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Original Article

Microbiological features of persistent nonspecific urethritis in men

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Background and Purpose: The role of opportunistic microorganisms in urethritis in men is controversial. The aim of this study was to determine the changes in urethral microflora in the presence of persistent nonspecific urethritis (PNU) in men by in vitro detection of decomplementary activity (DCA) phenotypes of urethral isolates and comparison of the isolates with those from patients with or without PNU.

Methods: The study included 18 healthy men and 24 men with PNU. Culture specimens were spread on various selective media. Bacterial DCA was tested by measuring the decrease in complement activity (CH_{50}) under the influence of culture supernatants.

Results: The most common isolates in both groups were coryneforms, coagulase-negative staphylococci, and streptococci. *Enterobacteriaceae*, enterococci, micrococci, and *Staphylococcus aureus* were isolated only from the PNU group. Simpson's diversity index (D) was significantly lower in the PNU group as compared to that in healthy men ($D = 1.37 \pm 0.4$ versus 3.5 ± 0.9, $p<0.05$). DCA of staphylococci from the PNU group was significantly higher than that of the control group (7.3 ± 1.4 versus 1.56 ± 1.05 anti-CH_{50}, $p<0.05$). DCAs of *Enterobacteriaceae* and enterococci were 16.7 ± 0.5 and 7.2 ± 2.9 anti-CH_{50}, respectively, while that of micrococci was 7.4 ± 3.2 anti-CH_{50}.

Conclusions: PNU is associated with a decrease in bacterial diversity and the occurrence of opportunistic microorganisms with pronounced pathogenic properties in the urethral microflora. These data suggest that PNU may be associated with microecological disorders.

Key words: Bacteriology; Complement system proteins; Urethra; Urethritis

Introduction

Urethritis is a common urologic condition that many clinicians find difficult to diagnose and treat effectively. Organisms such as *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and genital *Mycoplasma* spp. have been reported to cause urethritis [1]. A significant fraction of patients with urethritis, however, are not positive for these organisms [2,3]. In such cases, the clinical symptom is referred to as non-chlamydial non-gonococcal urethritis or persistent nonspecific urethritis (PNU) [2]. The role of opportunistic microorganisms in the pathogenesis of PNU is controversial [3]. Despite extensive microbiological studies, no single microbial agent has been identified as the main causative agent of PNU. One aspect that has not been investigated so far is the variability of normal urethral microflora in men with urethritis. This may be important because it is difficult to precisely establish the significance of various bacterial pathogens isolated from men with persistent urethral infection [1,3]. The definition of male urethritis in the absence of urethral inflammation has not been well established because information about the composition of the urethral flora in healthy men and men with urethritis is limited [3].

Phagocytic killing of bacteria represents the central mechanism of action against a microbial attack. The complement-dependent opsonization is a prerequisite for effective phagocytic uptake and killing of most bacteria [4]. Frick et al showed that the generation of a

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streptococcal inhibitor of complement by *Streptococcus pyogenes* strains correlates with the diagnosis of poststreptococcal glomerulonephritis [5]. However, the relationship between microbial decomplementary activity (DCA) and clinical source of PNU has not yet been addressed. The purpose of this study was to define the microbial communities present in the urethra of healthy men who had no history of sexually transmitted diseases and in patients with PNU. Here, we report the in vitro detection of DCA phenotypes of urethral isolates and their comparison with isolates from patients with or without PNU.

Methods

This study included 50 men ranging in age from 20 to 35 years. Primary clinical and bacteriological studies were carried out for urethral pathogens in all patients. *C. trachomatis* and *N. gonorrhoeae* antigens were determined in urethral specimens using the biochemical assays Chlamygen-M and Gonorgen-M (Syntron Bioresearch Inc., Carlsbad, CA, USA). *Ureaplasma urealyticum* and *Mycoplasma* spp. were cultured on selective media (A-7, bioMérieux, Marcy l'Etoile, France; Mycoplasma agar, BBL, Sparks, MD, USA). Giemsa staining of the urethral smears was done for pus cells and *T. vaginalis*. Venous blood (5 mL) was drawn for human immunodeficiency virus serological testing (enzyme-linked immunosorbent assay; Human GmbH, Wiesbaden, Germany). Positive results in these assays and the use of antibiotics in the 4 weeks preceding the study were considered as criteria for exclusion.

