Urol Res (1996) 24:51–54

ORIGINAL PAPER

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Potential contribution of optional urease-positive bacteria to idiopathic urinary calcium stone formation

I. Expression of urease activity in bacteria from the urinary tract that are commonly classified as urease-negative

Received: 29 December 1994 / Accepted: 15 August 1995

Abstract The aetiology of calcium oxalate stones, which are commonly believed to be sterile, has not yet been fully elucidated. Recent bacteriological studies and investigations using the scanning electron microscope have also shown microorganisms to be present in this type of stone. These microorganisms were assumed not to be able to split urea. To list the most common urease-negative bacteria established in the human urinary system, we isolated apparently urease-negative microorganisms from a consecutive series of 58 urinary stone-forming patients by using standard selecting agars. Pure strains were incubated in an inductive medium lacking all sources of nitrogen except urea. Induction of urease activity was monitored by a test based on the reaction of phenol/hypochlorite with ammonium ions. This test revealed whether the urease negativity of a strain indicated by the selective agar was optional or absolute. All strains we investigated by this method and which were classified by standard methods as urease-negative we found produced urease activity which was clearly measurable, though it was often comparatively small. In the light of these results, the matrix theory of calcium oxalate stone development will need some modifications.

Key words Calcium stones · Infection · Optional urease activity

Introduction

It is well established that struvite stones form as a result of infection with urease-splitting organisms [7]. However, the role of bacteria in the aetiology of calcium phosphate and calcium oxalate stones, commonly considered to be sterile, is undecided and several non-urea-splitting strains of organisms have been isolated from stones removed by surgery and from urine obtained under sterile conditions [6, 8]. Electron microscopic studies provide strong evidence for previous bacterial infection in a large number of calcium stones denoted by the presence of moulds from bacterial cells [4]. Experiments in vitro showed that several non-urea-splitting microorganisms are probably also able to trigger mineralization [2, 9]. However, it has not been certain that bacterial growth in cultures has not resulted from accidental contamination, but has been from the genuine participation of bacteria in stone genesis. Our aim was (1) to detect the extent and presence of organisms commonly classified urease-negative (*un*) in the urinary tract of calcium stone-forming patients, (2) to culture and characterize the strains of these organisms and (3) to show whether these organisms were obligatory or optional non-urease producers.

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Material and methods

Material

Bacteria colonizing the urinary system were isolated from midstream urine samples or collected from catheterized urine samples and/or stones or stone fragments from a consecutive series of patients ($n = 58$) undergoing extracorporeal shock wave lithotripsy (ESWL) and/or endoscopic treatment at local urological clinics. The urine samples were obtained prior to any treatment of the stone disease and were immediately processed. Stones or fragments were endoscopically or surgically removed after ESWL under sterile conditions. This material (non-struvite) was stored in sterile physiological NaCl solution at room temperature (max 20 h) until subjected to further investigations. Microorganisms were screened and cultivated with substrates from Merck and Bio-Merieux. These strains, which showed no reaction to urea nutrient medium according to Christensen [1], were selected and designated as "UAC-*un*" (*u*rea *a*gar Christensen *u*rease-*n*egative). The final identification of these UAC-*un* species was done by the diagnostic kits Api-E,

Api-Strep and Rapidec-Strep (bio-Merieux). All other reagents were of analytical grade.

Methods

The urine samples were appropriately diluted and plated on a selective solid medium (urea nutrient medium according to Christensen, UAC). Stone material with a mean diameter down to 5 mm was carefully decontaminated on its outside and broken. Surviving bacteria from the centre of the stone were transferred by stamping the fractures onto the solid nutrient medium. Stone debris of a smaller diameter than 5 mm was homogenized in the transport medium, and the resulting suspension was handled as for the urine samples. Sterile operating conditions were strictly observed during the whole period of handling the material, and any accidental contamination was carefully avoided.

Ability of urease expression was indicated when phenol red, a component of UAC, changed colour due to ammonium ion production. Urease-positive (*up*) bacteria were roughly identified by further culturing on differentiating media. UAC-*un* germs were cultured until purity, identified by the above-named biochemical test kits and stored in strain collection.

We developed a test method for quantifying the urease activity of the isolated microorganisms. This was based on the nitroprusside-hypochlorite reaction according to Fawcett and Scott [5], and we used some details from the Ingotest 553651 (Boehringer Ingelheim). Standardized jack bean urease (Boehringer Mannheim) was used to check the test conditions. The following reagents and solutions were applied: Solution 1, colour reagent: 0.7 mM sodium nitroprusside dihydrate/530 mM phenol/water; solution 2, developing reagent: 58 mM sodium hypochlorite solution/770 mM sodium hydroxide; solution 3, 0.5 M phosphate buffer, pH 6.8; solution 4, 0.5 M phosphate buffer, pH 6.8/0.04% urea; solution 5, ammonium chloride solution for calibration (ten concentration steps covering the range from 1.34 to 13.4 mM); solution 6, jack bean urease (seven concentration steps covering the range from 1.5 to 7.5 U/individual test series). Linearity of the reaction to ammonium ions was established by the following method: 1.2 ml solution 3 and 20 µl solution 5 were mixed and subsequently 1 ml solution 1 and 1 ml solution 2 were added. The well-mixed solutions were allowed to rest for 10 min at room temperature and were then diluted with 5 ml distilled water. Colour intensity was measured at $lambda_{456\ nm}$ against a blank.

