New Method for Differentiating Chronic Prostatitis/Chronic Pelvic Pain Syndrome IIIA From IIIB Involving Seminal Macrophages and Monocytes

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OBJECTIVES To evaluate a new method for differentiating inflammatory from noninflammatory prostatitis using the simple and rapid quantification of seminal macrophages and monocytes.

METHODS Patients affected with chronic pelvic pain syndrome (CPPS) were classified as having the IIIA (n = 11) and IIIB (n = 30) subtypes according to the peroxidase positive leukocyte concentration in semen; 18 healthy individuals served as controls. Seminal inflammatory markers, including polymorphonuclear elastase, interleukin (IL)-6 and IL-8, and numbers of macrophages/monocytes (MMs) per 50 fields of 1000× magnification (high-power field [hpf]), were determined for all patients.

RESULTS The numbers of MMs/50 hpf correlated significantly with the peroxidase positive leukocyte counts and IL-8, IL-6, and polymorphonuclear elastase levels (all $P < .001$). Data from the analysis of receiver operating characteristic curves (area under the curve 0.912 ± 0.073; $P < .001$) showed a sensitivity of 90.9% and specificity of 86.7% at a cutoff value of 5 MMs/50 hpf. The positive and negative predictive value was 71.4% and 96.3%, respectively. The median concentrations of IL-6, IL-8, and elastase in the patients with CPPS with ≥5 MMs/50 hpf differed significantly ($P \leq .002$) from those in the patients with <5 MMs/50 hpf.

CONCLUSIONS The results of our study have shown that the quantification of seminal macrophages and monocytes is a simple, rapid, and reproducible technique by which to differentiate chronic prostatitis/CPPS IIIA from IIIB. UROLOGY 78: 918–923, 2011. © 2011 Elsevier Inc.

According to the consensus classification of the National Institutes of Health (NIH), chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), characterized by urologic pain or discomfort in the pelvic region in the absence of bacterial infection, is subdivided into the subtypes of category IIIA (inflammatory) and category IIIB (noninflammatory). Patients with inflammatory CP/CPPS have leukocytes in the expressed prostatic secretions (EPSs), postprostatic massage urine, or semen. In contrast, patients with noninflammatory subtypes have no evidence of inflammation.[1]

Because of the uncertain etiology of CPPS, a wide variety of therapies have been used by urologists.[2] The efficacy of these treatments has recently been reviewed using a network meta-analysis.[3] That review has shown that the patients with predominant voiding dysfunction might respond best to α-blockers, those with a history of urinary tract infection might respond best to antibiotics, and those with pain/inflammation might respond best to anti-inflammatory drugs and/or gabapentinoids. Therefore, a fast method to rule out inflammation might be useful in choosing the most appropriate therapy.

Moreover, because antimicrobial treatment of chronic abacterial prostatitis, despite the absence of evidence of inflammation and the potential harm from, and side effects of, antibiotics, is common among urologists, the need for simple and clear diagnostic guidelines are desired.[2,4,5]

However, the critical cutoff values by which to differentiate inflammatory from noninflammatory prostatitis are still under discussion. Traditionally, microscopic examination of EPSs using a wet mount method uses a value of 10 white blood cells/high-power microscopic fields (hpf) to differentiate inflammatory from noninflammatory prostatitis.[6,7] However, the use of a wet mount

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technique for the detection of inflammation in EPSs is not recommended, owing to the high variability in the results and high sensitivity to volume.[8] Instead, the use of a hemocytometer, with a critical value of 500[9] or 1000[6] leukocytes/mm^3, and staining methods[8] are recommended for the accurate evaluation of the leukocytes.

The World Health Organization has set a cutoff value of $\geq 1 \times 10^6$/mL leukocytes to discriminate inflammatory and noninflammatory ejaculates.[10] Because this threshold value is not localized to the prostate gland and refers generally to "male accessory gland infection," Ludwig et al[11] has suggested a threshold value of 0.113×10^6 leukocytes/mL to discriminate CP/CPPS IIIA from IIIB. However, in a case-control study (a part of the NIH Chronic Prostatitis Cohort study), it was found that the number of white blood cells in the semen of men with CP/CPPS did not differ from that in the controls.[12]

The application of seminal inflammatory markers, such as polymorphonuclear (PMN) elastase and cytokines, including interleukin (IL)-6 and IL-8, to the differential diagnosis of CP/CPPS IIIA and IIIB has been reported.[11,13,14] A cutoff value of 280 ng/mL for PMN elastase has been suggested,[11] and IL-8 was reported to be a surrogate marker for discriminating inflammatory from noninflammatory CPPS at a seminal plasma concentration of 9645 pg/mL.[14]

Because macrophages and monocytes play an active role in the inflammatory process,[15-17] and because high levels of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) (chemokines that induce chemotaxis in monocytes and macrophages) are found in the EPSs[18] and seminal plasma[14] of patients with CP/CPPS, we hypothesized that the evaluation of these cells could be used to discriminate CP/CPPS IIIA from IIIB.

