Reversible Inactivation of Bladder Surface Glycosaminoglycan Antibacterial Activity by Protamine Sulfate

C. LOWELL PARSONS,* CHARLES W. STAUFFER, AND JOSEPH D. SCHMIDT

Department of Surgery, Division of Urology, University of California Medical Center, San Diego, California 92103, and the Veterans Administration Medical Center, La Jolla, California 92161

Received 27 April 1987/Accepted 2 February 1988

Prior studies in our laboratory have shown that the bladder surface is lined with glycosaminoglycans which appear to be an important antibacterial defense mechanism that operates by resisting bacterial adherence and infection. The present study further implicates bladder surface glycosaminoglycans as the key antiadherent factor and also suggests a potential model for diseases (such as urinary tract infections) whereby the antiadherent surface of the bladder is inactivated biochemically. Protamine sulfate treatment of bladder tissue was found to significantly increase bacterial adherence to the urinary bladder by approximately 2.3-fold. This effect was reversed by a second treatment of the bladder with pentosanpolysulfate (a polysaccharide known to duplicate the surface antiadherent effect). Protamine sulfate had no effect on bacterial viability or bacterial adherence when bacteria were pretreated with it.

Experiments conducted in our laboratory have shown that the bladder surface is lined with glycosaminoglycans (e.g., mucus, mucin, polysaccharide, and mucoprotein) that play an important role in the surface defense of the bladder. When present and intact at the bladder surface, this layer will resist adherence of bacteria, microcrystals, proteins, and calcium (1, 8–11, 15).

The glycosaminoglycan lining of the bladder is believed to be a nonspecific mechanism by which the bladder surface protects itself from potentially harmful substances in urine. An important discovery was the finding that the surface polysaccharide layer can be digested off of the bladder surface with acid solutions. This allows increased adherence of substances such as those mentioned above, but the antiadherent effect of the surface can be immediately restored by treatments with sulfated polysaccharides such as heparin and pentosanpolysulfate (5, 12, 13). Studies have also shown that removal of the surface polysaccharide layer led not only to increased bacterial adherence but also to bacterial infection (6). The increased adherence and infection can be reversed by treating glycosaminoglycan-deficient bladders with an exogenous sulfated polysaccharide such as pentosanpolysulfate (6). Obviously, digestion of the bladder surface with acid indiscriminately removes more of the bladder surface than just polysaccharides, and because of the low specificity of this treatment, it is difficult to say that glycosaminoglycans are the key factor responsible for the antiadherent surface. The fact that sulfated polysaccharides restore the antiadherence of the bladder was felt to be evidence that these compounds were the active agents. More-specific biochemical treatments of the epithelium could provide valuable support for this thesis. For this reason, the present study was conducted. Protamine sulfate, which is a known biochemical inactivator of sulfated polysaccharides (e.g., heparin), was used to see if it would alter the adherence of bacteria to the bladder surface. More important, after the bladder surface was treated with protamine sulfate, treatment with a sulfated polysaccharide was conducted to see if it would reverse the potential protamine effect. Protamine sulfate is multivalent with many branches containing quaternary ammonium compounds, and as such should, theoretically, first bind to the cell surface glycosaminoglycan and, after a second exposure to polysaccharide, should then bind the polysaccharide and essentially “sandwich” it onto the bladder surface, thus neutralizing itself.

**MATERIALS AND METHODS**

A quantitative bacterial adherence assay which has been previously reported was used. The following is a brief review of our methods.

**Initial preparation of rabbits.** Male New Zealand White rabbits weighing between 2 and 3 kg were secured and catheterized with 9 French pediatric feeding tubes as previously described (16). They were anesthetized with a mixture of Rompun and ketamine (5 mg/kg of body weight; intramuscular injection). To ensure that the bladders were emptied between treatments, they were exposed with a 2-cm lower-midline incision. The bladders were washed three times with 15 ml of 0.01 M phosphate-buffered saline (PBS) (pH 7.2).

**Control bladders.** After initial preparation, control bladders were incubated twice for 30 min with PBS and then given three washes with PBS (15 ml) and a final wash with 15 ml of PBS (pH 5.5). Bacteria were added as described below.