Based on the results of the primary study, 3 patients had used antibiotics, 4 were positive for *C. trachomatis* antigens, and 1 patient was positive for *U. urealyticum*. Thus, the study included 42 patients who were divided into 2 groups — 18 healthy men with no history and symptoms of sexually transmitted diseases and 24 men with nonspecific urethritis without symptoms of urethral inflammation.

For the cultures, specimens were spread on nutrient agar medium (Sifin, Berlin, Germany) supplemented with 10% horse serum and 5% sheep blood, Schaedler's agar with 10% sheep blood (BBL), Sabouraud's agar (Sifin), and MRS-agar (BBL) in accordance with the methods of Johnstone [6]. All the plates were incubated in 5% carbon dioxide for up to 2 days at 37°C except for Sabouraud's agar, which was left at room temperature for up to 1 week. For the anaerobic cultures, plates were incubated in GasPak anaerobic system (BBL) for up to 5 days. After incubation, the colonies were counted and subcultured for identification as described previously [7-9]. For the assessment of urethral microbiota, Simpson's diversity index (D) was used as described by Begon et al [10].

Inactivation of the complement system by microorganisms, so-called DCA, was tested by measuring the decrease in complement activity (CH_{50}) under the influence of bacterial products. Gelatin veronal buffer, complement sera of guinea pig, and antibody-sensitized sheep erythrocytes were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Agarose and trypticase soy broth (TSB) were purchased from Difco Laboratories (Detroit, MI, USA). Suspension of antibody-sensitized sheep erythrocytes in gelatin veronal buffer (approximately 1.25×10^8 cells/mL) was mixed with an equal volume of melted 2% agarose (in veronal buffer, cooled to 48°C). The agar was solidified (density of filling, 0.2 mL/cm^2) in polystyrene culture dishes (Corning Glass Works, Corning, NY, USA) and the sample wells were formed using a 4-mm-diameter borer. Prepared dishes were stored until use at 4°C. To obtain the bacterial culture supernatants, 3 mL TSB was inoculated with 0.1 mL cultures, incubated at 37°C for 24 h and then centrifuged at 3000 *g* for 20 min. Twenty microliters of culture supernatant was loaded into each well (in the control wells an equal volume of TSB was loaded). After incubation at 4°C for 3 h, 20 µL of the complement sera was added into the wells and the dishes were incubated overnight at 4°C. On the following day, the dishes were incubated at 37°C for 2 h. The diameters of the zones of hemolysis around the wells were measured. The initial CH_{50} of sera (CH_{50} per mL of sera) was determined as described by Kabat and Mayer [11]. Bacterial DCA was defined as the difference between the initial CH_{50} and the ratio of the square of the diameter of an experienced zone of hemolysis to the square of an average diameter of a control zone of hemolysis (this ratio corresponded to a part of the residual activity of complement in an experienced zone as compared with the control) and measured as anti-CH_{50}. The mean values and standard error of mean were calculated. Statistical analyses were performed using the unpaired Student's *t* test [12].

