Observations of the ultrastructure of infected kidney stones

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Summary. Struvite stones are formed as the result of urinary tract infection by urease-producing bacteria. Ultrastructural examination of calculi removed from a patient revealed bacteria incorporated throughout the stone matrix. Exopolysaccharide stained by ruthenium red was associated with most of the bacteria, but it represented only a small portion of the organic matrix in the stone. Localised deposits of calcium and phosphorus, components of carbonate-apatite, and magnesium, a struvite component, were detected in close proximity to the cells. Histochemical examinations revealed that several of the gram-negative bacteria within the stone matrix possessed high levels of urease activity. We propose that bacterial slime production, intimately involved in the initiation of stone matrix deposition, is less prominent in mature stones because of the increased incorporation of host-derived mucoproteins and mucopolysaccharides.

Introduction

Putrefying urine has been associated with the occurrence of kidney stones since the time of Hippocrates (Blath, 1977). The 10–20% of the patients with kidney stones that develop these struvite ($\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$) and carbonate-apatite ($\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3$) calculi commonly have an alkaline urine due to the presence of ureolytic bacteria in the urinary tract (Griffith, 1978; Fowler, 1984).

Urinary calculi caused by infection consist of two major components—an inorganic mineral component, and an organic matrix (Vermeulen *et al.*, 1965; Vermeulen and Lyon, 1968; Griffith, 1978). In-vitro experiments have established the role of bacterial urease in the production of the struvite and carbonate-apatite mineral components of these stones (Griffith *et al.*, 1976; Hedelin *et al.*, 1985). Ammonia, produced by the bacterial hydrolysis of urea, raises urine pH and causes precipitation of Mg^{2+} , Ca^{2+} , CO_3^{2-} , and PO_4^{3-} as struvite and carbonate-apatite (McLean *et al.*, 1988). A third mineral component of these stones, newberyite ($\text{MgHPO}_4 \cdot 3\text{H}_2\text{O}$) has recently been described (Uebelhart *et al.*, 1984). The origin of the organic

matrix components is less clear. Chemical analysis of the stone matrix reveals the presence of mucoproteins, mucopolysaccharides, and glycosaminoglycans (Boyce and Garvey, 1956; Wickham, 1975, 1982).

Two theories of matrix deposition have been proposed. The first states that matrix deposition occurs solely as a consequence of struvite crystallisation (Vermeulen *et al.*, 1965; Vermeulen and Lyon, 1968). However, Boyce and Garvey (1956) proposed that matrix deposition was a prerequisite for stone growth. As crystal-free regions or “matrix” stones are quite often found at the periphery of struvite stones (Wickham, 1975, 1982), it is probable that matrix formation precedes crystallisation.

In previous in-vitro (McLean *et al.*, 1985*b*) and animal model experiments (Nickel *et al.*, 1987) of struvite stone production, we proposed that bacterial exopolysaccharide production plays an initial important role in establishing the organic stone matrix. In this report we confirm our hypothesis of infection stone aetiology with in-vivo observations of bacterial slime and urease activity within a stone taken from a patient.

Materials and methods

Patient history and isolation of calculi

Struvite and carbonate-apatite calculi were obtained from a nephrostomy tube in an elderly male patient. This

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patient had a long history of recurrent struvite stone production and changing catheter flora in spite of continuous antibiotic therapy. Urease-producing strains of gram-negative *Providencia rettgeri*, and gram-positive *Staphylococcus aureus* and *Streptococcus* group D were isolated. Examination by polarised light microscopy (Prien and Frondel, 1947) revealed that these calculi contained approximately equal amounts of struvite and carbonate-apatite.

Transmission electronmicroscopy (TEM)

Stone samples were processed for conventional transmission electronmicroscopy, as follows. The organic crystal-containing material which lined the luminal surface of the nephrostomy tube was removed with a sterile scalpel blade, fixed in glutaraldehyde 2% in 1% w/v N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer, pH 7.0, and mounted in agar. Samples were then washed in HEPES buffer, stained with OsO_4 2% w/v in HEPES buffer, washed again in HEPES buffer, and dehydrated in a graded acetone series. Infiltration, embedding and polymerisation with Spurr (1969) resin were performed according to the method of Marrie and Costerton (1984). Thin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-600 TEM at an accelerating voltage of 50 kV.