**Protamine sulfate-treated bladders.** After initial preparation, the bladders were treated for 30 min with 4.0 ml of protamine sulfate dissolved in sterile distilled water to a final concentration of 10 mg/ml (pH 7.2). Control bladders were treated with 4.0 ml of PBS (pH 5.5). At the end of the incubation period, all bladders were washed three times with PBS (pH 5.5) and one time with 0.01 M PBS (pH 5.5). A second 30-min incubation with PBS was done to match the treatment of bladders also receiving pentosanpolysulfate. The prepared bladders then received the labeled bacteria as described below.

**Protamine sulfate and SP54-treated bladders.** The bladders were washed and prepared with protamine sulfate as described above, washed with PBS three times, and then given a second treatment for 30 min with 4.0 ml of pentosanpolysulfate (SP54) (20 mg/ml) suspended in distilled water. Control bladders and protamine-treated bladders simultaneously
TABLE 1. Effect of protamine on bacterial adherence to bladder tissue

<table>
<thead>
<tr>
<th>Treatment of bladder tissue</th>
<th>n</th>
<th>No. of bacteria/mg of mucosa (mean ± SE) (10^3)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>18</td>
<td>1.8 ± 0.52</td>
<td></td>
</tr>
<tr>
<td>Acid</td>
<td>10</td>
<td>12.3 ± 3.8</td>
<td>0.0003</td>
</tr>
<tr>
<td>Protamine</td>
<td>26</td>
<td>4.3 ± 1.1</td>
<td>0.003</td>
</tr>
<tr>
<td>Protamine and pentosanopoly sulfate</td>
<td>26</td>
<td>2.4 ± 0.62</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* Compared with control group by Mann-Whitney rank sum test.

received 4.0 ml of PBS. After 30 min, the bladders were washed with PBS (pH 5.5) as previously indicated.

Acid-treated controls. To remove the luminal mucin layer, a fourth group was treated by placing 7.0 ml of 0.6 M hydrochloric acid intravesically for 1 min. This was followed by a 15.0-ml wash with 0.01 M PBS (pH 10.0). The bladders were then washed again with PBS as described above. This group was included to act as a positive control for bacterial adherence.

(i) Preparation of bacteria. Escherichia coli type O4 was grown and passed in Davis minimal broth medium. After several passages, 2% (vol/vol) of an overnight culture was used to inoculate 10 ml of Davis minimal broth medium containing 50 μCi of 14C-labeled sodium bicarbonate. This culture was incubated overnight at 37°C. Approximately 20 ml of medium was used in each experiment to achieve a desired ratio of bacteria to radioactive counts (between 200 and 600). This was determined by viable colony counts and by measuring radioactivity with a liquid scintillation counter.

The bacteria were sedimented at 1,000 × g for 20 min, washed three times with PBS (pH 5.5), and suspended in a volume that allowed a 10-fold concentration of the bacteria. Viable colony counts were done and radioactivity was determined to obtain the ratio of bacteria per radioactive count.

(ii) Bacteria pretreated with protamine. After being prepared as described above, bacteria were incubated with 20 mg of protamine sulfate per ml for 30 min, removed by centrifugation, and suspended in PBS. They were examined for viability as described below.

Incubation of bacteria in bladders. Concentrated 14C-labeled E. coli (0.4 ml) were placed into each prepared rabbit bladder and allowed to interact with the surface for 15 min. The reaction was then terminated by injecting 10 ml of PBS (pH 7.2) into the bladder. The rabbits were allowed to make 50 ml of urine under hydration with intravenous normal saline. The rabbits were sacrificed, the bladders were removed, and the mucosa were stripped from the muscle and assayed for radioactivity.

Determination of radioactivity. Bladder tissue samples were placed into tared-glass scintillation vials and desiccated overnight at 50°C. The dry weight of each sample was determined. The tissue was dissolved in a solution of 0.2 ml of 70% perchloric acid and 0.4 ml of 30% hydrogen peroxide at 50°C for 4 h. To assay for radioactivity, the samples were mixed with 15 ml of Ecolite (Westchem Products, San Diego, Calif.). The sample vials were chilled, and radioactivity was measured with a liquid scintillation counter. The data was analyzed by the Mann-Whitney rank sum test. Differences between means were considered significant when P was <0.05.

RESULTS

The acid-treated tissue showed greater bacterial adherence than did the controls (Table 1). Treatment with protamine sulfate also resulted in significantly higher bacterial adherence in treated bladders than in the controls (Table 1). This effect was reversed by the addition of pentosanopoly sulfate.

