, 4.6; Na_2SO_4, 2.3; sodium citrate, 0.65; sodium oxalate, 0.02; KH_2PO_4, 2.8; KCl, 1.6; NH_4Cl, 1.0; urea, 25.0; creatine, 1.1 (Sigma, St. Louis, MO, USA) and tryptic soy broth, 10.0 (BTL, Poland). Prior to the experiment, pH was adjusted to 5.8 and the urine was sterilized by passing through a 0.2 μm pore-size filter.

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