The reaction of jack bean urease under the test conditions was examined as follows: 200 µl solution 6 was added to 1 ml solution 3. The reaction was started by the addition of 20 µl solution 4. After exactly 5 min incubation time at room temperature, the reaction was stopped by the addition of solution 1 and solution 2 (see above).

Optional urease activity of the isolated UAC-*un* strains was quantified by the following method: a bacterial culture in an "inductive medium" (see below) was homogenized (Ultraturrax, Janke & Kunkel, 8000 rpm/20 s) in the presence of a proteinase inhibitor cocktail. The resulting suspension was adjusted to an optical density (OD) of 0.1 ($lambda_{578\ nm}$). An aliquot of 200 µl bacteria suspension was added to 1.020 ml solution 4 (prewarmed to 37 °C). The mixture was incubated for 5 min in a water bath (37 °C) and then the reaction was stopped by addition of the reagents solution 1 and solution 2. Colour intensity was measured against a blank without urea substrate.

Induction of optional urease activity

Strains which had demonstrated no positive reaction with UAC were revitalized on CLED agar (cystine-lactose-electrolyte-deficient medium). After having formed a dense lawn (after about 3 days of incubation), the cells were carefully removed from the agar surface and suspended into the "inductive medium" (a physiological NaCl solution containing 0.04% urea, pH 6.8). The cells were starved by incubation for 24 (48, 72, 96) h in a shaking water bath at 37 °C. This NaCl incubate was used as an "enzyme solution" when determining urease activity of UAC-*un* strains.

Results

The subject of our investigations were the bacteria colonizing the urinary system which showed no positive reaction to a commonly used medium for identifying urea-splitting microorganisms, i.e. UAC agar. These UAC-*un* bacteria have been commonly believed not to express the enzyme urease. From a series of 58 specimens we were able to select 32 infected ones (55.2%). This group comprised 19 cases infected each with an UAC-*un* bacterium (32.8%), one case of multiple UAC-*un* infection (1.7%), 9 cases infected by an *up* organism (15.5%), and 2 mixed *up*/UAC-*un* infections (3.5%). In total, there were two-thirds UAC-*un* infections versus one-third *up* infections (Table 1). Thirteen different species contributed to 22 infections involving UAC-*un* bacteria (Table 2). Cocci (62.2%) were well to the fore, compared with gram-negative rods in only 34.4% of the infections. The above-mentioned UAC-*un* bacteria were examined to determine whether their failure to exhibit urease activity was obligate or

Table 1 Type of infection in urine samples and stone material from stone-forming patients

Total number of investigated cases	58
Sterile cases	26
Sterile cases treated with antibiotics	11
Total infected cases	32
Infected with one "urease-negative" organism	19
Multiple "urease-negative" infection	1
Infected with one "urease-positive" organism	9
Mixed "urease-positive/urease-negative" infection	2
Infected with unidentified organisms	1

Table 2 UAC–urease-negative strains from urine and stone material from stone-forming patients (*UAC*, no reaction on urea substrate according to Christensen)

Species	Frequency
Alcaligenes spp.	1
Coccales spp.	1
Enterobacter agglomerans	1
Enterobacter cloacae	2
Enterococcus faecalis	2
Escherichia coli	3
Klebsiella pneumonia	1
Staphylococcus aureus	1
Staphylococcus spp.	2
Staphylococcus epidermis	3
Staphylococcus xylosis	3
Streptococcus agalactiae	1
Streptococcus spp.	2

optional, i.e. whether there were any circumstances forcing biosynthesis or activation of urease. The low growth rate of bacteria in different artificial urine samples was a limiting factor to the choice of test systems similar to natural urine. Pre-breeding of cells on CLED agar supplied the most suitable bacterial material. Subsequently, a situation deficient in ionic nitrogen (and carbohydrates), i.e. incubation in physiological saline/urea over 24 h, stimulated (in some cases strong and lasting) urease activity in all the strains isolated (Table 3). These bacterial ureases exhibited cyclical activity variations dependent on the incubation time, as had been noted by previous authors [10].

The colour reaction to ammonium ions in our test for measurement of bacterial urease activity showed good linearity and reproducibility throughout the range (1.34–13.4 mM NH_4Cl equivalent to 13.4–134 nmol urea). The standard deviation value for the slope of the calibration curve ($n = 3$) was 0.05 when reagents of the same charge were used. Reagents from different charges ($n = 6$) altered this value to 0.76.