Bacterial suspensions employed for the adherence assays had a viability of between 1.0 × 10^9 and 2.0 × 10^9 CFU/ml (0.4 ml was used for each rabbit). The ratio of bacteria to radioactivity was between 100 and 300. There was no difference in the viability of the bacteria pretreated with protamine compared with those that were not treated. Pretreatment of bacteria with protamine had no effect on adherence of bacteria to bladder tissue (Table 2).

DISCUSSION

The bladder surface has previously been found to be lined with glycosaminoglycans which appear to be an important surface defense mechanism for resisting adherence of bacteria, microcrystals, proteins, and calcium (1, 8-10, 15, 17, 18). The mechanism for this resistance to adherence was felt to rest in the hydrophilic nature of the glycosaminoglycans. Sulfated polysaccharides, when bound to the bladder surface, are so hydrophilic that they will absorb water micelles to the sulfate moieties. This saddles a micelle layer of water between the cells of the bladder surface and any potentially harmful substances in the urine (2-4). The effect, surface changes (such as possible cell surface receptors) are masked. Thus, this glycosaminoglycan lining is a relatively undiscriminating surface antiadherence mechanism that may be important in preventing urinary tract infection, calculus disease, and possibly even the action of carcinogens which might be in the urine.

The present study was conducted to find a specific inactivation of the bladder surface polysaccharide layer that would impair this layer as the principal defense mechanism of the bladder. Such an inactivation could also demonstrate a potential model for disease in that some type of protamine-like substance, if present in urine, could interfere with epithelial defense. The ability of sulfated polysaccharides to reverse the effect of protamine was also investigated for two principal reasons. First, it would provide further information on whether the surface polysaccharide layer was the principal antiadherence defense mechanism and might point to a way to prevent the damaging effect of urinary protamine-like substances either by recoating the bladder surface or by acting as an "antibody" and scavenging urine compounds. Second, in some disease states the ability of protamine to sandwich polysaccharides between itself and

TABLE 2. Effect of protamine pretreatment on bacterial viability and adherence to transitional epithelium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>No. of bacteria/mg of mucosa (mean ± SE) (10^3)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated) bacteria</td>
<td>10</td>
<td>0.6 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>Acid-treated mucosa</td>
<td>10</td>
<td>8.6 ± 5.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Protamine-treated bacteria</td>
<td>10</td>
<td>0.5 ± 0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Compared with untreated bacteria by Mann-Whitney rank sum test. NS, Not significant.
the bladder surface may be a way to attach additional sulfated polysaccharides to the bladder surface. We have found that this combination treatment triples polysaccharide binding to the bladder surface (unpublished data). This effect has therapeutic possibilities in some urologic diseases, such as interstitial cystitis (5a) and radiation cystitis, in which epithelial defenses may be inadequate (7, 14). The potential for protamine-like substances to initiate infectious diseases is supported by the data reported here in that protamine sulfate increased bacterial adherence to the bladder surface. This effect was reversed by sulfated polysaccharides.

It is important to point out that in vitro studies investigating bacterial adherence as a potential mechanism for predisposition to human urinary tract infections consistently show that, whether one is studying these alterations in the host cell or in the different species of bacteria (which use different adherence mechanisms), individuals who are predisposed to infection show an approximately twofold-greater bacterial adherence to the urothelial cells than normal subjects do (16). This certainly is consistent with our findings of a 2.3-fold rise in adherence (Table 1) seen following treatment of the bladder surface with protamine sulfate. It is possible that bacteria use such substances as protamine sulfate to enhance virulence and that individuals who are infection prone excrete such agents into their urine.

In summary, the current study supports the concept that sulfated polysaccharides of the bladder surface are the principal antiadherent defense mechanism of the bladder and at the same time implies a potential model for disease. It may be that a urinary protamine-like substance produced by either the host or the microorganism initiates disease. Our findings also imply that a urinary polysaccharide, such as that excreted by the kidneys, may have additional protective effects by reversing the damage caused by protamine-like agents at the bladder surface.

ACKNOWLEDGMENTS

This study was supported by the Medical Research Service of the Veterans Administration, by the Urological Research and Education Foundation, and by Public Health Service grant 1RO1DK39239-01 from the National Institutes of Health.

LITERATURE CITED