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Antibacterial Activity of Bladder Surface Mucin Duplicated by Exogenous Glycosaminoglycan (Heparin)

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We have previously shown that the transitional cells lining the urinary bladder are capable of producing glycosaminoglycan (GAG). By use of a quantitative in vivo method of measuring bacterial adherence, we demonstrated that bacterial adherence to the mucosal cells is diminished in the presence of this GAG, rises when it is removed (by acid), and returns to normal when the GAG is resynthesized (in less than 24 h). We also found that this mucin layer could be removed (with a corresponding rise in bacterial adherence) and that addition of exogenous GAG (heparin) to the bladder prevented the expected rise in bacterial adherence. This study analyzed in depth the manner by which heparin prevents the rise in adherence seen when the mucin is removed. Pretreatment of bacteria with heparin had no effect on adherence, whereas pretreatment of the bladder with heparin inhibited adherence. To corroborate our impression that the heparin was coating the transitional cells, ([3]H]heparin was added to bladders after removal of mucin. Autoradiography revealed the heparin to be adherent to the surface of the transitional cells.

Much attention has recently been devoted to the concept that bacterial adherence is important in determining a strain's virulence at a mucous surface, including those of the genitourinary tract, the gastrointestinal tract, and the oral cavity (5-7, 19, 22-26). We became intrigued by the fact that little is known concerning possible host immunodefenses directed against this bacterial virulence factor. More specifically, we were interested in how the urinary tract was capable of maintaining itself in a sterile state despite its direct contact with environmental organisms. It seemed to us that an "anti-adherence factor" directed against bacteria Inight be operating at the surface of the transitional cell epithelium.

We developed an in vivo model to measure bacterial adherence (16). By use of this assay, we were able to discover that the transitional cells lining the urinary bladder appeared capable of synthesizing a glycosaminoglycan (GAG) which was able to prevent bacterial adherence to the mucosal cells (15, 17, 18, 21). We called it anti-adherence factor (AAF). It also was found that endogenous bladder GAG could be removed (with acid) with a corresponding rise in bacterial adherence (15, 22), but that addition of exogenous GAG (heparin) to such a mucin-deficient bladder prevented bacterial adherence (11).

The current study was conducted to determine the mechanism by which the addition of heparin blocked bacterial adherence in the urinary bladder that has had its natural mucin removed.

MATERIALS AND METHODS

Preparation of bacteria. *Escherichia coli* type 04 was accommodated to Davis medium (Difco Laboratories, Detroit, Mich.) by serial passage using 2.5% inocula (vol/vol) and incubation at 37°C overnight. To label the organisms with [14]C, a 2.5% inoculum (vol/vol) was added to fresh Davis medium containing 10 I'Ci of [[14]C]bicarbonate (Amersham/Searle, Des Plaines, Ill.) per ml, and the mixture was incubated overnight at 37°C. The next morning the bacteria were spun in a centrifuge at 3,000 x *g* for 5 min and washed once with an equal volume of0.9% NaCl (physiological saline solution; PSS). After washing, the bacteria were suspended in the respective solutions as described in step 3 in a total volume that was 10% of their original. For the control rabbits (step 2a), the suspension was into a solution containing 0.1 M sodium phosphate monobasic (adjusted to pH 5.5 with sodium phosphate dibasic). This buffered solution (BS) was the basic one used for all the experiments described in step 3.

Basic model. Male New Zealand White rabbits weighing 2 to 3 kg were used. All animals were anesthetized with pentobarbital, 18 mg/kg of body weight.

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Step 1. Urethral catheter. Each rabbit was secured and given 100 ml of PSS intravenously over a 30-min period. The intravenous infusion was turned off. A pediatric feeding tube no. 8 French (C. R. Bard, Murray Hill, N.J.) was inserted into the urethra and secured with a 4-0 silk purse-string suture tied around the penis. The abdomen was open prior to step 2 to expose the bladder. Only in this fashion could one be assured of totally emptying the bladder of its contents between rinses or treatments. Between treatments the bladder was returned to the abdomen and the overlying fascia was secured.

