Bladder Surface Mucin

Its Antibacterial Effect Against Various Bacterial Species

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We previously reported the results of quantitative and histochemical studies implicating the surface mucin of the bladder mucosa as an important antibacterial defense mechanism, which functions by preventing bacteria from adhering to the bladder wall. We call the mucin "anti-adherence factor" and we feel this is a previously undocumented role for mucin as a type of host antibacterial defense. These experiments were conducted with *Escherichia coli*. In an effort to determine whether the anti-adherence ability of the vesical mucin was a generalized phenomenon, we repeated these studies using unrelated bacterial species, including *E coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*. The ability of the vesical mucosa to resist bacterial adherence to its surface was found to be independent of the bacterial species that was investigated. (Am J Pathol 93:423–432, 1978)

ANTIBACTERIAL DEFENSE MECHANISMS of mucosal surfaces are felt to involve antibodies or enzymes which exert a bactericidal effect or some factor preventing bacterial adherence to the mucosal cells.[1-6] We have been interested in the latter concept in regard to the urinary tract. A mechanism that could prevent bacterial adherence to transitional cells (an "anti-adherence factor") would be extremely efficient at disposing of large numbers of microorganisms. This would make it possible for urine flow to easily remove the organisms, explaining both the need and the efficiency of the "urine washout effect" in preventing infection.[3,7-11] Previously, the existence of an anti-adherence factor had been suggested but had not been well documented at any mucosal surface,[5,6,12] but we reported its possible existence in the urinary bladder.[1,2] Our studies included quantitative and histochemical data which showed that transitional cells secrete a mucopolysaccharide located at the bladder surface (surface mucin). This mucin layer was found to be capable of preventing bacteria from adhering to mucosal cells.[1,2] Additional and more extensive histochemical studies have corroborated these findings.[13] All the experiments were conducted with *Escherichia coli*. To determine if the antibacterial activity of the bladder mucin was a generalized phenomenon and not confined to our species of *E coli*, the current study was conducted. We examined the

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Accepted for publication July 5, 1978.

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0002-9440/78/0119-0423/$01.00

anti-adherence effect of mucin against three unrelated species of bacteria: *E coli, Klebisella pneumoniae,* and *Staphylococcus aureus.*

Materials and Methods

Preparation of Bacteria

E coli type 04 was cultured and labeled with ^{14}C as previously described.[1]

S aureus was inoculated into heart infusion broth (Difco Laboratories, Detroit) and incubated at 37 C overnight. A 2.5% inoculum (v/v) was transferred to 5.0 ml of fresh heart infusion broth containing 10 μCi/ml of ^{14}C-bicarbonate (Amersham/Searle, Des Plaines, Ill.) and the mixture was incubated at 37 C. Klett readings were obtained at 30-minute intervals until the culture reached the end of the log phase. The bacteria were then sedimented at 3000*g* for 10 minutes, washed once with 5.0 ml of 0.9% NaCl (PSS), and again sedimented at 3000*g* for 10 minutes. The bacteria were resuspended in 1.0 ml of PSS, and this culture was assayed for both radioactivity (see Step 5) and viability. Colony-forming units were determined using brain heart infusion agar (Difco Laboratories) as the growth medium. The 1.0-ml bacterial suspension was divided into two 0.5-ml aliquots for introduction into rabbit bladders, one aliquot to a control rabbit (as described under Step 2a) and one to an experimental rabbit (as described under Step 2b). The same procedure was used to prepare *K pneumoniae.*

Basic Model

Male New Zealand white rabbits weighing 2 to 3 kg were used. All animals were anesthetized with Innovar (Pitman–Moore, Englewood, N.J.), 0.2 mg/kg body weight.

Step 1—Cystostomy. Each rabbit was secured and given 100 ml of PSS intravenously over a 30-minute period. The intravenous infusion was turned off. A polyethylene tube (PE 205, Clay–Adams, Parsippany, N.J.) was inserted suprapubically, secured with a 4-0 silk purse-string suture, and brought out through the wound. Care was taken to manipulate the bladder as little as possible.

Step 2—Preparation of bladder. a) Control rabbits. The bladder was flushed through the cystostomy tube 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 were irrigated with four 15-ml aliquots of PSS and were slowly infused (over 20 seconds) with 7 ml of 0.3N HCl through the suprapubic tube with the penis clamped. When the acid had remained in the bladder for 60 seconds, it was aspirated and the bladder was flushed with one 15-ml aliquot of 0.5 M K_2HPO_4 followed by three additional rinsings with 15-ml aliquots of PSS. The introduction of bacteria was then performed as in the *Basic Model (Step 3).*

Step 3—Introduction of bacteria. Both sets of rabbits as described in Steps 2a and 2b received 0.5 ml of bacteria, either *E coli, S aureus,* or *K pneumoniae.* As described under *Preparation of Bacteria,* this bacterial suspension was then rinsed into the bladder with 0.5 ml PSS, and the cystostomy tube and penis were clamped.