Analytical electronmicroscopy

Specimen preparation for analytical electronmicroscopy was similar to that for conventional electronmicroscopy except that OsO_4 was not used as a fixative and thin sections were examined unstained with a Philips model 400T scanning transmission electronmicroscope with an energy dispersive X-ray analysis (EDX) analytical attachment. During specimen examination, the microscope was operated in the transmission mode with an electron beam current of 80 μA at an accelerating voltage of 100 kV. The electron beam spot size used during TEM-EDX elemental analysis gave an effective resolution of 100 nm.

Histochemical urease localisation

The buffer used during histochemical urease localisation was HEPES buffer 0.85% w/v, pH 7.0 (McLean *et al.*, 1985a). Specimens to be examined for urease activity were washed for 1 h at room temperature in HEPES buffer, to remove traces of urine and ammonia. The specimens were then split into "reaction" and "substrate control" treatments. Urease was localised histochemically by incubating the "reaction" specimens in a trapping solution containing 5 mM sodium tetraphenylboron, 1 mM EDTA and 100 mM urea dissolved in HEPES buffer, for 4 h at room temperature. During this treatment, ammonia, produced by the enzymic hydrolysis of urea, was precipitated with the sodium tetraphenylboron trapping

reagent. After incubation, the samples were washed in HEPES buffer, and stained with AgNO_3 2% w/v dissolved in HEPES (McLean *et al.*, 1986). Samples were then embedded in agar and processed for TEM as described by McLean *et al.* (1986). "Substrate control" samples, which were used to monitor chemical contamination during urease localisation, were treated in a similar fashion except that the sodium tetraphenylboron trapping solution lacked urea.

Results and discussion

TEM of conventionally processed material from infective stones (fig. 1) revealed that both gram-negative and gram-positive bacteria were present. Most observed bacteria possessed ruthenium red-staining glycocalyxes. Additional matrix material, probably composed of urinary mucoproteins and mucopolysaccharides, was also seen. Although the three bacterial cultures isolated from these stones were all ureolytic, reaction product deposition due to urease activity was seen only in several highly ureolytic gram-negative bacteria, presumed to be *Prov. rettgeri* (fig. 2). Transmission electronmicroscopy coupled with energy dispersive analysis of X-rays (TEM-EDX) on unstained stone samples, revealed localised deposits of Ca, P (presumably as PO_4), and Mg in the vicinity of bacterial cells (fig. 3). The elemental constituents of CO_3^{2-} or NH_4^+ cannot be resolved by this technique.

Infective kidney stones are interesting ecological phenomena; the bacteria responsible for their pathogenesis exhibit a commensal or symbiotic relationship with their human host when present in other regions of the body (McLean *et al.*, 1988) such as the skin (Pillsbury and Rebell, 1952) or the gastrointestinal tract (Hespell and Smith, 1983). Urea, which enters the gastrointestinal tract by diffusion from the blood (Cheng and Wallace, 1979), is hydrolysed by urease-producing bacteria. Ammonia, resulting from this hydrolysis or from the deamination of amino acids, represents the major source of nitrogen for gastrointestinal bacteria which assimilate it into microbial protein. This microbial protein, some of which was originally derived from urea, can be used as a secondary protein source for the human host (Hespell and Smith, 1983; Leng and Nolan, 1984). In contrast to the gastrointestinal tract, the upper urinary tract (i.e., bladder and kidneys) is considered to be sterile (Kunin, 1979). The lack of an autochthonous microflora in the urinary tract probably enhances the pathogenicity of any ureolytic bacteria present, because excess ammonia generated by urease activity cannot be removed by microbial assimilation (McLean, 1986; McLean *et al.*, 1988).

