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Evaluation of urinary IL-1α and IL-1β in gravid females and patients with bacterial cystitis and microscopic hematuria

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Abstract *Objectives*: to determine IL-1α and IL-1β levels in patients with bacterial cystitis, microscopic hematuria, and gravid females relative to a control group of normal subjects. *Methods*: enzyme immunoassays were used to measure concomitantly urinary IL-1α and IL-1β in clean catch urine samples from normal subjects ($n = 31$) and study patients ($n = 46$). All normal subjects and patients underwent urinalysis, urine culture, and urine creatinine level determination. Since the IL-1α assay was developed for serum, the utility of the assay for urine specimens was unknown. The key parameters of urine collection, processing and sample storage for IL-1α were evaluated in detail. *Results*: mean values ± SEM (pg/mg) for IL-1α/Cr and IL-1β/Cr were control group (0.25 ± 0.10 and 0.17 ± 0.06), bacterial cystitis (9.97 ± 1.15 and 42.45 ± 1.86), and microscopic hematuria (2.81 ± 0.65 and 2.82 ± 0.70). Differences in cytokine levels between the control group and patients with either bacterial cystitis or microscopic hematuria were statistically significant for both IL-1α/Cr ($P < 0.026$; $P < 0.007$, respectively) and IL-1β/Cr ($P < 0.0004$; $P < 0.014$, respectively). IL-1β/Cr correlates better with pyuria than IL-1α/Cr ($P = 0.02$ vs $P = 0.44$). In gravid females, only IL-1α was significantly elevated relative to non-pregnant females (IL-1β elevation approached statistical significance). Gravid females with positive urine cultures could not be distinguished from those with negative cultures based on either interleukin ($P > 0.05$). *Conclusions*: Significant elevations of IL-1α and IL-1β occur in patients with bacterial cystitis and microscopic hematuria. Correlation between pyuria and cytokine elevation was stronger for IL-1β than for IL-1α. Changes in IL-1α may reflect changes in the bladder epithelium rather than in the inflammatory leukocytes. The ability of IL-1α and IL-1β to serve as markers for bacterial cystitis in gravid females is diminished due to high basal levels during pregnancy.

Key words Cytokines · Cystitis · IL-1

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Introduction

Investigation of interleukin (IL-1) has revealed its central regulatory function in the immune system. Lipopolysaccharide (LPS), the coat of Gram-negative organisms, stimulates macrophages and neutrophils to produce IL-1 which causes a release of prostaglandin E_2. The prostaglandin resets the thermoregulatory center and thus produces fever. It is now widely acknowledged that IL-1 is the endogenous pyrogen and is critically important in the pathogenesis of bacterial infections [8]. In addition, IL-1 acts as a mediator in immune cascades. It is one of the earliest interleukins to be expressed by macrophages and regulates IL-2 expression and the growth and differentiation of macrophages and T and B lymphocytes [8].

Because of its central role in immunity, IL-1 has become an area of interest in urologic research. Bacillus Calmette-Guerin (BCG) treatment is known to provoke an IL-1 response. We previously reported that IL-1β is elevated by bacterial cystitis, but not by interstitial cystitis (IC) suggesting etiologic differences between the two diseases [17].

IL-1 is expressed in two distinct forms [8, 15]. IL-1β is a secretory cytokine and regulates the immune processes. IL-1α is membrane-associated and is thought to be involved in cell-to-cell communication between macrophages, T lymphocytes, and B lymphocytes. Therefore, IL-1α may be expected to be elevated in urologic disease states.

Based on these findings we examined IL-1α and IL-1β in bacterial cystitis to see if they are simultaneously elevated. Noting that several of the patients with bacterial cystitis were pregnant, we became interested in studying this group. IL-1 is known to be increased during pregnancy and is thought to be an important physiologic regulator [18, 20]. It is unclear whether both IL-1α as well as IL-1β are elevated. Whether bacterial infections during pregnancy can be discerned against the background of basal cytokine elevation that characterizes pregnancy is of particular interest. The study of gravid females was a small subset of the total number of patients included in the study. The microscopic hematuria group was studied because our previous studies, carried out on a limited number of patients, suggested that IL-1β was elevated in bacterial cystitis but not in patients with microscopic hematuria.

Upon initiating these studies we became aware that no information was available defining the methodology for IL-1α measurement. Currently available assays were designed for use on serum samples and their applicability to urine was unknown. We have previously reported validation of the serum IL-1β assay for use on urine samples [17]. Following these guidelines, we have determined methods for urine collection, sample preparation, and urine storage for IL-1α assays.