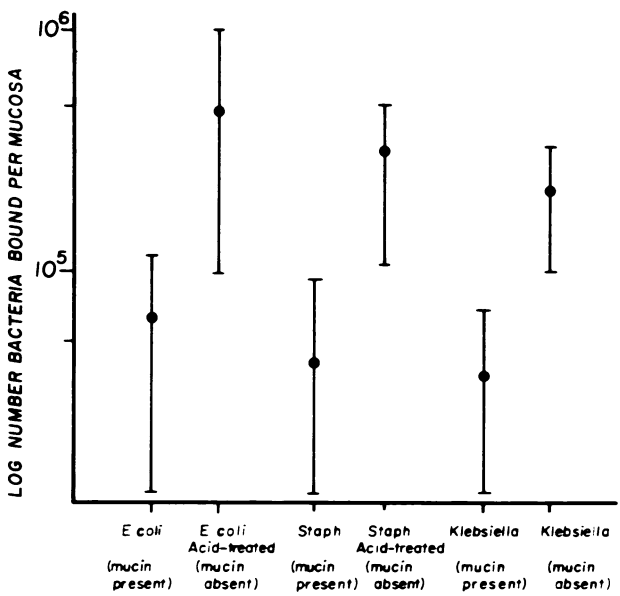
Step 4—Interaction of bacteria with the bladder mucosa. After the penis was clamped and the bacteria were introduced into the bladder through the suprapubic catheter, it was then also clamped for 15 minutes while PSS was given intravenously at a rate of 200 ml/hr. At the end of 15 minutes, 10 ml of the respective solutions in which the bacteria were suspended was introduced into the bladder through the suprapubic tube to dilute the bacteria and terminate the reaction. The suprapubic tube was then 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. Bladder tissue was homogenized overnight in 3 ml of 1.5 M NaOH at 60 C and neutralized with 2.0 ml of 2N HCl. The next morning the solution was bleached by adding 2.0 ml of perchloric acid and 0.4 ml of 30% hydrogen peroxide to remove color that might quench bacterial counts. After 30 minutes, two or three drops of 15% ascorbic acid were added to oxidize remaining hydrogen peroxide. This whole solution was titrated to neutrality with 2N HCl. Fifty percent of the mucosal samples and 25% of the muscle samples were suspended in 20 ml of Aquasol (New England Nuclear, Boston). Radioactive counts were recorded by a Packard-Bell liquid scintillation counter. Bacterial samples were suspended in 5 ml of Aquasol for radioactive counting.

Results

All three bacterial species adhered approximately 10 times greater to the acid-treated bladders (Text-figure 1). Fifteen separate experiments were performed on different days; each experiment involved 6 rabbits. Two rabbits (one bladder was acid-treated and the other was normal) received either *E coli, S aureus,* or *K pneumoniae.* A summary of these results is shown in Table 1.

Viable cell counts for all three bacterial species studied were between 9.0×10^8 and 2.0×10^9 viable colony counts (VCC) per milliliter, and the ratio of VCC to radioactive counts per minute was between 50 and 200 for *E coli* and between 200 and 500 for *K pneumoniae* and *S aureus.* On each day of experimentation both the acid-treated and control rabbits received the same number of bacteria; the variation in VCC was on a day-to-day



TEXT-FIGURE 1—These data summarize the absolute numbers of microorganisms that bound to both normal and acid-treated vesical mucosas (removes mucin layer at bladder surface). Three species of bacteria are compared and all three bound significantly better (10-fold higher) to acid-treated, mucin-deficient mucosas.

Table 1—Adherence of *E coli*, *S aureus*, and *K pneumoniae* to Control Rabbit Bladders and to Bladders Whose Surface Mucin Has Been Removed by Acid Treatment