One possible advantage of this method would be the simple and rapid laboratory workup involved, because the leukocytes could be stained using routine staining methods. In contrast, the use of seminal inflammatory markers, such as elastase and cytokines in the routine diagnosis of prostatitis IIIA and IIIB would be more complex and, hence, more expensive.

The aim of the present study was therefore to establish whether a simple and rapid method of quantifying macrophages and monocytes in stained semen smears could be used to differentiate inflammatory from noninflammatory ejaculate in men with CP/CPPS. The seminal inflammatory markers, including PMN elastase, IL-6, and IL-8, were also measured to test our hypothesis.

MATERIAL AND METHODS

Study Populations and Semen Samples

The patients were recruited from men attending our andrology unit (for reasons, including an unfulfilled wish for a child and prostatitis) from February to June 2009. All patients completed the validated German version of the NIH Chronic Prostatitis Symptom Score.[19] Men with CP/CPPS were classified as having subtype IIIA or IIIB, according to the peroxidase positive leukocyte (PPL) concentrations in semen using a cutoff value of 1 million/mL.[10] Ejaculated samples from healthy men showed a good sperm density and progressive motility and morphology ($\geq$ 20%) and were considered normal ejaculates according to World Health Organization criteria.[20]

All semen samples were obtained at the hospital by masturbation, after a period of sexual abstinence of 4 days. The samples were collected in a sterile container and underwent ejaculate analysis according to World Health Organization criteria[20] after liquefaction at 37°C. Standard microbial investigations (eg, for aerobic and anaerobic bacterial infections, urea and mycoplasmal infections, and *Chlamydia trachomatis*, *Trichomonas vaginalis*, and *Candida* infections) were performed for all semen samples. Seminal plasma samples for the determination of IL-6, IL-8, and granulocyte elastase were obtained by centrifugation of the samples at 1800*g* for 8 minutes.

Leukocytes

Two different methods were used for counting leukocytes: the hemocytometer method and a stained smear technique. In the hemocytometer method, the presence of PMN granulocytes in semen samples was assessed according to the Endtz method,[21] commercially produced as Leucoscreen (FertiPro NV, Beernem, Belgium). The working solution was prepared by adding 30-μL hydrogen peroxide to 1 mL of benzidine cyanosine solution. All samples were diluted 1:10 with phosphate-buffered saline, and 50 μL of working solution was added to 50 μL of a prediluted sample. A 10-μL aliquot was then placed in a Neubauer Improved C-Chip, a disposable plastic hemocytometer (PEQLAB Biotechnologie, GmbH, Linz, Austria), and peroxidase positive cells (ie, dark brown round cells) were counted under a Reichert biovar microscope at 400× magnification.

In the stained smear technique, 10 μL of ejaculate was applied to a prestained Testsimplets slide (Diagonal, GmbH, Münster, Germany). A cover slide (24 mm × 36 mm) was placed on the sample and the slide examined after a 15-minute staining period under a Reichert biovar microscope using oil immersion to identify leukocytes at 1000× magnification. The leukocyte cell types, in particular, macrophages, monocytes, and PMN leukocytes, were distinguished by cytoplasmic and nuclear characteristics (Fig. 1). A total of 50 fields were examined, and the sum of the macrophages/monocytes (MMs) was used to determine the number of MMs/50 hpf. To increase precision and reduce the counting error, enumeration of the MMs was performed in duplicate using 2 different slides and then averaged.