Materials and methods

Urine collection and criteria for patient selection

Clean catch spot urine samples were collected from three groups of patients in the urology and hematology clinics: a microscopic hematuria group ($n = 12$), a bacterial cystitis group ($n = 18$), and a group consisting of gravid females ($n = 16$). Each sample underwent a dipstick and routine urinalysis, urine culture, and creatinine level. Urine cultures growing more than 100 000 bacterial organisms were classified as bacterial cystitis. Normal urine samples ($n = 31$), constituting the control group, were collected from patients seen in the Employee Health Center after an informed consent was obtained. Normal subjects were defined as those patients with a negative: urologic history, dipstick analysis, urinalysis, and urine culture. The microscopic hematuria group included patients who had negative urine cultures, but had more than five red blood cells per high power field on urinalysis. As microscopic hematuria was an incidental finding on urinalysis, its etiology is currently being investigated. Urine samples from gravid females were obtained as part of their routine prenatal care. Upon collection of the urine samples, they were placed on ice, centrifuged at 4°C and then frozen at −70°C to ensure cytokine stability (see results below) until assays could be performed [17].

Cytokine assays

Enzyme immunoassays (EIA; R & D Systems, Minneapolis, Minn.) were used to measure IL-1α and IL-1β levels. The assay employs solid phase, surface bound antibodies which bind to the cytokine. The bound cytokine is further reacted with a horseradish peroxidase-conjugated monoclonal antibody. Color development following the horseradish peroxidase reaction was monitored using a Biotek EIA reader. A standard curve was constructed using a purified cytokine supplied in the assay kit. The discriminating level between IL-1-positive and IL-1-negative patients was chosen on the basis of four methods for determination of normal ranges. These include (1) mean ± 2 SD, (2) log-normal distribution method, (3) cumulative percentage method, and (4) non-parametric method. In addition the ROC method which optimizes sensitivity/specificity combinations was used [7].

Since the IL-1α assay was developed for serum, the utility of the assay for urine specimens was unknown. The key parameters of urine collection, processing and sample storage for IL-1α were evaluated in detail. The methodology and results of these experiments are reported in the results section.

Statistical analysis

The interleukin levels of the control, bacterial cystitis, and microscopic hematuria patients were compared using the Kruskal-Wallis test and pairwise analysis was carried out using Newman-Keuls test. Although parametric tests are probably less desirable, a conventional ANOVA analysis, followed by Newman-Keuls test, was also performed. The application of non-parametric versus parametric testing did not affect the statistical conclusions reached.

Gravid females were separated into two groups: those with bacterial infection ($n = 6$) and those without infection ($n = 10$). Student's *t*-tests were performed to detect differences in IL-1α or IL-1β levels between the two groups.

Analysis was performed to evaluate the correlation between leukocyte positive (more than five leukocytes per high power field) and negative groups with IL-1α and IL-1β levels. Normal ranges of IL-1α and IL-1β were determined using standard methods described above, and the individual values were classified as elevated or within the normal range. The association of these groups with leukocyte positive or negative subsets was then determined using the Fisher's exact test.

Results

Sample preparation and validation assays

Researchers have advocated various steps in the preparation of urine samples prior to cytokine determination. These steps include centrifugation, filtration, and the addition of sodium azide, a bacteriostatic agent used in the storage of urine. To evaluate each of these steps, validation assays were performed as follows. Prior to each validation assay, urine samples were augmented with purified IL-1α as supplied by the manufacturer. The concentrations of IL-1α tested ranged from a low of 4.5 to a high of 24.5 pg/ml. To validate the effects of centrifugation, samples were centrifuged for 10 or 30 min. Sodium azide was added to other samples at 0.1% or 0.5%. The effects of filtration were evaluated using a syringe filtration apparatus with a 0.22 μm filter. The level of IL-1α after each of these manipulations was then determined using an EIA. IL-1α levels from various conditions were then compared with the results obtained from assays of urine samples that were not manipulated.

