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REFERENCES

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RELAPSE OF URINARY TRACT INFECTION IN THE PRESENCE OF URINARY TRACT CALCULI: THE ROLE OF BACTERIA WITHIN THE CALCULI

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The association between infection of the urinary tract and urinary calculi is well known and has been documented for many years (Harris, 1900; Vermeulen, 1960). Calculi enhance the susceptibility of the urinary tract to infection (Vermeulen; Rocha and de Almeida, 1965), and infection can predispose to stone formation (Harris; Vermeulen).

Attempts to sterilise an infected urinary tract in the presence of stones almost always meet with failure (Lindemeyer, Turck and Petersdorf, 1963). Relapse or reinfection is the rule under these circumstances and this suggests that calculi may serve as a nidus of infection in which bacteria are protected from the action of antimicrobial drugs.

The purpose of the present study was to determine whether bacteria within urinary calculi remain viable in spite of exposure to antibacterial drugs, and whether bacteria within a calculus can infect surrounding culture media.

MATERIALS AND METHODS

Bladder calculi from 6 patients, admitted to Hospital Prof. Edgard Santos, were collected under sterile conditions at time of surgery. Quantitative urine cultures were performed in all patients before surgery and at the time of the removal of calculi. Two patients (cases no. 1 and 6) had 2 calculi in the urinary bladder. The calculi from patients no. 2, 3, 4, 5 and 6 were put into a 3 per cent. solution of iodine in absolute alcohol for periods of 2–6 hr, after which they were washed twenty times, each time in about 50 ml sterile distilled water. The last portion of distilled water in contact with the calculus was cultured by inoculating 1 ml into 9 ml of trypticase soy broth (Baltimore Biological Laboratories), and also by direct streaking with a platinum loop on to the surface of a blood agar plate subsequently incubated.

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for 48 hr at 37°C. After washing, the intact calculi were incubated at 37°C in trypticase soy broth until bacterial growth was detected.

The single calculi from patients no. 2 and 5, and 1 calculus from patient no. 6 were crushed under sterile conditions, after exposure to the solution of iodine in alcohol, and 4-6 samples of internal layers were incubated each in 10 ml of trypticase soy broth at 37°C for 48 hr. Subsequently, the 2 calculi from patient no. 1, which had not been exposed to iodine-alcohol, and the intact iodine-alcohol-treated calculi from patients no. 3, 4 and 6, which had yielded bacteria during incubation in trypticase soy broth, were incubated at 37°C in 100-ml volumes of trypticase broth containing 10 μg per ml of kanamycin or chloramphenicol or colistin. The concentration of the antibiotic was between two and more than ten times the minimum inhibitory concentration for the isolated bacteria. Daily subcultures were performed for up to 7 days. When subcultures became positive, or after 7 days if subcultures remained negative, all stones were again thoroughly washed with sterile distilled water and crushed under sterile conditions with a mallet, and 4-6 samples of inside layers were cultured as described above.

Bacteria recovered from urine and stones were identified by standard bacteriological procedures, and antibiotic sensitivity tests were performed by both a disk method and a two-fold serial dilution technique with broth.

RESULTS

Persistence of infection after exposure of calculus to iodine in alcohol

The sequence and results of a typical experiment with a 3 per cent. solution of iodine in alcohol (case no. 5) are given in the figure. Bacteria could be recovered from the stones in all instances after exposure to the iodine-alcohol for 2-6 hr (table I). In one instance (case no. 3) the same calculus was exposed to the antibacterial solution for three consecutive periods of 2 hr, and each time a subsequent incubation in trypticase soy broth yielded a growth of Proteus mirabilis in the culture medium.

Persistence of infection after exposure to antibiotics

As shown in table II, the exposure of bladder calculi to antibiotic solutions did not sterilise 3 of 5 calculi tested in this way. Bacteria could not only be cultured from fragments...
of inside layers, but also from the whole calculus incubated in trypticase soy broth. In one instance (case no. 1) the patient presented a mixed urinary flora (*P. mirabilis* and *Escherichia coli*), but only one organism (*P. mirabilis*) could be recovered from the fragments of inside layers of the 2 calculi that he had. The incubation of stones from cases no. 3 and 4 in an kanamycin solution led to apparent sterilisation of the calculi. In both instances, the calculi had been previously incubated in a 3 per cent. solution of iodine in alcohol without success (table I). Kanamycin was entirely ineffective in sterilising the calculi of case no. 1.