PMN Elastase and Cytokine Quantification

Granulocyte elastase was determined in frozen-thawed seminal plasma samples using an enzyme-linked immunosorbent assay (Human PMN Elastase ELISA [enzyme-linked immunosorbent assay], Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. The IL-8 levels in seminal plasma were quantified using a lateral flow immunoassay designed for the semiquantitative measurement of human IL-8 (Milenia QuickLine, Milenia Biotec, GmbH, Gieβen, Germany) according to the manufacturer's instructions. The IL-6 levels were determined using a solid-phase, 2-site sequential chemiluminescent immunometric assay performed using the IMMULITE 2000 automated analyzer (Siemens AG, Vienna, Austria). The assessment of IL-6 in the seminal plasma was performed at the Institute for Medical and Chemical Laboratory Diagnostics of

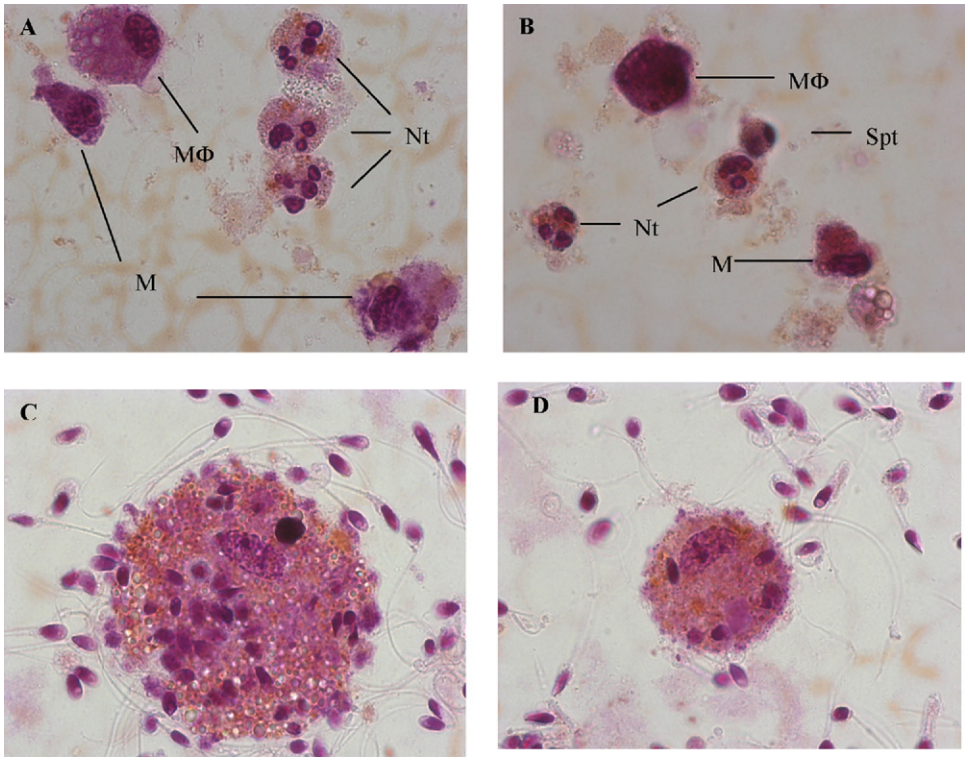

Figure 1. (A, B) Leukocytes in semen smears stained with cresyl violet acetate and new methylene blue using 1000× magnification. Neutrophils (Nt) identified by segmented nuclei and granules in cytoplasm; monocytes (M) and macrophages (MΦ) characterized by round, reniform, or irregular nuclei, usually placed eccentrically. Cytoplasm can also be spread out; spermatid (Spt). **(C, D)** Macrophages in semen smears stained with cresyl violet acetate and new methylene blue using 1000× magnification. Macrophages contained several cytoplasmic vacuoles with degenerated cellular debris. Phagocytosed sperm heads also seen in cytoplasm (color figure available online).

Krankenhaus Hietzing mit Neurologischem Zentrum Rosenhügel, either on the same day as harvest (kept at 3°-8°C) or within 5 days, after thawing from being frozen at −20°C.

Statistical Analysis

Statistical analysis was performed using Predictive Analytics SoftWare, version 18 (SAS Institute, Cary, NC). The Shapiro-Wilk test was used to verify a normal or non-normal distribution of values. Significant differences among the groups were determined using the Mann-Whitney *U* test. Statistical significance was set at $P < .05$. To determine the predictive value of MMs in the detection of inflammation in patients with CP/CPPS, the receiver operating characteristic test was used. Spearman's coefficient of correlation was calculated to detect a statistical correlation between 2 variables.

RESULTS

A total of 59 men (mean 39 years, range 22-63) were enrolled in the present study. Of the 59 men, 41 with CPPS had a mean ± standard error Chronic Prostatitis Symptom Score of 19.37 ± 1.17 and 18 healthy controls had a mean Chronic Prostatitis Symptom Score of 3.11 ± 0.82. Of the 41 men with CPPS, 11 had the CPPS IIIA (inflammatory) subtype and 30 had the IIIB (noninflammatory) subtype on the basis of the semen PPL concentrations. No patient demonstrated pathologic growth in the standard bacterial cultures of their seminal plasma.