The results of our evaluation of each processing step are shown in Table 1. Centrifugation for either 10 or 30 min has no effect on the recovery of IL-1α. Addition of sodium azide at a concentration of 0.1% is without effect. By contrast 0.5% sodium azide had a significant negative effect on IL-1α detection. Thus the concentration of sodium azide used has been restricted to levels

Table 1 Stability of IL-1α: recovery after centrifugation, addition of sodium azide and filtration (% of untreated)

Untreated (pg/ml)	Centrifugation (1000 *g*)		Sodium azide		Filtration[a]
	10 min	30 min	0.1%	0.5%[a]	
19.56	18.73 (95.9%)	18.45 (94.3%)	16.44 (84.0%)	12.28 (62.8%)	0
4.47	4.77 (100.0%)	4.73 (100.0%)	4.13 (92.4%)	2.11 (47.2%)	0
24.53	22.59 (92.1%)	23.23 (94.7%)	21.0 (85.6%)	14.25 (58.1%)	0
7.1	7.06 (99.4%)	6.39 (90.5%)	6.37 (73.3%)	3.57 (50.3%)	0

[a] Recovery of cytokine is significantly different from untreated ($P < 0.05$)

below 0.1%. This concentration of sodium azide was sufficient to maintain culture free urine for long periods of time. Filtration has been commonly used to clear urine samples. We find that virtually all the IL-1α is lost after filtration, and have not employed it in our standard urine preparation.

The effects of urine storage were closely examined because the assay's utility is ultimately determined by the ability to evaluate stored samples. Both the cost and assay flexibility are compromised if the cytokine has to be evaluated on fresh urine samples. A clean catch urine sample was collected in a sterile urine cup using standard collection procedures. The sample was immediately cooled to 4°C. It was then centrifuged at 1000 *g* for 15 min and then placed in a −70°C freezer. Samples were aliquoted prior to freezing, thawed once at room temperature, and the remnants discarded. At a period of 6 weeks, essentially complete recovery of IL-1α was noted, and for a period of up to 17 weeks the samples were predominantly stable with the recoveries (with the exception of sample 9) ranging from 85% to 100% (Table 2). These results illustrate that frozen urine samples can be evaluated for IL-1α, and that short term storage does not alter the results of the assay.

Table 2 Stability of IL-1α during storage

Specimen no.	IL-1α after storage (pg/ml)		
	0 day	6 weeks[a]	17 weeks[a]
1	14.15	14.34 (100.0%)	13.39 (94.6%)
2	5.71	7.03 (100.0%)	6.30 (100.0%)
3	9.51	7.81 (82.1%)	8.46 (90.0%)
4	47.88	44.18 (92.3%)	38.09 (79.6%)
5	12.44	11.36 (91.3%)	10.99 (88.3%)
6	17.05	17.87 (100.0%)	17.97 (100.0%)
7	22.74	25.52 (100.0%)	28.02 (100.0%)
8	7.31	9.11 (100.0%)	8.30 (100.0%)
9	16.84	11.91 (70.7%)	8.43 (50.1%)

[a] Not significantly different between 0 day and 6 weeks or 17 weeks using paired Student's *t*-test ($P = 0.58$ for 6 weeks; $P = 0.36$ for 17 weeks) and Pearson's regression coefficient (0.96 for 6 weeks; 0.87 for 17 weeks)

Assay specificity

IL-1α and IL-1β are both derived from a common precursor IL-1. Therefore, a potential for cross-reactivity between the monoclonal antibodies used in the respective assays might exist. To test this hypothesis, IL-1α was added in increasing amounts to assay diluent and to a culture- and urinalysis-negative urine specimen. These samples were then tested with the IL-1β assay. The reciprocal procedure was carried out after IL-1β was added to assay diluent and a urine sample and then tested with the IL-1α assay. No cross-immunoreactivity between the IL-1α and IL-1β-directed antibodies used was detectable in these experiments (unpublished data).

Following an accepted tradition in the literature, we present interleukin values as the ratio of cytokine to creatinine concentrations. However, since creatinine levels in urine are sensitive to changes in diet and exercise, we verified that, for statistical analysis, the use of this ratio versus the absolute cytokine value rendered the same result.

The results (Figure 1) indicate that IL-1α is markedly elevated in patients with bacterial cystitis as well as in those with microscopic hematuria. IL-1β is considerably elevated in bacterial cystitis and marginally elevated (*P* values slightly above 0.01) in microscopic hematuria. The patients' individual values are shown as well as the means. The data demonstrate that the majority of patients with bacterial cystitis have elevations in both IL-1α and IL-1β, although, IL-1β is elevated to a higher degree than IL-1α in comparison with the control. For microscopic hematuria patients, IL-1α distribution is markedly more dispersed than is observed for IL-1β.

The possible correlation of IL-1α or IL-1β elevation with pyuria is shown in Table 3. A striking finding is that IL-1α is elevated in some patients whose urine samples are negative for leukocyte presence. The data do not consider possible patient heterogeneity which may underlie the sample. The data suggest that IL-1β is more intimately associated with leukocyte-mediated inflammation while IL-1α may be associated with organic changes in the bladder itself.

