

# A DIAGNOSTIC IN VITRO URINE ASSAY FOR INTERSTITIAL CYSTITIS

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## ABSTRACT

**Objectives.** A low molecular weight urine factor that inhibits the proliferation of normal bladder epithelial cells in vitro was previously shown to be present significantly more often in the urine of patients with interstitial cystitis (IC) than in the urine of asymptomatic age-, race-, and sex-matched control subjects. We sought to determine the specificity of this finding for IC by determining whether the urine of patients with other urogenital inflammatory disorders also contains a factor that inhibits bladder epithelial cell proliferation.

**Methods.** Urine was collected from women with IC, acute bacterial cystitis, or vulvovaginitis, as well as from asymptomatic control women. The proliferation of primary normal adult bladder epithelial cells was determined by measuring  $^3\text{H}$ -thymidine incorporation in vitro.

**Results.** Osmolality- and pH-corrected urine specimens from 50 (86%) of 58 women with IC significantly inhibited human bladder epithelial cell proliferation compared with 3 (8%) of 36 asymptomatic control women, 7 (12%) of 58 women with bacterial cystitis, and 0 (0%) of 12 women with vulvovaginitis ( $P < 0.001$  for the comparison of mean percent change in  $^3\text{H}$ -thymidine incorporation with IC urine versus urine from each of the control groups). Optimal sensitivity and specificity values of 91.4% and 90.6%, respectively, were achievable at a cutoff of 25% inhibition of  $^3\text{H}$ -thymidine incorporation, using all three control groups.

**Conclusions.** The measurement of urine antiproliferative activity may be a useful noninvasive means for diagnosing IC in women. UROLOGY 52: 974-978, 1998. © 1998, Elsevier Science Inc. All rights reserved.

**I**nterstitial cystitis (IC) is a chronic bladder disease that affects at least 20,000 to 90,000 women

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*Three of the authors (S.K., M.K.H., and J.W.W.) are described as inventors on a pending patent application for an IC diagnostic test based on the antiproliferative factor.*

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in the United States<sup>1</sup>; approximately one tenth as many men also suffer from this disease. Women with IC often present with pain, urgency, and increased frequency of urination, symptoms common to other urogynecologic disorders, including bacterial cystitis and vulvovaginitis. Currently, IC must be diagnosed by the demonstration of bladder epithelial abnormalities at cystoscopy, such as petechial hemorrhages (glomerulations) or ulcers that extend into the lamina propria (Hunner's ulcers).<sup>2</sup> No one specific infectious or other etiology for IC has yet been identified.<sup>1-5</sup>

Previous studies in our laboratory noted that the urine of patients with IC inhibits the proliferation of normal adult bladder epithelial cells in vitro significantly more often than urine specimens from age-, race-, and sex-matched control subjects.<sup>6</sup> The agent responsible for this antiproliferative activity in IC urine appears to be a low molecular weight, heat stable, trypsin-sensitive factor. Because damage to the epithelium is the predominant histologic finding in IC,<sup>2,7,8</sup> the factor that inhibits normal bladder epithelial regeneration may be causally re-

lated to IC and/or may prove to be a useful diagnostic marker for this disease.

Therefore, to better determine the sensitivity and specificity of urine antiproliferative activity for IC, we screened urine specimens from women with IC, asymptomatic control women, women with acute bacterial cystitis, and women with vulvovaginitis for their ability to inhibit  $^3\text{H}$ -thymidine incorporation by normal bladder epithelial cells *in vitro*.