	Escherichia coli			*Staphylococcus aureus*			*Klebsiella pneumoniae*		
Experiment	Control ($\times 10^5$)	Acid-treated ($\times 10^6$)	Ratio A/C	Control ($\times 10^5$)	Acid-treated ($\times 10^6$)	Ratio A/C	Control ($\times 10^5$)	Acid-treated ($\times 10^6$)	Ratio A/C
1	1.7	1.7	10	3.8	2.3	6.1	14	2.0	1.4
2	3.0	2.2	7.3	0.34	0.17	5	5.4	12	22
3	2.2	2.0	9.1	1.0	0.77	7.7	1.7	0.14	0.82
4	13	4.6	3.5	7.3	1.1	1.5	6.4	0.57	0.89
5	3.0	2.9	9.7	5.3	1.9	3.6	3.5	1.1	3.1
6	11	3.9	3.5	1.3	2.7	21	2.9	3.4	11.7
7	20	11	5.6	0.16	0.31	19.4	3.4	1.0	2.9
8	3.2	2.0	6.3	2.5	3.8	15.2	5.3	0.98	1.8
9	8.7	7.6	8.7	3.4	0.43	1.3	3.9	1.2	3.1
10	1.2	0.77	6.4	19	2.8	1.5	2.1	2.9	13.8
11	5.8	2.9	5	1.5	3.9	26	1.5	0.65	4.3
12	0.4	0.87	22	0.79	1.0	12.7	0.6	2.3	38.3
13	1.1	4.4	40	3.0	2.7	9.0	2.3	2.4	10.4
14	2.2	20	91	9.8	9.3	9.5	0.65	2.0	30.7
15	4.3	1.5	3.5	2.7	0.47	1.7	0.39	0.12	3.1
	$\bar{x}$ = 5.4 SD = 5.5*	$\bar{x}$ = 4.8 SD = 3.9	$\bar{x}$ = 15.4 SD = 22.9	$\bar{x}$ = 4.1 SD = 4.9	$\bar{x}$ = 2.2 SD = 2.3	$\bar{x}$ = 9.4 SD = 7.9	$\bar{x}$ = 3.6 SD = 3.4	$\bar{x}$ = 2.2 SD = 2.9	$\bar{x}$ = 9.9 SD = 11.7

* All standard deviation (SD) values = $\times 10^5$

Summarized above are the absolute number of bacteria bound per mucosa for each experimental point. Reading across, the six points were all obtained on the same day. The column "Ratio A/C" represents the ratio of bacteria bound to the acid-treated control and the bacteria bound to the normal bladders on that day of experimentation. In almost all instances the ratio is greater than 1.0.

basis. To arrive at the total number of bacteria bound per mucosa (live + dead bacteria), the radioactive counts that were present on each mucosa were converted to bacteria based on the ratio of bacteria to radioactive counts for that particular day, and the computed data are summarized in Table 1.

No bacteria were detected in the muscle layer in any of the experiments.

Discussion

Mucosal membranes are felt to employ several antibacterial defense mechanisms to control microorganisms at their surfaces. These mechanisms are thought to involve either a bactericidal effect, promote phagocytosis, or inhibit bacterial adherence to the mucosal cells. Bactericidal mechanisms that have been reported include various combinations of IgG, IgA, or enzymes and have been recently reviewed.[4]

Bacterial adherence has long been known to be important in virulence for many bacterial species, including streptococci, gonococci, *Vibrio cholerae*, *E coli*, and *Mycoplasma pneumoniae*.[9,13-29] Microappendages on the bacteria, called "pili" or "fimbriae," are felt to be the means by which the bacteria attach to tissue they are to infect.[17,20,23,30] On the other hand, little is known concerning any host defense mechanism that might be directed against this form of bacterial virulence.

Antibodies have been suggested to play some role in host resistance, although this role is not well understood. Recent *in vitro* studies with IgA, however, suggest one possible role for antibodies, ie, that secretory IgA may be responsible for decreasing bacterial adherence.[5] Additional *in vitro* experiments have implied that glycoproteins may also function in this regard.[6] We have been interested in the possible existence of an anti-adherence factor in the genitourinary tract. Such a mechanism would confine large numbers of microorganisms to the urine and eliminate the need for bactericidal substances or reticuloendothelial responses. The urine "washout" effect could then readily remove the bacteria.[7,8,10,11,31]

Little has been previously known concerning bacterial defense of the urinary bladder. Secretory IgA may be locally produced,[3,32-37] but no antibacterial activity has been ascribed to it. The only other bladder mechanism studied was a possible bactericidal effect,[37-39] but data supporting its existence have been challenged.[40,41] As mentioned, our research efforts have been concerned with the possible existence of an anti-adherence factor in the urinary tract. We have previously reported data in-

dicating that the transitional cells of the vesical mucosa secrete a mucopolysaccharide (mucin) that is bound to the surface cells.[1] This layer was previously demonstrated by Monis and Dorfman.[42] If the mucin layer is removed by acid, the ability of bacteria to adhere to mucosal cells rises markedly.[1,2] When the mucin is replaced, bacterial adherence is greatly reduced.[1,2] These histochemical and physiologic studies have been corroborated by additional experiments.[13] It is our contention that the surface mucin layer in the bladder is or contains an anti-adherence factor active against bacteria.