Analysis of bacterial infection during pregnancy is shown in Table 4. The results indicate that IL-1α is elevated due to pregnancy alone, whereas IL-1β elevation approaches significance. The presence of infection however may be difficult to predict based on cytokine elevation alone, since in gravid women further elevation due to positive urine culture is statistically insignificant relative to the pregnant, culture-negative group.

The IL-1α/Cr value for the non-pregnant culture-positive group is different from that shown for the bacterial cystitis group of Figure 1. The reasons for this may include (a) the use of the creatinine ratio which can

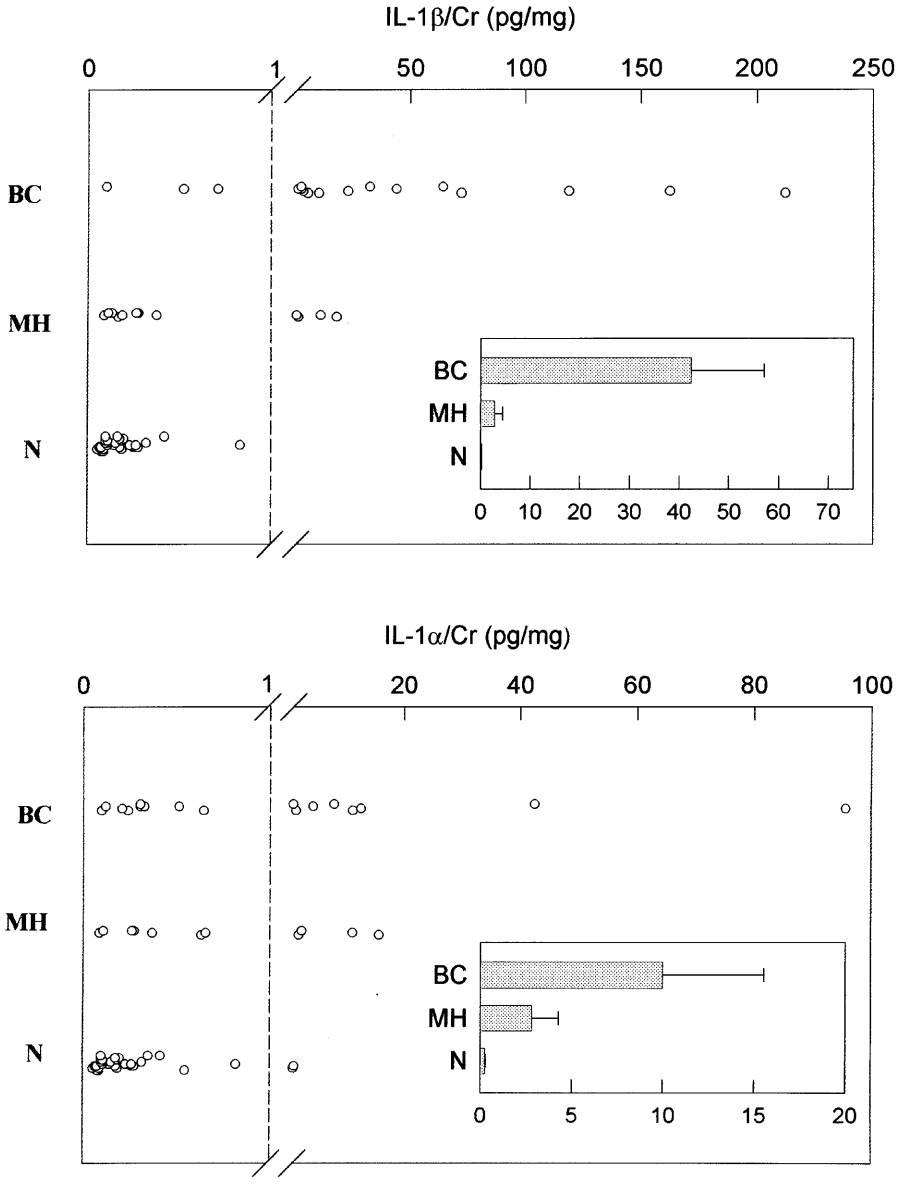

Fig. 1 Urinary cytokine levels in normal (*N*), microscopic hematuria (*MH*) and bacterial cystitis (*BC*). Data are grouped according to clinical status and are presented as the ratio of IL-1α and IL-1β to creatinine. The *dashed lines* indicate the cutoff point distinguishing IL-1-positive values from IL-1-negative values. It was determined as the point at which sensitivity and specificity of the assay are optimal, and is close to the observed upper limit of the normal range. The inset indicates the mean ± SEM of the observed values for each group. The hematuria group had significantly higher IL-1α/CR ratio compared with normal ($n = 12$, 2.81 ± 1.48 vs $n = 31$, 0.25 ± 0.05 respectively, $P = 0.007$) and the bacterial cystitis group was also significantly higher ($n = 18$, 9.97 ± 5.58, $P = 0.026$). Likewise the hematuria group had a significantly higher IL-1β/CR ratio compared with normal (2.82 ± 1.68 vs 0.17 ± 0.02 respectively, $P = 0.014$) and the bacterial cystitis group was also significantly higher (42.45 ± 14.70, $P = 0.0004$). *P* values were determined using Student's *t*-test

occasionally provide outliers, IL-1α/Cr ratio being more sensitive to outliers than IL-1α values expressed alone, and (b) small and different patient populations, the data being derived from different clinics.

Discussion

Our study has addressed several aspects of the evaluation of IL-1α methodology in urine. Centrifugation is an adequate method for separation of debris from urine prior to evaluation using an EIA. By contrast, filtration, which has been extensively used for other cytokines, is not acceptable for IL-1α because all of this cytokine is lost during the filtration process. The etiology of this loss is not clear, but probably involves the non-specific adherence of the cytokine to the filter. Such binding has been noted for a number of hydrophobic hormones and cytokines. Use of a bacteriostatic agent needs to be considered when urine samples have to be stored for long periods of time. The agent of choice is sodium azide. Our studies show that while sodium azide can be used, its concentration has to be maintained below 0.1%. Above these levels sodium azide interferes with detection of IL-1α levels in the urine.