## MATERIAL AND METHODS

### PATIENTS

All patients with IC had previously undergone diagnostic cystoscopy, and fulfilled the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) diagnostic criteria for IC.<sup>9</sup> Urine was collected at the University of Maryland School of Medicine from women with IC at least 3 months after the most recent known bacterial urinary tract infection and 1 month after the last antibiotic use. Urine specimens collected at the University of Pennsylvania were obtained from women during routine office visits for management of IC. Asymptomatic controls were female volunteers with no history of IC or other urologic disease; each asymptomatic control was required to have no symptoms of urinary tract infection or antibiotic use for at least 1 month. Urine was also collected at the time of diagnosis from female patients with acute bacterial cystitis at the University of Maryland School of Medicine and the University of Maryland-College Park (diagnosis based on the presence of bacteriuria [more than  $10^3$  bacteria/mL with a single type of bacterium isolated] plus pyuria in combination with appropriate symptoms); 50 of the 58 patients had more than  $10^3$  bacteria/mL. Patients with vulvovaginitis were diagnosed clinically on the basis of appropriate findings on physical examination (labial erythema and vaginal tenderness with abnormal discharge). All participants were at least 18 years old and enrolled in accordance with guidelines of the Institutional Review Boards at the University of Maryland School of Medicine, the University of Maryland-College Park, and Allegheny University and the University of Pennsylvania.

### URINE SPECIMENS

Urine was collected by the clean catch method in which each patient wiped the labial area with 10% povidone iodine solution and then collected midstream urine into a sterile container, as previously described.<sup>6</sup> Specimens obtained at the University of Maryland were initially kept at 4°C, then transported to the laboratory where cellular debris was removed by low-speed centrifugation at 4°C. Specimens obtained at the University of Pennsylvania (from patients with IC) and University of Maryland-College Park (from patients with bacterial cystitis) were frozen at -20°C for up to 4 weeks, then transported to the University of Maryland School of Medicine on ice. All specimens were subsequently aliquoted under sterile conditions and stored at -80°C until used.

### CELL CULTURE

Normal adult human bladder epithelial (HBE) cells were grown from tissue obtained at autopsy from a single patient who had no history of bladder disorder, according to previously published methods.<sup>10</sup> The explanted cells were grown in Eagle's minimal essential medium (MEM) containing 10% heat inactivated FBS, 1% antibiotic/antimycotic solution, 1% glutamine, and 1.0 U/mL insulin (all from Sigma). Cells were cultured at 37°C in a 5% carbon dioxide atmosphere.

### $^3\text{H}$ -THYMIDINE INCORPORATION

HBE cells explanted from bladder tissue were plated at a density of  $1 \times 10^4$  cells/well onto 96 well tissue culture plates and incubated at 37°C overnight (resulting in approximately 60% confluence the next day). The medium was then changed to MEM containing only 1% glutamine and 1% antibiotic/antimycotic solution, and the cells were incubated at 37°C overnight. On the third day, urine specimens from patients with IC or controls were corrected to pH 7.2 and 300 mOsm, filtered through a 0.2- $\mu\text{m}$  pore filter (Gelman Sciences, Ann Arbor, Mich), diluted 1:2 in MEM (with only glutamine and antibiotic/antimycotic solution) and applied to the cells. After 48 hours of incubation at 37°C, the cells were pulsed with 1  $\mu\text{Ci}$ /well  $^3\text{H}$ -thymidine (NEN DuPont, Wilmington, Del) and incubated for another 4 hours at 37°C. Cells were then trypsinized, and insoluble cell contents harvested and methanol-fixed onto glass fiber filter paper, as previously described<sup>6</sup>; the amount of radioactivity incorporated was determined as counts per minute using a Beckman LS 3801 scintillation counter.

### STATISTICAL ANALYSIS

Comparisons of the percentage of patients with IC or control patients whose urine significantly inhibited bladder epithelial cell proliferation was performed using Fisher's exact test. Comparisons of the mean change in  $^3\text{H}$ -thymidine incorporation caused by urine specimens from patients with IC versus asymptomatic controls or patients with bacterial cystitis or vulvovaginitis were performed using a two-tailed analysis of covariance with age as the covariate. To assess the usefulness of measuring urine antiproliferative activity as a potential diagnostic assay for IC, logistic regression analysis (Stata Corp., College Station, Tex) was performed with case or control status (ie, presence or absence of clinical IC) serving as the dependent variable and the percent change in  $^3\text{H}$ -thymidine incorporation serving as the independent variable. Sensitivity and specificity derived from the logistic regression model were determined for various cutoff values.