Results

All specimens yielded some bacterial growth. Anaerobes were not found in either group. The most common aerobic isolates (Table 1) were coryneforms, coagulase-negative staphylococci, and streptococci. These

Table 1. Bacterial isolates from urethra of healthy men (n =18) and patients with persistent nonspecific urethritis (PNU; n = 24)

Organism	Number of cases (%)		Bacterial count (log_{10} CFU/mL)	
	Healthy men No. (%)	PNU No. (%)	Healthy men Mean (± SE)	PNU Mean (± SE)
Staphylococcus epidermidis	4 (22.2)	9 (37.5)	5.1 (0.3)	4.0 (0.3)
Staphylococcus haemolyticus	9 (50.0)	7 (29.2)	3.2 (0.1)	3.7 (0.3)
Staphylococcus saprophyticus	8 (44.4)	0 (0)	5.1 (0.3)	-
Staphylococcus capitis	7 (38.9)	0 (0)	4.2 (0.3)	-
Staphylococcus hominis	8 (44.4)	5 (20.8)	4.3 (0.2)	6.0 (0.8)
Staphylococcus aureus	0 (0)	2 (8.3)	-	4.0 (0.5)
Corynebacterium genitalium	18 (100)	6 (25)	4.6 (0.4)	5.0 (0.5)
Corynebacterium pseudogenitalium	5 (16.6)	0 (0)	5.0 (0.5)	-
Corynebacterium equi	0 (0)	4 (16.6)	-	3.8 (0.8)
Corynebacterium seminale	0 (0)	4 (16.6)	-	4.8 (1.8)
Corynebacterium xerosis	0 (0)	2 (8.3)	-	4.5 (1.5)
Lactobacillus spp.	18 (100)	0 (0)	4.5 (0.5)	-
Enterococcus faecalis	0 (0)	4 (16.6)	-	3.7 (0.5)
Streptococcus spp.	12 (66.7)	7 (29.2)	4.3 (0.9)	4.0 (0.5)
Micrococcus spp.	0 (0)	6 (25.0)	-	5.0 (0.4)
Escherichia coli	0 (0)	5 (20.8)	-	4.0 (0.5)
Enterobacter spp.	0 (0)	2 (8.3)	-	3.0 (0.5)

Abbreviations: CFU = colony-forming units; SE = standard error

organisms occurred widely in the urethra of all patients. *Enterobacteriaceae*, enterococci, micrococci, and *Staphylococcus aureus* were not isolated from the control group. However, lactobacilli were isolated only from the urethra of healthy men. The occurrence of some spp. of Gram-positive cocci was rare in men with PNU. *Corynebacterium* spp., in general, and *Corynebacterium genitalium*, in particular, were isolated more frequently from the control group than from the PNU group, while *Corynebacterium seminale*, *Corynebacterium equi*, and *Corynebacterium xerosis* were found only in specimens obtained from patients with urethritis. We found no particular difference in the concentration of microorganisms between these groups. Specimens from men with PNU had a mean bacterial count of log_{10} 4.8 colony-forming units (CFU)/mL which was similar to that of the control group (log_{10} 4.6 CFU/mL).

The mean number of species isolated from the anterior urethra was significantly lower in men with urethritis than in the control group (2.3 ± 0.7 versus 5.7 ± 0.9, $p<0.05$). To estimate the biocenotic changes of species in urethral microbiota, we determined Simpson's diversity index for each group. As was established, Simpson's diversity index was significantly lower in the PNU group as compared to that in the control group (D = 1.37 ± 0.4 versus 3.5 ± 0.9, $p<0.05$).

The extracellular products of bacteria reduced the CH_{50} in the hemolytic system (Table 2). The organisms from the urethra of healthy men exerted DCA at 1.56 ± 1.05, 3.47 ± 2.03, and 3.56 ± 2.2 anti-CH_{50}

Table 2. Decomplementary activity (DCA) of bacterial isolates from urethra of healthy men and patients with persistent nonspecific urethritis (PNU)

No. of organisms (healthy men/PNU)	No. of strains (healthy men/PNU) with different levels of DCA (anti-CH_{50})			
	0	0.1-5.0	5.1-10.0	>10.0
Staphylococcus spp. (36/23)	24/0	12/0	0/23	0/0
Corynebacterium spp. (23/16)	4/0	18/4	1/8	0/4
Lactobacillus spp. (18/0)	ND	-	-	-
Micrococcus spp. (0/6)	-	0/4	0/1	0/1
Streptococcus spp. (12/7)	4/7	4/0	4/0	ND
Enterobacteriaceae (0/7)	-	-	-	0/7
Enterococcus faecalis (0/4)	-	0/2	0/2	-