Step 2. Preparation of bladder. (a) Control rabbits: The bladder was flushed through the catheter with four aliquots of 15 ml of PSS, (b) Acid-treated bladders: Before the introduction of bacteria (step 3), the bladders of the animals which were to receive acid treatment (remove mucin layer) were flushed with four aliquots of 15 ml of PSS, after which they were slowly infused (over 20 s) with 7 ml of 0.6 N HCl through the catheter. When the acid had remained in the bladder for 60 s, it was aspirated and the bladder was flushed with one 15-ml aliquot of 0.5 M potassium phosphate dibasic (pH 9.4), followed by three additional rinsings with 15-ml aliquots of BS. The introduction of bacteria was then performed as in the basic model, step 3.

Step 3. Introduction of bacteria. Rabbits from step 2a (control rabbits; vesical mucin layer present) received 0.5 ml of BS followed immediately with 0.4 ml of bacteria as described in Preparation of Bacteria, and the catheter was clamped. Prior to addition of bacteria, the bladders were emptied of contents under direct vision.

Heparin and bacterial experiments. Bacteria from Preparation of Bacteria were suspended in BS. Also suspended in BS was heparin (Upjohn Company, Kalamazoo, Mich.) in concentrations of either 2,000, 1,000, 500, 250, 125, or 34 U/ml. To each empty bladder (step 2b), under direct vision, was added 0.5 ml of this suspension, followed immediately with 0.4 ml of bacteria, and the catheter was clamped.

Pretreatment of bacteria with heparin. Bacteria from Preparation of Bacteria were suspended in BS containing 1,000 U of heparin per ml and incubated for 15 min at 37°C. The bacteria were sedimented from this mixture and resuspended in an equal volume of BS and washed once. To each empty bladder under direct vision was added 0.4 ml of bacteria (step 2b), and the catheter was clamped. Control rabbits (step 2a) received 0.4 ml of bacteria also, as did acid-treated bladders (step 2b), which were used as positive controls.

Pretreatment of bladder with heparin. Prior to adding bacteria to the rabbit bladders (from step 2b), the bladders were treated with 1.0 ml of BS containing 1,000 U of heparin per ml for 15 min. This solution was then removed, and the bladder was washed twice with 15.0-ml aliquots of BS. At that time the 0.4 ml of bacteria from Preparation of Bacteria which had been suspended in BS was added to the empty bladder, and the catheter was clamped.

Autoradiography. To an empty rabbit bladder from step 2b was added 1.0 ml of [^{3}H]heparin (New England Nuclear, Boston Mass.), 1,000 U/ml, for 15 min. The heparin was removed, and the bladder tissue was irrigated with two 15.0-ml aliquots of BS. After the rabbit made 50.0 ml of urine, the animal was killed and the bladder was fixed in Bouin solution and prepared as described in Localization of Heparin.

Step 4. Interaction of bacteria with the bladder mucosa. The bacteria were introduced into the bladder through the penile catheter and the catheter was clamped for 15 min while PSS was given intravenously at a rate of 200 ml/h. At the end of 15 min, 10 ml of BS was introduced into the bladder through the penile catheter to dilute the bacteria and terminate the reaction. The penile catheter was left to straight drainage and after the rabbit had made 50 to 70 ml of urine, the rabbit was killed and the bladder was removed. The mucosa was dissected free from the muscle layer, and both were assayed for ^{14}C activity.