The excellent stability of IL-1α in urine was a pleasant surprise. The assumption that urine adversely affects the stability of all cytokines seems to be incorrect. For a period of up to 17 weeks at −70°C, IL-1α was measurable with minimal losses. We have previously found that both tumor necrosis factor (TNFα) and IL-1β are similarly stable in urine (unpublished data). The stability of these cytokines in urine allows the use of the EIA assays in a cost-effective fashion.

The theoretical potential for loss of assay specificity arising from cross-reactivity of the antibodies was also

addressed. There is no cross-reactivity detectable between the IL-1α and IL-1β antibodies. The standardization of the cytokine methodology in this paper and previous studies [4, 17] should contribute to a wider use of these assays in the urology laboratory.

Our study calls into question many of the traditional views of IL-1α's role and source in bacterial infections of the bladder. We have found that the simultaneous elevations of both IL-1α and IL-1β in bacterial cystitis patients are statistically significant. These results are surprising in light of the prevailing view that IL-1α is membrane associated while IL-1β is a secretory cytokine. Based on this hypothesis, one would expect markedly different levels of IL-1α and IL-1β in the urine of bacterial cystitis patients from those we report. In addition, IL-1α is elevated in gravid women with and without bacterial cystitis, whereas IL-1β approaches statistical significance in gravid women in the absence of cystitis. These results suggest that IL-1α should be considered along with IL-1β as a marker of potential clinical utility. The view that IL-1α is membrane-associated and thus not a candidate for secretion, may have to be modified in the future. A possible explanation for the elevation of IL-1α in the urine may relate to the concept of post-translational processing of IL-1α. Another possibility is that IL-1α may be released from the plasma membrane during cell death or via some other specialized process. Still another possible explanation is that IL-1α is directly secreted by, and acts on bladder cells rather than leukocytes. This theory is supported by the difference between IL-1α and IL-1β levels and their correlation with pyuria during bacterial infection. While IL-1β clearly correlates with leukocyte levels, IL-1α does not. Recent results show that IL-1α is increased in response to LPS treatment of bladder cancer cells, and may in part act as a hormone modulating growth and secretion in bladder cancer cell lines [9]. The question of whether IL-1α plays an important cellular role in bladder cell lines needs to be addressed.

Other recent studies have also demonstrated unexpected roles for IL-1α. IL-1α and TNFα have been shown to be released from BC31ad cell lines and are autocrine or paracrine factors in bladder cells [14]. IL-1α is a potent regulator of epidermal growth factor receptor expression in bladder cell lines RT4 and RT112 [1]. Once again, the lack of correlation with leukocytes may imply that IL-1α is at least in part, produced from stimulated bladder epithelium rather than the leukocyte population. If this is the case, then the simultaneous study of IL-1β and IL-1α may provide a marker set of additive value with potential clinical applications.