## RESULTS

To determine whether urine specimens from patients with IC were more likely to inhibit bladder epithelial cell proliferation than specimens from any of the three control groups, a significant inhibition of  $^3\text{H}$ -thymidine incorporation was defined as a mean decrease in counts per minute of greater than 2 standard deviations from the mean of control cells for each plate. Osmolality- and pH-corrected urine specimens from women with IC were significantly more likely to inhibit human bladder epithelial cell proliferation (50 [86%] of 58) than specimens from any of the three control groups (3 [8%] of 36 asymptomatic control women, 7 [12%] of 58 women with bacterial cystitis, and 0 [0%] of 12 women with vulvovaginitis [ $P \leq 0.001$  for a comparison of patients with IC versus each control group]). The slight differences in the methods of urine storage between the centers did not significantly affect the percent of patients with IC whose urine significantly inhibited  $^3\text{H}$ -thymidine incorporation, with 82% of IC specimens obtained at the University of Maryland in Baltimore having antiproliferative activity compared with 88% of IC

specimens obtained at Allegheny University. Similarly, there was no significant difference in the percent of patients with bacterial cystitis whose urine had antiproliferative activity between specimens obtained at the University of Maryland in College Park (12%) and specimens obtained at the University of Maryland in Baltimore (10%).

Although the mean age of the patients with IC ( $44.2 \pm 2.2$  years) did not differ significantly from the mean age of either the asymptomatic control group ( $42.3 \pm 2.9$  years) or the patients with vulvovaginitis ( $39.6 \pm 6.7$  years), the patients with acute bacterial cystitis were significantly younger than the patients with IC ( $22.3 \pm 1.3$  years,  $P < 0.001$ ). We therefore compared the mean percent change in  $^3\text{H}$ -thymidine incorporation in cells cultured with IC urine to the mean percent change in  $^3\text{H}$ -thymidine incorporation in cells cultured with urine from each control group using a two-tailed analysis of covariance with age as the covariate. This analysis indicated that urine from patients with IC also caused a significantly greater inhibition of bladder cell proliferation than urine from any of the control groups, with a mean percent inhibition of  $^3\text{H}$ -thymidine incorporation in cells cultured with IC urine of  $69.4 \pm 3.5$ , compared with  $5.1 \pm 1.4$  for asymptomatic controls,  $9.9 \pm 2.6$  for patients with bacterial cystitis, and  $0 \pm 0$  for patients with vulvovaginitis ( $P < 0.001$  for patients with IC versus each control group) (Fig. 1).

To assess the usefulness of measuring urine antiproliferative activity as a potential diagnostic assay for IC, logistic regression analysis was performed with case or control status serving as the dependent variable and the percent inhibition of  $^3\text{H}$ -thymidine incorporation (in cells cultured with urine specimens diluted in medium as compared with cells cultured with medium alone) serving as the independent variable. A receiver operator characteristic curve generated for a comparison of data from patients with IC and asymptomatic controls indicated an area under the curve of 0.9588 (Fig. 2A); a similar comparison of data from patients with IC versus data from all three control groups yielded an area under the curve of 0.9453 (Fig. 3A).

Sensitivity and specificity derived from the logistic regression model were determined for various cutoff values. As shown in Figure 2B, optimal sensitivity and specificity values of 93.1% and 94.4%, respectively, were achievable at a cutoff of 23% inhibition of  $^3\text{H}$ -thymidine incorporation when patients with IC were compared with the asymptomatic control group alone. A comparison of patients with IC to persons in all three control groups yielded optimal sensitivity and specificity values of 91.4% and 90.6%, respectively, at a cutoff value of 25% inhibition (Fig. 3B), with a positive predictive