Step 5. Recording of radioactivity. The bladder tissue was placed in an incubator at 65°C until desiccated, and dry weight was determined. The bladder tissue was then homogenized overnight in 2 ml of 1.5 M NaOH at 60°C. The solution was bleached to remove color that might quench radioactive counts, by adding 0.2 ml of 70% perchloric acid and 0.4 ml of 30% hydrogen peroxide. The next morning, two or three drops of 15% ascorbic acid were added to oxidize remaining hydrogen peroxide. This entire solution was titrated to neutrality with 70% perchloric acid and 15% ascorbic acid. One hundred percent of the mucosal samples and 25% of the muscle samples were suspended in 15 ml of Aquasol (New England Nuclear, Boston, Mass.). Radioactive counts were recorded by a Searle liquid scintillation counter. Bacterial samples were suspended in 5 ml of Aquasol for radioactive counting.

Localization of heparin. The fixed bladder tissue, exposed to [^{3}H]heparin (step 3), was embedded and thin-section-stained with hematoxylin and eosin. Afterwards, the slides were dipped in full- and one-half-strength autoradiographic emulsion NTB2 (Kodak) and left in the dark. Slides were removed and developed at weekly intervals until silver grains were noted.

Statistical analysis of data. All data were subjected to variance analysis employing Student's *t* test. Differences in mean bacterial adherences are reported as significant when $P < 0.05$ and as not significant when $P > 0.05$.

RESULTS

Bacteria. The bacterial solution injected into the rabbits ranged between 1.0×10^9 colony-forming units per ml and 2.0×10^9 colony-forming units per ml with a ratio of bacteria to counts per minute of between 300 and 500.

Bladder muscle. No bacteria were detected in the bladder muscles.

Heparin and bacteria. The data are expressed in two ways: first, the bacteria per milligram (dry weight) of mucosa (Tables 1 and 2); and second, the ratio of bacteria per milligram of mucosa of the experimental rabbits (acid treated) to that of the control rabbits (non-acid treated) for each given day of experimentation

(Tables 1 and 2). This latter ratio is very important since we have long since noted that bacterial ability to adhere varies greatly from day to day (perhaps reflecting drifts in pili production), but the ratio in adherence between experimental and control rabbits on any day is consistent.

As can be seen from Table 1, the heparin exerts its maximum effect at blocking adherence at 250 U/ml. For this reason, the bladder and bacteria were pretreated with heparin at a concentration of 1,000 U/ml, four times the minimum needed to ensure an effect. There were 35 rabbits in the acid-treated group, 35 in the control group, and 15 in the heparin-treated group.

In general, after acid treatment of the bladder mucosa there was a 50- to 60-fold rise in bacterial adherence when one obtained the ratio of bacteria bound per milligram of mucosa of experimental to control rabbits. Bacterial adherence seen after acid treatment was significantly greater ($P < 0.0001$) than that in control rabbits but not for acid-treated bladders that received heparin in concentrations of 1,000, 500, and 250 U/ml ($P > 0.05$). However, bacterial adherence to bladders at concentrations of heparin of 125 U/ml and 34 U/ml was significantly greater than that in control rabbits ($P < 0.001$) but was not different from that in the acid-treated controls (Table 1) ($P > 0.05$).

Pretreatment of bacteria with heparin. There were 12 rabbits each in the control and acid-treated groups, as well as in the group with bacteria pretreated with heparin. Table 2 summarizes these data. As can be seen, pretreating bacteria with 1,000 U of heparin per ml did not interfere with bacterial adherence to acid-treated bladders, with a rise in bacterial adherence of 55-fold (Table 1), comparing the ratio of experimental bladders to the control bladders on each day of experimentation. Statistically, no significant difference was found between the acid-treated bladder and the experimental. The higher adherence seen in the acid-treated, positive control rabbits as compared to those in the heparin and bacteria experiments reflects the fact that the bacteria had been previously diluted with additional BS at the time of addition to the heparin and bacteria bladders only, which was not done here.