Abbreviations: CH_{50} = complement activity; ND = not determined

for staphylococci, streptococci, and diphtheroids, respectively. DCA of lactobacilli was not found. In contrast to bacteria isolated from the control group, the strains isolated from patients with PNU showed more intensive inhibition of CH_{50} of sera. The culture supernatants of staphylococci from the PNU group decreased complement-induced hemolysis more actively than those from the control group (7.3 ± 1.4 anti-CH_{50}, $p<0.05$). DCAs of *Enterobacteriaceae* and enterococci were 16.7 ± 0.5 and 7.2 ± 2.9 anti-CH_{50}, respectively. Micrococci demonstrated DCA at 7.4 ± 3.2 anti-CH_{50}. In contrast to the control group, inhibition of CH_{50} by streptococci isolated from men with PNU was not found. DCA of diphtheroids from men with PNU was not significantly different from that of corynebacteria strains isolated from the anterior urethra of healthy men (7.3 ± 2.7 anti-CH_{50}, $p>0.05$).

Discussion

Our results suggest that the greater part of the persistent urethral infection is due to bacteria usually considered normal constituents of the urethra and hence often disregarded and discarded as "contaminants" in the routine laboratory [1]. It is shown that in healthy men, the urethral microflora is characterized by Gram-positive microorganisms. Lactobacilli, coagulase-negative staphylococci, and streptococci have been reported as part of the normal urethral flora [1,2]. The high occurrence of diphtheroids in the urethra of healthy men in contrast to men with PNU suggests the participation of these microorganisms along with lactobacilli in preventing invasion of the genital tract by opportunistic microorganisms [13].

Gram-positive organisms did constitute major bacterial flora of urethra in both groups, but *C. seminale* and *S. aureus* were detected only in the PNU group. The role of these pathogens in urethritis has been suggested by some workers [2,9]. Fecal microorganisms, such as enterococci and enterobacteria, were isolated only from the urethra of men with PNU. Our results suggest that the normal urethral microflora (lactobacilli, diphtheroids, etc.) was replaced by other organisms with pronounced pathogenic properties in men with persistent infection of the urethra. Presumably, PNU results from the synergistic interaction of different bacterial pathogens.

The pronounced ability of opportunistic microorganisms to inhibit CH_{50} may contribute to the formation of seroresistance of different pathogens that may result in disseminated infections, especially in immunocompromised patients [14]. On the other hand, it is possible that the inactivation of complement by pathogenic strains may reduce the effectiveness of the interaction of bacterial pathogens with mononuclear phagocytes via opsonization [4]. If this hypothesis is correct, it would suggest that organisms are capable of pronounced inhibition of CH_{50}; further, prolonged persistence in the urethra may alter the antimicrobial host defense following the development of local immunodeficiency of the genital tract in the presence of the chronic infections, as demonstrated for infected pleural effusions [15]. In contrast to pathogenic bacteria, normal microflora had low levels of DCA. Hypothetically, the constituents of normal flora must exhibit basal levels of resistance to the antimicrobial host defense factors. It is possible that low levels of inactivation of CH_{50} by normal organisms are sufficient to protect them from complement-dependent killing, thus providing stability of urethral microflora.

Concurrently, the properties of normal flora could likely alter in the presence of PNU. The constituents of normal microflora are capable of causing diseases, as has been shown for coagulase-negative staphylococci [16]. On the other hand, the change in properties of normal bacteria reduces their ability to provide microbial defense against genital colonization by pathogens [13].

Thus, we have shown that PNU is associated with decreased bacterial diversity and the occurrence of opportunistic microorganisms with pronounced pathogenic properties. The data obtained in this study point to the occurrence of microecological disorders in the urethral microbiota in PNU. However, the pathogenesis of these disorders and the mechanisms by which microorganisms produce the symptoms of PNU are not yet clear.

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