It is interesting to ask whether cytokines are elevated in patients with microscopic hematuria with negative urine cultures. We have previously shown that only IL-1β is significantly elevated in patients with microscopic hematuria [17]. Here, in a larger series, we show that IL-1α is elevated to statistically significant levels, while IL-1β may be elevated in a subset of these patients. Certainly the elevation of IL-1β in bacterial cystitis patients is far greater than that in the culture negative patients with microscopic hematuria. The significance of these studies is obscured by the fact that the origin of the microscopic hematuria is unknown at this time. We have previously shown that urinary IL-1β is elevated in many urologic conditions associated with microscopic hematuria including benign prostatic hypertrophy, pelvic prolapse, and renal cysts [4]. IL-6 has been shown to be elevated in pyelonephritis and bacterial cystitis, and is directly released from epithelial cells in response to infection [10–12, 19] and in acute phase responses [13]. The subtypes of various pathologies and their relationship to IL-1α elevation in urine is being actively studied.

Studies of the immune system during pregnancy have indicated that cytokines can be elevated during pregnancy. Our studies demonstrate that IL-1α as well as IL-

Table 3 Correlation of pyuria with cytokine elevation

White blood cells	Elevated cytokine (%)	
	IL-1β/Cr	IL-1α/Cr
Presence	94	47
Absence	0	33
P value[a]	0.02	0.44

[a] Fisher's exact test

Table 4 Statistical analysis of IL-1α/Cr and IL-1β/Cr in pregnancy

Comparison groups	IL-1α/Cr (mean ± SEM)	IL-1β/Cr (mean ± SEM)
Pregnant culture negative ($n = 10$)	27.20 ± 13.54	41.69 ± 35.29
vs pregnant culture positive ($n = 6$)	vs 31.65 ± 16.91	vs 20.61 ± 6.96
P value	>0.05	>0.05
Non-pregnant culture negative ($n = 15$)	0.22 ± 0.66	0.17 ± 0.22
vs non-pregnant culture positive ($n = 13$)	vs 2.82 ± 1.26	vs 40.56 ± 19.10
P value	<0.05	<0.05
Non-pregnant culture negative ($n = 15$)	0.22 ± 0.66	0.17 ± 0.22
vs pregnant culture negative ($n = 10$)	vs 27.20 ± 13.54	vs 41.69 ± 35.29
P value	<0.05	>0.05
Non-pregnant culture positive ($n = 13$)	2.82 ± 1.26	40.56 ± 19.10
vs pregnant culture positive ($n = 6$)	vs 31.65 ± 16.91	vs 20.61 ± 6.96
P value	<0.05	>0.05

1β are both significantly elevated during the course of a normal pregnancy. This finding implies that IL-1α is processed and appears outside the cell. Again, the biochemical mechanisms involved are unknown. Whether IL-1α plays a role in pregnancy is an important question which merits further study. Because both IL-1α and IL-1β are sharply elevated in pregnancy, they provide high background levels, obscuring the significance of further elevations due to bacterial infection. Thus these cytokines may not be useful in identifying bacterial infections during pregnancy. Defining low grade bacterial infections with certainty during pregnancy is an important goal. Whether other cytokines may be useful in this regard remains to be determined.

These results collectively suggest that IL-1α is a promising candidate for urologic study. An obvious application would be to bladder cancer patients where the role of inflammation and immunologically derived tumor killing processes remain to be defined in the context of BCG therapy [2]. Recent studies suggest that IL-2 and TNFα may be helpful in predicting outcomes of BCG therapy [3, 5, 6, 21]. Whether IL-1α and IL-1β may be useful in separating inflammation from immunologically derived tumor cellular reactivities remains to be seen. Patients with IC are another potential study group. We have previously shown that histamine is increased, and IL-1β is not elevated in some patients with IC prior to treatment and after hydrodistension [23], but not after DMSO therapy [22]. The finding that IL-1β was not elevated in these patients may be significant in excluding low levels of bacterial cystitis. Another observation helpful in the differentiation of IC and bacterial cystitis is that glycosaminoglycan may be shed to a greater degree in IC [16]. Whether IL-1α may similarly distinguish between bacterial cystitis and IC is currently being examined. In view of our study and the work of other researchers, IL-1α is a useful urinary cytokine in research studies, and potentially one of clinical value.

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