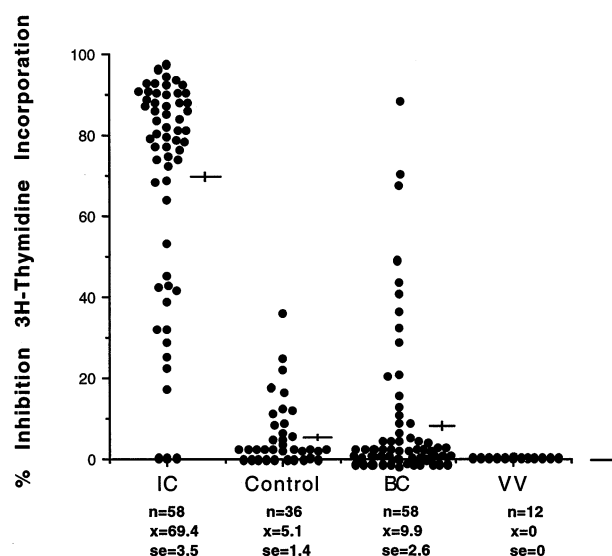


FIGURE 1. Inhibition of  $^3\text{H}$ -thymidine incorporation in normal adult human bladder epithelial cells by IC urine specimens. Data are expressed as the percent inhibition of  $^3\text{H}$ -thymidine incorporation in cells incubated for 48 hours with either IC urine specimens or control urine specimens diluted in serum-free cell culture medium compared with cells incubated with serum-free cell culture medium alone. Each data point is the mean of 3 to 6 determinations. Horizontal bars indicate the mean value of the data points for each group; vertical bars indicate the standard error of this mean. IC = interstitial cystitis, BC = bacterial cystitis, VV = vulvovaginitis.

value of 83% and a negative predictive value of 95%.

## COMMENT

In this report, we present evidence that the antiproliferative factor previously identified in IC urine specimens is related specifically to that disease. The identification of this factor may therefore provide a noninvasive method for the diagnosis of IC, as suggested by logistic regression analysis.

A sensitive and specific marker or diagnostic test for IC would be very useful. Although certain markers, such as urinary histamine levels after bladder distention,<sup>11</sup> urinary interleukin-6,<sup>12,13</sup> or flow cytometry profiles of urinary cells,<sup>14</sup> have been suggested for IC, these are unlikely to be clinically useful because they require cystoscopy,<sup>11</sup> are unlikely to be able to distinguish between IC and other urinary tract diseases,<sup>12</sup> and/or are potentially useful for only a small percentage of patients with IC.<sup>13,14</sup> Similarly, increases in spot urine histamine,<sup>15</sup> methylhistamine,<sup>15</sup> and tryptase levels,<sup>16</sup> or decreases in certain components of the glycosaminoglycan layer that lines the bladder lumen,<sup>17-19</sup> have been shown to occur in IC. Although these differences may be very important for a greater understanding of the disease process, the

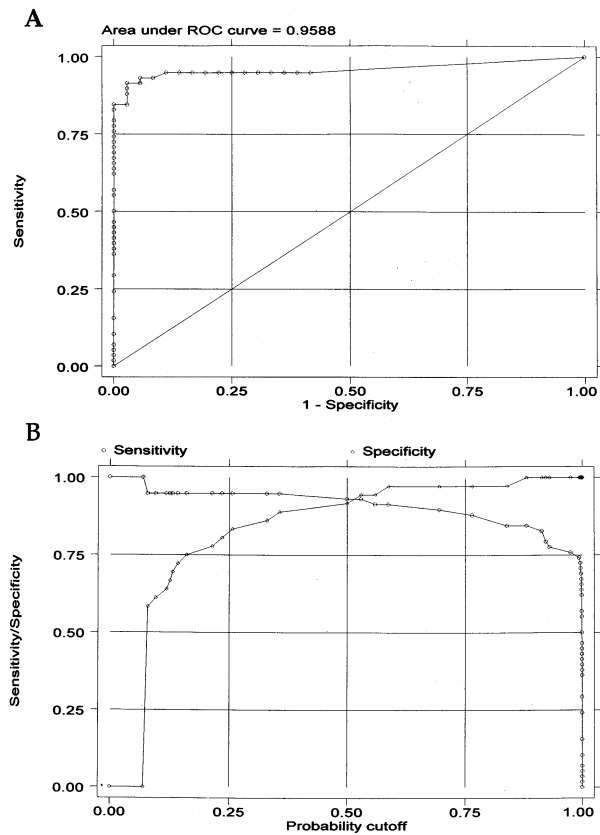


FIGURE 2. Logistic regression analysis comparing percent inhibition of  $^3\text{H}$ -thymidine incorporation in primary bladder epithelial cells by urine from women with IC compared with urine from asymptomatic control women. (A) receiver operating characteristic curve; (B) sensitivity/specificity curves at an optimal cutoff value of 23% inhibition of  $^3\text{H}$ -thymidine incorporation.

general overlap between patients with IC and controls makes the usefulness of these substances as markers for IC currently uncertain.