TABLE 1. *Bacteria bound per milligram (dry weight) of mucosa and influence of heparin on adherence*

Mucosal group	Bacteria ($\times 10^3$)/mg of mucosa(± SD)	Ratio of acid-treated bladders to control[a]
Control[b]	4.0±8	1.0
Acid control[c]	51± 6.2	60
Heparin[c] at concn (U/ml):		
2,000	0.9 ± 1.2	1.7
1,000	2.7 ± 5.1	3.3
500	4.7 ± 5.7	7.3
250	8.9 ± 10.5	3.8
125	41.3 ± 9.4	36
34	79.4 ± 147	67

[a] Ratio of acid-treated bladders to controls on each separate day of experimentation.
[b] Bladder mucin present; no acid treatment.
[c] Bladder mucin absent; pretreated with acid.

TABLE 2. *Mean bacteria adherent per milligram (dry weight) of bladder mucosa: effect of pretreatment of bladder and bacteria with heparin on bacterial adherence*

Mucosal group	Bacteria (xlo")/mg of mucosa (±SD)	Ratio of acid-treated bladders to control[a]
Control[b]	2.6 ± 4.1	1.0
Acid[c]	170 ± 270	60
Heparin plus bacteria[b]	2.7 ± 5.1	3.3
Bacteria pretreated with heparin[b]	150 ± 200	55
Bladder pretreated with heparin[c]	15.0 ± 21	5.5

[a] Ratio of acid-treated bladders to controls on each separate day of experimentation.
[b] Bladder mucin present; no acid treatment.
[c] Bladder mucin absent; pretreated with acid.

Pretreatment of bladders with heparin. Twelve rabbits were in this group. Table 2 shows that the pretreatment of the bladder with heparin partially blocked bacterial adherence to the experimental bladder wall with only a 5-fold rise (Table 2), compared to the 55-fold rise seen in the acid-treated bladders without heparin. This difference was statistically significant ($P < 0.001$).

Autoradiographs of bladder treated with [^{3}H]heparin. Figure 1 shows sample radiographs. As is evident, after 4 weeks there was found a distinct and well-localized layer of silver grains right at the surface of the superficial epithelial cells. No nonspecific layers of silver grains were seen.

DISCUSSION

The urinary bladder has long been known to be remarkably resistant to infection when unobstructed. Little has been known concerning any intrinsic antibacterial defense mechanism; the only one suggested has been a vesical mucosal bactericidal activity (3, 4, 14), but its existence