Fig. 1. TEM of a stone sample taken from a patient with a chronic history of struvite stone production. Microbiological analysis of this stone revealed a mixed infection of *P. rettgeri*, *S. aureus*, and *Streptococcus* sp. Note the presence of gram-positive (g+) and gram-negative (g-) bacteria, some of which are surrounded by ruthenium red-staining glycocalyx (arrows). Matrix material (M), probably composed of urinary mucoproteins and mucopolysaccharides, is quite evident. Bar, in this and all micrographs, represents 0.4 μ m.

We have recently shown that struvite and carbonate-apatite crystals formed *in vitro* as a result of bacterial urease activity in artificial urine, do not become encased in slime-like material if bacterial exopolymers are physically excluded by a dialysis membrane (Clapham *et al.*, unpublished observations). Such loose, sand-like crystals could be easily voided with the urine in the absence of a cementing organic matrix. Thus, it would appear that bacterial slime production is involved in the initial formation of the stone organic matrix components.

TEM of conventionally prepared stone material (fig. 1) revealed that ruthenium red-staining bacte-

rial exopolysaccharides constitute a relatively minor component of the organic matrix of mature infective stones. This finding agrees with biochemical analyses of the organic matrix components of these calculi (Boyce and Garvey, 1956; Wickham, 1975, 1982; Nishio *et al.*, 1985). Therefore, we conclude that bacterial exopolysaccharide production, intimately involved in the initiation of stone matrix deposition, is less prominent in mature stones because of the increased incorporation of host-derived mucoproteins and mucopolysaccharides (McLean *et al.*, 1985b).

The condition of the urinary tract also influences

Fig. 2. Stone sample stained for urease activity (McLean *et al.*, 1985a, 1986); large deposits of urease reaction product are shown in a few of the *P. rettgeri* cells (fig. 2a; arrows). No reaction product was seen in the gram-positive cocci. Note the absence of reaction product in the substrate control preparation (fig. 2b).

stone development. In a recently completed animal-model study (Nickel *et al.*, 1987), we have shown that disruption of the urinary immune system by the presence of scar tissue in the bladder, or of a foreign object, enhances bacterial colonisation and resultant stone formation.

We propose the following aetiological process for infective kidney stone formation (outlined in fig. 4): invading ureolytic pathogens ascend the urinary tract and colonise epithelial tissue and inert surfaces as slime-enclosed microcolonies; exopolysaccharide production by these bacteria facilitates adhesion of these pathogens to surfaces, protects the organisms from urinary defence mechanisms, antibiotics and urease inhibitors, and binds and nucleates

the growth of struvite and carbonate-apatite crystals that result from bacterial urease activity. Continued growth of these encrusted bacterial microcolonies into mature calculi is characterised by continued bacterial growth, urease activity by some or all the organisms present, and an increasing incorporation of urinary organic components into the stone matrix. This mechanism of stone formation would account for the well-documented presence of bacteria within the stones and the persistence of infection (Rocha and Santos, 1969; Nickel *et al.*, 1985).

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Fig. 3. Examination of an unstained stone specimen by TEM-EDX shows the majority of Mg, Ca and P mineral components to be localised in electron-dense crystalline regions often found in association with bacterial cells. One such region (S) contained the struvite component, Mg. Carbonate-apatite components Ca and P were found in a different region (C).

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Fig. 4. Schematic representation of infective urinary stone pathogenesis. Infective kidney stones arise when urinary epithelial cells (E) are colonised by glycocalyx-secreting pathogens which form microcolonies (fig. 4a). Urease activity (U) by some of these pathogens (fig. 4b) raises urine pH, resulting in a precipitation of struvite (S) and apatite (A) crystals. The glycocalyx (outlined by ----) traps these crystals and other components such as mucoproteins present in the urine. The resulting organic matrix protects the pathogens from antibiotics (*) and host-mediated immune responses such as lymphocytes (L). Growth of these encrusted microcolonies into mature stones is characterised by continued bacterial growth, urease activity and crystal deposition, and an increasing incorporation of urinary mucoproteins into the stone matrix. The mature stone (fig. 4c) resembles an enlarged, mineralised, bacterial microcolony (Reprinted with permission from McLean *et al.*, 1988).

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