**TABLE I**

_The in-vitro effect of a 3 per cent. solution of iodine in alcohol on the bacterial flora of bladder calculi_

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Bacteria in urine</th>
<th>Time of exposure of calculus to iodine in alcohol (hr)</th>
<th>Bacteria recovered from calculus</th>
<th>Whole calculus</th>
<th>Fragments (positive/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td><em>Proteus mirabilis</em></td>
<td>2</td>
<td>None</td>
<td>None</td>
<td><em>P. mirabilis</em> (6/6)</td>
</tr>
<tr>
<td>3</td>
<td><em>Proteus mirabilis</em></td>
<td>2</td>
<td><em>P. mirabilis</em></td>
<td><em>P. mirabilis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td><em>P. mirabilis</em></td>
<td><em>P. mirabilis</em></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>Klebsiella aerogenes</em></td>
<td>2</td>
<td><em>K. aerogenes</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Coliform, intermediate</td>
<td>6</td>
<td>Coliform, intermediate</td>
<td>Coliform, intermediate (4/6)</td>
<td></td>
</tr>
<tr>
<td>6*</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>a 4, b 6</td>
<td>a <em>Ps. aeruginosa</em>, b <em>Ps. aeruginosa</em></td>
<td><em>Ps. aeruginosa</em> (4/4)</td>
<td></td>
</tr>
</tbody>
</table>

* Patient had two bladder calculi (a and b).

_Bacterial flora of the bladder calculi_

In spite of a prior exposure to antibacterial agents, the culture of inside layers of crushed calculi revealed the presence of micro-organisms in most of the samples taken from different parts (tables I and II). It is of interest that the bacterial population was not uniformly distributed inside the calculi, and it was always the same as that encountered in the urine or in the broth culture after incubation of the whole stone. The predominant organism was *P. mirabilis*.

**DISCUSSION**

These studies show that infected bladder calculi could not be sterilised by exposure to a 3 per cent. solution of iodine in alcohol for 2–6 hr and, in the majority of cases, exposure of the calculi to antibiotic solutions was ineffective. Of great interest, moreover, was the demonstration that bacteria surviving inside the stones were able to infect the surrounding broth during incubation of the calculi. These observations made in vitro strongly suggest that a similar mechanism may operate in vivo and may be important for the persistence of urinary tract infection in the presence of calculi. In fact, a similar phenomenon has been demonstrated in gallstones of chronic carriers of *Salmonella* spp. Tynes and Utz (1962) were able to produce the chronic carrier state in rabbits by surgically placing gallstones from human carriers of *Salmonella* spp. in the gall-bladders of these animals. These workers also demonstrated that artificially infected gallstones incubated in broth can contaminate...
the surrounding culture medium. Moreover, after exposure of gallstones to bacterial cultures, micro-organisms that eventually penetrated into the stone were protected against high concentrations of antibiotics to which they were otherwise sensitive.

Urinary calculi may harbour a mixed bacterial flora. In fact, *Escherichia coli* and *Proteus mirabilis* were isolated from one of the calculi of patient no. 1. Also, two types of *Proteus* (*P. mirabilis* and *P. rettgeri*) were isolated from different parts of a calculus produced in a patient not included in the present series; the calculus was not experimentally exposed to antimicrobial drugs in this case.

Kaye *et al.* (1962) recovered organisms from a large solitary gallstone of a chronic carrier of *Salmonella typhi*, apparently cured by previous antibiotic therapy; cultures of bile and the wall of the gall-bladder were sterile. By analogy, it seems conceivable that a patient could have a negative urine culture and still have bacteria within a urinary tract calculus.

The results of the present study may help to explain the ineffectiveness of therapy of urinary tract infection in the presence of calculi.