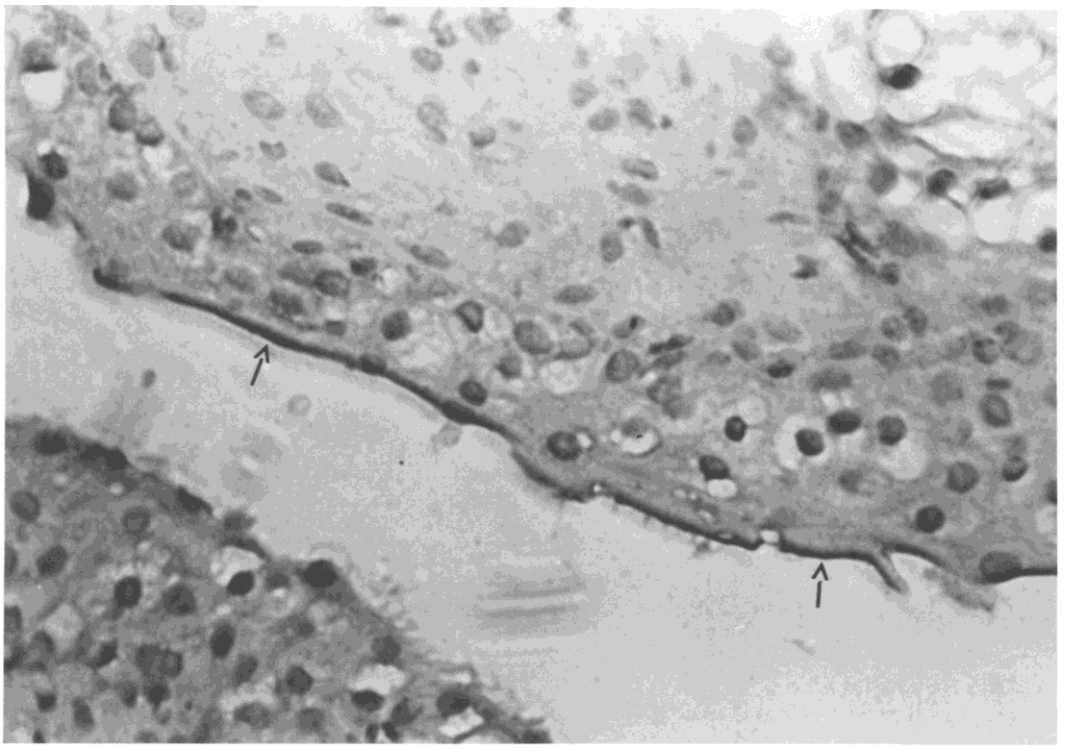

FIG. 1. *Section of bladder treated with acid (to remove natural mucin), exposed to [^{3}H]heparin. The arrows point to the layer of silver grains seen at the surface of the epithelium, the only area of localization.*

has not been corroborated by other investigators (12, 13). In view of the fact that the bladder maintains a sterile mucosa in the face of direct continuity with environmental organisms, we felt that chance alone could not account for this; rather, that antibacterial defense mechanisms actively maintain this equilibrium. In this regard, we became interested in the concept of bacterial adherence as an important function for bacterial virulence.

Adherence has been reported to play a role in bacterial virulence at many mucous surfaces including the gastrointestinal tract, the genitourinary tract, and the oral cavity (5-7, 19, 22-26), the main theme being that microbial ability to infect a surface is directly proportional to its ability to adhere to mucosal cells. What intrigued us was the idea that if adherence were important to bacterial virulence, the body may produce AAFs as a counter measure. In particular, an AAF preventing bacteria from adhering to the bladder wall would explain both the need and the efficiency of the urine washout factor (4). Human immunoglobulin A and glycoproteins have been studied as AAFs perhaps acting in an antibody-like fashion inactivating bacterial virulence factors such as pili or the glycocalyx (12, 13).

To explore the possible existence of an active AAF in the urinary tract, we developed and reported an in vivo method of quantitating bacterial adherence to the vesical mucosa (16). It was discovered that pretreatment of the bladder with acid could raise bacterial adherence 20- to 50-fold to the transitional cells. This increased adherence was found to be pH dependent and independent of the bacterial species employed (17, 18). Histochemical studies revealed that bacterial adherence was increased in the absence of bladder surface GAG (which is removed by acid treatment) and the ability of the bladder surface to resist bacterial adherence correlated with the return of the GAG after its removal with acid (15). Additional histochemical studies corroborated these findings (21).

It is important to note that the manner in which we remove the surface layer of mucin, namely, with 0.6 N HCl, is, of course, harsh treatment; several important points are to be made in this regard. First, grossly no effect was noted on the bladders. Microscopically, we found that, while doing the histological studies (15, 16), the mucosa was basically intact, with the only consistent fmding being the loss of the

mucin layer (15, 16). In fact, the time of acid treatment and the concentration of acid were reduced in a stepwise fashion to a minimum level which would produce a rise .in bacterial adherence. It is at this point that we also discovered the loss of the mucin layer (15). This layer turned out to be remarkably resistant to attempts at removal. We attempted to hydrolyze it with trypsin, hyaluronidase, neurominidase, and mucomyst; the latter three produced a modest but significant rise in bacterial adherence, but only acid removed the mucin layer histologically (S. H. Shrom, C. L. Parsons, and S. G. Mulholland, Abstr. Annu. Meet. Assoc. Acad. Surg., Cleveland, Ohio, 1978).

From these data, we formulated the thesis that the surface GAG (or its contents) in the urinary bladder has the ability to act as an AAF. Subsequently, it was found that we could remove the endogenous GAG (with a corresponding rise in bacterial adherence) and, by placing intravesically into these mucin-deficient bladders the sulfonated GAG, i.e., heparin, the bacterial adherence was returned to control levels (11). The manner in which heparin interfered with bacterial adherence had not been defmed.