Table 1. Correlations between MMs/50 hpf and PPL counts, cytokines, and PMN elastase for all subjects (n = 59)

Variable	Correlation Coefficient	*P* Value
PPL count (million/mL)	0.685	<.001
IL-8 (pg/mL)	0.698	<.001
IL-6 (pg/mL)	0.653	<.001
PMN elastase (ng/mL)	0.714	<.001

MMs, macrophages/monocytes; hpf, high power field; PPL, peroxidase positive leukocytes; IL, interleukin; PMN, polymorphonuclear.

Positive and statistically significant associations between the MMs/50 hpf and PPL counts and the IL-8, IL-6, and PMN elastase levels were apparent for all patients (Table 1).

To establish a cutoff value for the MM counts in the detection of inflammation in the patients with CP/CPPS, data from the analysis of the receiver operating characteristic curves (area under the curve 0.912 ± 0.073; $P < .001$) showed a sensitivity of 90.9% and specificity of 86.7% at a cutoff of 5 MMs/50 hpf (Fig. 2). The positive and negative predictive value was 71.4% and 96.3%, respectively.

To verify this cutoff value, the patients were divided into 2 groups: inflammatory (≥5 MMs/50 hpf, n = 14) and noninflammatory (<5 MMs/50 hpf, n = 27). The median concentrations of IL-6, IL-8, and PMN elastase were to

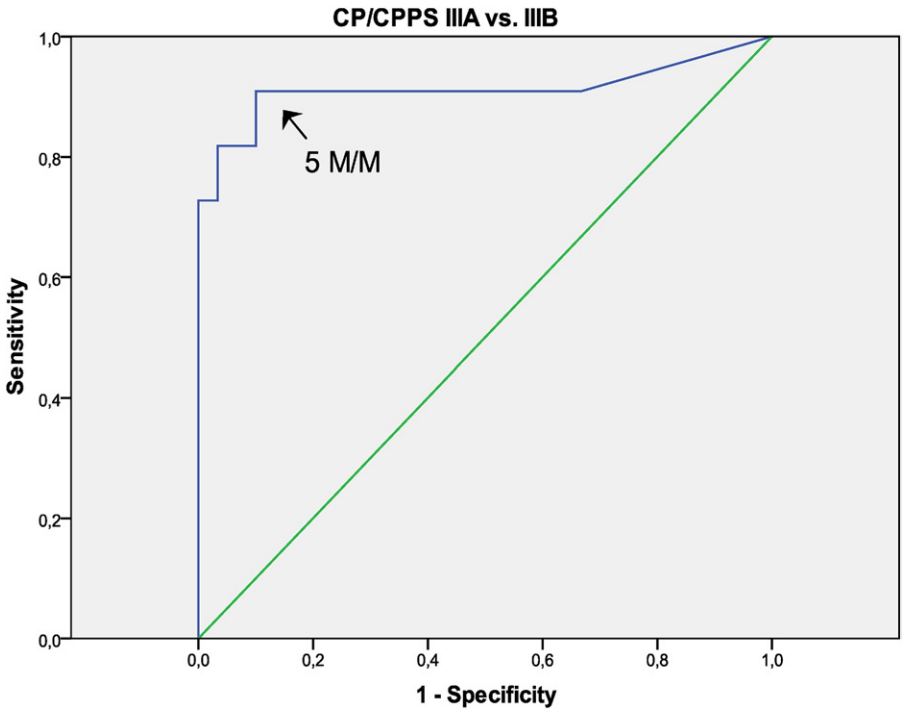

Figure 2. Receiver operating characteristic curve to determine cutoff value for MM count for detecting inflammation in patients with CP/CPPS.

Table 2. Concentrations of IL-8, IL-6, and PMN elastase in MMs <5/50 hpf and MM ≥5/50 hpf groups and control group

Variable	IL-8 (pg/mL)	IL-6 (pg/mL)	PMN Elastase (ng/mL)
Control group (n = 18)			
Median	741.50	16.8	40.72
25%-75%	465.25-1547.25	10.07-31.93	9.74-96.01
MM <5/50 hpf (n = 27)			
Median	1220	31	100
25%-75%	787-2776	14.2-61	40-375
P value versus control	.022	.69*	.01
MM ≥5/50 hpf (n = 14)			
Median	5348.5	197.5	632
25%-75%	2391.25-9987.25	65.35-420.75	467.5-957
P value versus control	<.001	<.001	<.001
P value versus MM <5/50 hpf group	.002	.002	<.001

Abbreviations as in Table 1.
* No significant difference.

differ significantly between the 2 groups and between each group and the control group, with 1 exception. The IL-6 levels in the noninflammatory group did not differ significantly from those in the control group (Table 2).