**SUMMARY**

Bacteria inside urinary bladder calculi were shown to survive after exposure of the calculi to a 3 per cent. solution of iodine in alcohol, or a concentration of an antibiotic far in excess of that required to inhibit the growth of the isolated organism. Calculi were shown to be able to infect fluid culture media in which they were incubated, even if they had first been exposed to antimicrobial agents. The findings may help to explain the persistence of infection and the ineffectiveness of therapy of urinary tract infection in the presence of urinary tract calculi.

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**TABLE II**

*The in-vitro effect of some antibiotics on the bacterial flora of bladder calculi*

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Bacteria in urine</th>
<th>Incubation of calculus in broth containing antibiotic (10μg per ml)</th>
<th>Bacteria recovered from calculus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Antibiotic</td>
<td>Result</td>
</tr>
<tr>
<td>1*</td>
<td><em>P. mirabilis</em></td>
<td>a Kanamycin</td>
<td><em>P. mirabilis</em> (after 2 days)</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td>b Chloramphenicol</td>
<td><em>P. mirabilis</em> (after 2 days)</td>
</tr>
<tr>
<td>3</td>
<td><em>P. mirabilis</em></td>
<td>Kanamycin</td>
<td>No growth (up to 7 days)</td>
</tr>
<tr>
<td>4</td>
<td><em>K. aerogenes</em></td>
<td>Kanamycin</td>
<td>No growth (up to 7 days)</td>
</tr>
<tr>
<td>6†</td>
<td><em>Ps. aeruginosa</em></td>
<td>Colistin</td>
<td><em>Ps. aeruginosa</em> (after 1 day)</td>
</tr>
</tbody>
</table>

* Patient had two bladder calculi (*a* and *b*).
† Patient had two bladder calculi, but only one was incubated in colistin.
PRODUCTION OF RUBELLA ANTISERUM IN HAMSTERS

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BHK 21 cells chronically infected with rubella virus can be serially passed in culture (Professor K. McCarthy, personal communication). The observation that most BHK cells that have been continually passed are oncogenic in hamsters (Foley and Handler, 1957), and the demonstration of viral antibodies in sera of hamsters with tumours induced with adenovirus type 12 (Huebner, Rowe and Lane, 1962), prompted us to study the effects of inoculating rubella virus-infected cells into hamsters. As a comparison with the prolonged stimulus of these infected cells, control animals were inoculated with a single dose of cell-free antigen.

MATERIALS AND METHODS

BHK 21 clone 13 cells, chronically infected with rubella virus, were grown in suspension culture in Eagle's medium containing 5 per cent. (v/v) inactivated foetal calf serum, 0·3 per cent. (v/v) tryptose phosphate broth, 0·1M glucose, 0·01 µg per 100 ml (w/v) ferric nitrate, 100 units per ml of penicillin and 100 µg per ml streptomycin at 35°C. The presence of virus antigen was checked by indirect (sandwich) immunofluorescent staining of acetone-fixed coverslip preparations of the cells with an immune human serum (haemagglutination-inhibition titre 1 in 512), with the usual tests for specificity (Nairn, 1964).

Fourteen hamsters, each weighing approximately 80 g, were given an inoculation into the interscapular space of 10^7 infected cells, 69 per cent. of which contained rubella antigen. Tumours developed in 2-3 wk, and when these were beginning to break down, 3-4 wk later, the animals were bled and killed. In order to test for the continued presence of antigen, smears were made of 11 of the tumours, and the cells of 1 tumour were cultured.

Cell-free antigen was obtained by alternately freezing and thawing the rubella virus-infected cells 3 times, followed by centrifugation and filtration. The antigen from 10^7 cells, which contained 5 × 10^5 ID50 of infectious virus, was inoculated into the interscapular space of each of 10 hamsters as a control. After 6 wk the animals were bled and killed.

Estimation of rubella antibody in sera. Two methods were employed to assay rubella antibody in the hamster sera.

(i) Haemagglutination-inhibition test. Antigen was produced from the rubella virus-infected cells suspended in serum-free medium. A modification of the technique of Stewart and his colleagues (1967) was used. Pigeon red cells were substituted for chick red cells (Holmes and Warburton, 1967); cells from a single pigeon were used throughout. The dextrose gelatin barbitone diluent was adjusted to pH 6·2 (Clarke and Casals, 1958; Holmes

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