All of the previous experiments were conducted with a method that quantitatively measured bacterial adherence of *E coli* to rabbit bladder mucosas.[1,2,13] The question arises whether the anti-adherence factor is active against other unrelated bacterial species or merely a phenomenon unique to the species we investigated. The current study was conducted to explore this question. Adherence experiments were performed using *E coli*, *S aureus*, and *K pneumoniae*, and the results are summarized in Text-figure 1. As is seen, all three organisms sorbed to the normal mucosas at approximately equal efficiency. These data were subjected to analysis by the Student *t* test, and no significant difference was found between the means of bacteria bound to the normal bladders. Each organism was approximately 10 times more efficient at adhering to bladders after surface mucin had been removed by acid (Table 1), which agrees with previous data.[1,2,13] These values were also subjected to variance analysis, and the differences of the means between the acid-treated and control bladders (Table 1) were significant for all three bacterial species ($P < 0.01$).

One theoretic source of error in our adherence assay is variation in rabbit bladder size. For this reason, we randomly assigned rabbits to all six points in Text-figure 1 and examined 15 rabbits at each point. And, as Table 1 shows, we had very consistent results that again are significant when the means of the acid-treated rabbits and control rabbits were subjected to variance analysis ($P < 0.01$). In addition, we compared the ratio of bacteria adherent to the acid-treated bladders with bacteria adherent to normal bladders for each experiment; in only 2 of 45 sets of rabbits (Table 1) were the ratios less than 1.0. Means of these ratios were not significantly different for the three bacterial species according to the student *t* test ($P > 0.05$). We feel that the consistency of the rise in bacterial adherence after acid destruction of the surface mucin again speaks for the reliability of our assay as performed without determining the dry weights of the mucosas. The same rise in bacterial adherence and the absolute numbers of bacteria bound per mucosa are also identical to previously published data.[1,2,13]

The manner with which we remove the surface layer of mucin, ie, 0.3N HCl, is, of course, harsh treatment and it is important to point out several things. Grossly no effect is noted on the bladders. Microscopically, we found while doing the histologic studies [1,2] that the mucosa was basically intact, with the only consistent finding being the loss of the mucin layer.[1,2] In fact, the time of acid treatment and the concentration of acid were reduced in a step-wise fashion to a minimum level which would produce a rise in bacterial adherence. It is at this point we also discovered the loss of the mucin layer.[2] This layer turned out to be remarkably resistant to attempts at removal. We tried to hydrolyze it with trypsin, hyaluronidase, neurominidase, and mucomyst; the latter three produced a modest but significant rise in bacterial adherence, but only acid consistently removed the mucin layer histologically.[43]

It seems likely to us that the anti-adherence property of the vesical mucin layer is a generalized phenomenon. It not only functions to prevent *E coli* and mucosal cell interactions but also prevents other unrelated species from binding to transitional cells, including gram-negative and gram-positive bacteria. Teleologically, it should be expected that anti-adherence factor would be universally active; otherwise, it would not be effective as a method to control bacteriuria against a host of bacterial species. Activity against *Staphylococcus* was also expected for this reason, despite the fact that it does not cause urinary tract infections (but its inability to grow in normal urine probably is the major reason for this).

The manner by which vesical mucin interrupts bacterial and mucosal cell interaction is, of course, speculative. Whatever mechanism the bacteria employ to adhere is present in both metabolically poisoned (using NaF) and dead bacteria (formalin-treated) since both groups adhere as effectively as live bacteria.[44] This makes it unlikely that mucin destruction of microorganisms will prevent adherence. We believe bladder mucin prevents bacterial adherence by blocking the transitional cell receptor sites to which the bacteria might attach. And as such, its presence over the entire mucosal layer as a coating would be important. By preventing bacterial–mucosal cell interactions, the bladder can thus rely on the urine "washout" effect to remove the microbes, an effect long known to be important in preventing urinary infections and known to depend on voiding frequency, residual urine, and bacterial growth rate.[4,5,12,28,45] Bladder surface mucin (or contents) acting as anti-adherence factor helps explain both the importance of this effect and the relative resistance of the normal urinary bladder to infection.

The *in vivo* quantitative assay that we have developed to measure bacterial adherence to transitional cell epithelium in the intact bladder

has provided data indicating that mucin acts as an anti-adherence factor active against bacteria. No one, to our knowledge, has shown mucin to have antibacterial activity in this regard. We feel that these are the first data to suggest such a role for mucin in host antibacterial defense, a role perhaps not confined to the mucous surface in the genitourinary tract.

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