The current study provides insight into the mechanism. Bacteria were preexposed to concentrations of heparin four times that necessary to block bacterial adherence, as were mucin-deficient bladders. Tables 1 and 2 show that pretreatment of bacteria did not affect adherence, whereas pretreatment of bladders with heparin reduced it. It is our feeling that the anti-adherence activity of heparin depends not on an antigen-antibody-like reaction (inactivating bacterial attachment organelles), but rather by coating the transitional cells. Autoradiography with [3H]heparin corroborated this (Fig. 1).

It is our belief that the data presented here offer significant support for our thesis that surface GAG acts as an AAF directed against bacteria and is perhaps the most important antibacterial defense mechanism of the lower urinary tract. In the study reported herein, the exogenous GAG, heparin, seems capable of binding to the transitional cells denuded of their natural mucin layer and blocking bacterial adherence, the important point being that one can remove the natural mucin and specifically reproduce its anti-adherence effect with an exogenous and purified GAG. Since the mucin lines the entire genitourinary tract, appears to be synthesized rapidly (15), and binds to transitional cells (even when placed intracavitarily), it could conceptually provide a diffuse and effective barrier to bacterial adherence. An AAF acting in this fashion would preclude the specificity (and hence limited activity) required of an antigen-antibody response, as has been suggested for immunoglobulin A and glycoprotein (12, 13). The fact that such specificity would not be required seems logical, since an efficient AAF should ideally act in a nonspecific and generalized manner to be effective against a host of environmental organisms. This explains why various bacterial species were all found to behave similarly (17) in our model and why it was found that adherence of live and dead bacteria and colloid particles was also controlled by the presence of mucin (20).

This function of GAG may also be an important antibacterial defense at other mucosal surfaces. Additionally, since sulfonated GAGs are produced by the endothelial lining of blood vessels (1, 2), perhaps they are important AAFs at that site also, keeping blood elements from adhering to vascular walls and becoming obstructive. To our knowledge, GAGs have not had ascribed to them this role as an AAF in the immunodefense mechanism of the host.

A mechanism for how the sulfonated GAG may prevent molecular adherence to surfaces is provided by data obtained in the field of synthetic membrane technology (8-10; T. W. Jeffries, D. R. Omstead, R. R. Cardenas, and H. P. Gregor, Biotechnol. Bioeng., in press); specifically, that sulfonated GAGs act as "nonfouling" polymers. The sulfonate groups (SO) of the GAGs are extremely hydrophilic and will preferentially bind several molecules of water in preference to even heavy metal cations and hydrogen (even at low pH values). What the sulfonate group does, in effect, is place water between the polymer and the environment and prevent fouling of the surface, since no net electrical charge is available to bind ions or molecules. Such sulfonated polymers are also called "wetting agents," since they specifically bind water molecules and "wet" the surface.

We feel that heparin (and mucin) may operate in an analogous fashion in the urinary tract, preventing bacterial adherence by providing a tightly bound molecular layer of water between the bacterium and the transitional cell. In fact, by acting in such a fashion, the GAG could conceivably act as a nonspecific AAF and explain other phenomena in the urinary tract. For example, the presence of a molecular layer of water bound to the surface of the transitional cells could be why calcium, supersaturated in the urine, does not encrust the epithelium as it will foreign bodies. Moreover, the mucin could also deny access to mucosal cells of various carcinogenic promoters.

In essence, we suggest that the ability of mucin

to block adherence in the urinary tract may not be limited to those materials we have already studied, i.e., live bacteria, dead bacteria, and colloid particles. The simplicity of such a model, that is, a substance capable of blocking adherence at many levels (ionic, molecular, and cellular), could explain its remarkable efficiency, since its action is not hampered by the restraints of specificity.

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