COMMENT

In the present study, a cutoff of 5 MMs/50 hpf was found to distinguish between the men with inflammatory (IIIA) and noninflammatory (IIIB) subtypes of CP/CPPS, with a sensitivity of 90.0% and a specificity of 86.7%. This finding was supported by data showing significant differences in the levels of seminal markers of inflammation between men with ≥5 MM/50 hpf and those with <5 MM/50 hpf. Hence, we suggest that the simple quantification of monocytes and macrophages in the stained smears of ejaculate could play a diagnostic role in CP/CPPS. This method could be particularly crucial for andrologic laboratories that need to establish a rapid diagnosis on the day of analysis and for those laboratories that are unable to evaluate inflammatory markers such as PMN elastase and cytokines.

Little information is available regarding the biologic significance of monocytes and macrophages in the male genital tract. It is hypothesized that these cells originate mainly from the epididymis and rete testis[22]; a role in the removal of abnormal spermatozoa from the ejaculate has also been suggested.[23] Activated macrophages have frequently been observed in the nonleukocytospermic ejaculates of infertile men.[24]

In general, monocytes and macrophages represent 20%-30% of all seminal leukocytes and, compared with

granulocytes, which represent 50%-60% of seminal leukocytes, constitute a minority in the ejaculate.[22] Counting and differentiating monocytes and macrophages in the ejaculate can be performed by immunocytochemistry using monoclonal antibodies,[10] flow cytometry,[25] or transmission electron microscopy.[26] However, these techniques are time-consuming and expensive and are usually not performed on a routine basis in an andrologic laboratory. In addition, the use of peroxidase staining, which is recommended for the counting of leukocytes, enables only the identification of PMN granulocytes and not monocytes and macrophages. However, these so-called neglected leukocytes (ie, macrophages and monocytes) can be stained and quantified in stained semen smears during routine laboratory workup.

However, it must also be considered that the use of staining techniques to differentiate leukocytes is associated with some drawbacks. It might be difficult to differentiate multinucleated spermatids in semen samples from neutrophils with a segmented nucleus[27] and lymphocytes or monocytes resemble spermatocytes.[28] Another major drawback of stained smears is the unexpected variation owing to the unequal distribution of cells.

To minimize the problems concerning unexpected variations, the counting should be performed under defined and similar conditions for all samples, using well-mixed and liquefied ejaculates, a constant volume, and applying uniform cover glasses. Because variations in magnification can also influence the interpretation of results,[8] the use of a single microscope is also advisable when counting leukocytes. To overcome problems regarding the possible misidentification of macrophages and monocytes, it is also suggested that counting be performed by a skilled technician trained in leukocyte morphology.

Elevated levels of MCP-1 and MIP-1α in the EPSs and seminal plasma of the patients with CPPS have been previously reported in 2 studies.[14,18] Although the seminal plasma levels of MIP-1α were significantly greater in the patients with CPPS IIIA than in those with CPPS IIIB in 1 study, a finding in line with our results, no statistically significant differences in the MCP-1 and MIP-1α levels of the EPSs between those with CPPS IIIA versus IIIB were seen in the other study. The association between the MCP-1 and MIP-1α levels and the presence of monocytes and macrophages in ejaculate, therefore, remains to be elucidated.

CONCLUSIONS

Our results indicate that the quantification of seminal macrophages and monocytes is an easy, rapid, and reproducible technique by which to differentiate CP/CPPS IIIA from IIIB. Because the differentiation of CP/CPPS into inflammatory and noninflammatory subtypes might have an influence on the further treatment of patients,[3,29] and because urologists rarely use the 4-glass test in the diagnosis and treatment of prostatitis,[4,5] we suggest that this simple technique could easily be incorporated into the routine workup performed by clinical laboratories, without the need for new, complex, or expensive instrumentation. However, our recommended cutoff value of 5 MMs/50 hpf should not necessarily be regarded as a reference value for other laboratories. We suggest that these laboratories should establish their own cutoff values for macrophages and monocytes.

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