Discussion

Expression of urease activity in bacteria is traditionally exposed by the catabolism of urea, which is added to the culturing substrate as the sole nitrogen source. A very familiar medium is the urea-based substrate according to Christensen (UAC) [1]. Ammonium ions resulting from the hydrolysis of urea cause a colour change of the pH indicator included (phenol red, phenolsulphonphthalein). We found this test method to be rather insensitive, since in the case of a weak urea turnover small amounts of ammonium ions become buffered, not counting the part which escape in the form of the gaseous NH_3. Thus, the proof of urease using UAC is only suitable for screening microorganisms splitting urea intensively.

The part of the Api-E enzymatic identification test (bio-Merieux) which proves urease activity is also of low sensitivity, and this is pointed out on the instruction sheet: all the optional *up* (*oup*) enterobacteria strains we identified by this kit showed a negative reaction. Also considering that it is not only a depletion in inorganic nitrogen which triggers urease activity but that there are a multitude of different factors which may be decisive [11] depending on the strain or organism, it is not surprising that potential urease activity in *oup* bacteria which infect the urinary tract has often remained undetected. In our experiments, even when cultured under identical controlled conditions, different strains of the same species and, moreover, the control strain, which was always the same, each time showed variations in the time-dependent urease activity pattern as shown in Table 3.

Bacteria which are originally *un* may apparently be transformed: Recently Dorazio and Collins [3] detected a plasmid-encoded urease gene cluster in uropathogenic members of the Enterobacteriaceae

Table 3 Optional urease activity of uropathogenic microorganisms as a function of incubation time in the inductive medium. The data represent mean values from $n = 2$ determinations. A series of optional urease-positive strains was always co-incubated with a constitutional urease-positive control strain (*indicated by underlining*)

Patient no.	Species	Milliunits after *n* hours of incubation			
		24 h	48 h	72 h	96 h
48/92	*Streptococcus* sp.	0.3	4.8	4.7	
52/92	*Streptococcus* sp.	4.1	3.3	5.1	
41/92	*Escherichia coli*	3.5	5.9	6.5	7.6
59/92	*Pseudomonas paucimobilis*	2.7	7.7	15.5	19.9
12/92	*Escherichia coli*	0.6	1.8	6.4	
36/92	*Alcaligenes* spp.	0.1	2.7	9.0	
20/92	*Escherichia coli*	1.5	2.5	6.7	
36/92	*Proteus mirabilis*	3.6	7.7	22.9	
53/92	*Staphylococcus* sp.	10.6	21.9	15.9	
19/92	*Enterococcus faecalis*	5.9	4.0	3.8	
29/92	*Enterococcus faecalis*	3.5	4.3		
37/92	*Streptococcus agalactiae*	4.2	6.5	5.9	
59/92	*Pseudomonas paucimobilis*	4.2	9.6		
58/92	*Staphylococcus* sp.	1.6	1.5		
60/92	*Staphylococcus epidermis*	13.0	21.2		
59/92	*Pseudomonas paucimobilis*	3.4	3.1		
2/92	*Enterobacter agglomerans*	6.8	6.7	9.9	
8/92	*Enterobacter cloacae*	7.0	7.1	11.9	
13/92	*Staphylococcus epidermis*	4.5	2.2	2.7	
32/92	*Staphylococcus xylosis*	5.5	5.0	7.3	
35/92	*Staphylococcus xylosis*	4.3	1.3	2.4	
59/92	*Pseudomonas paucimobilis*	7.7	8.6	16.0	138.9

family. There was some hint that this originated from the *Proteus mirabilis* genome by gene transfer.

Our incubation results suggest that urease activity in our UAC-*un* strains while resting in the "inductive medium" was induced by the total lack of nitrogen (and carbohydrate) sources other than urea. As a rule (except for some cocci, cf. also part B of this paper, Fig. 2) urease activity increased as a function of incubation time (Table 3). Pre-breeding of bacteria on CLED agar is not expected to trigger expression of urease gene as it fairly provides with peptones and carbohydrates.

We isolated *oup* bacteria from the urine and concrements of stone-forming patients. It would have been desirable to obtain bacteria from the centres of larger urinary tract calculi (>5 mm in diameter) to be sure that the infection had existed during initiation of the stone. Unfortunately, since the employment of ESWL, undamaged concrements are virtually no longer available, but only very small or minute remnants. In addition, there are good grounds for treating patients with antibiotics prior to ESWL. Thus, it was not possible to ascertain in 11 cases whether the sterility of stone fragments (Table 1) was real or artificial, i.e. the number of infected cases we determined represents the minimum.

Our finding that none of the strains isolated from the urine of stone-forming patients was really urease-negative, i.e. if necessary would be able to split urea when starved by a minimal medium, should stimulate efforts to revise the matrix theory of urinary calcium stone formation. Anion exchange events on matrices, whether originating from urinary secretions or bacterial glycocalices, and resulting in mineralization, may be supplemented by crystal seed production induced by some urease activity, thus also providing heterogeneous nucleation centres for calcium stone formation. In part B of this paper we investigate this idea and discuss further whether it may be a factor in vivo.

Acknowledgements We thank Mrs. U. Berning-Mader for skilfull and dedicated technical assistance. This project was supported by the Deutsche Forschungsgemeinschaft (DFG).

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