Preliminary characterization of the antiproliferative factor(s) we identified suggests it is a relatively heat stable, low molecular weight, soluble protein (less than 10,000 daltons).<sup>6</sup> Our laboratory is in the process of purifying this factor and determining its identity and source. When fully characterized, this low molecular weight factor could serve as the basis for subsequent *in vitro* antiproliferative assays or immunoassays. That urine from patients with documented acute bacterial urinary tract infections did not inhibit epithelial cell proliferation to the same extent as urine from patients with IC indicates that this factor does not result from an inflammatory response involving polymorphonuclear leukocytes. Whether it results from inflammation involving lymphocytes, as is sometimes evident in the interstitium of bladder tissue from patients with IC,<sup>2,20</sup> remains to be determined.

We have proposed a model of IC, centered on one or more antiproliferative urinary factors, that

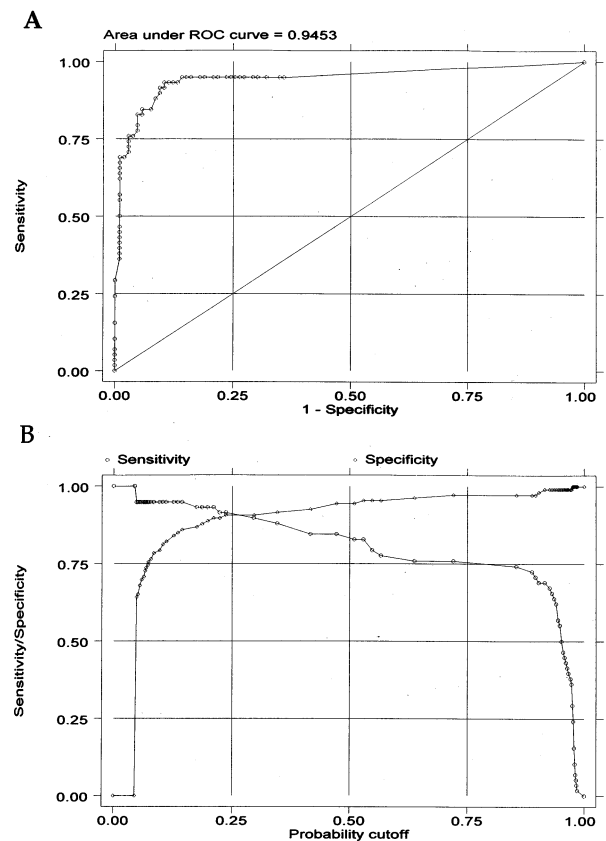


FIGURE 3. Logistic regression analysis comparing percent inhibition of  $^3\text{H}$ -thymidine incorporation in primary bladder epithelial cells by urine from women with IC compared with urine from asymptomatic control women, women with acute bacterial cystitis, and women with vulvovaginitis. (A) receiver operating characteristic curve; (B) sensitivity/specificity curves at an optimal cutoff value of 25% inhibition of  $^3\text{H}$ -thymidine incorporation.

explains many of its epidemiologic, clinical, and histologic characteristics.<sup>21</sup> We speculate that IC begins with initial bladder epithelial damage, and the presence of an antiproliferative factor(s) in the urine of patients with IC prevents appropriate regeneration of bladder epithelium. The chronically damaged epithelium is more permeable to urinary constituents, prompting a low level bladder inflammatory response, including mast cells, and the development of autoantibodies to usually sequestered antigens in urine and/or bladder epithelial cells.

Because a chronically damaged epithelium is a central finding in IC, the factor that inhibits bladder epithelial cell proliferation may provide clues to the pathogenesis of this disease. However, whether the mucosal changes found in IC represent the initiating abnormalities or whether they result from abnormalities in the submucosa remains to be determined. Also, whether the low molecular weight antiproliferative protein is neces-

sary or sufficient to cause bladder epithelial cell damage such as the damage seen in IC awaits the development of suitable animal models. A study correlating the amount of this protein and the severity of patient symptoms at different time points is in progress to help determine the role of this factor in IC.

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