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Role of gram-positive bacteria in chronic pelvic pain syndrome (CPPS).

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Abstract

Introduction—Chronic pelvic pain syndrome (CPPS) is a complex disorder that affects a large proportion of all men. A limited understanding of its etiology and pathogenesis is reflected by the absence of effective therapies. Although CPPS is deemed clinically non-infectious with no well-defined etiological role for microbes, bacteria is readily isolated from both healthy and patient prostate secretion and urine samples. Our laboratory has previously demonstrated that a specific gram-negative bacterial isolate can induce CPPS-like symptoms in mice. Here we aimed to expand on these findings examining the role of gram-positive patient-derived bacteria in CPPS.

Methods—A retrospective analysis of bacterial cultures from CPPS patients from a single center was performed. Gram-positive bacteria were isolated from the expressed prostatic secretion (EPS) of three CPPS-patients (pain inducers, PI) and one from a healthy volunteer (non-pain inducer, NPI). These bacteria were inoculated intra-urethrally in two mouse backgrounds and analyzed for their ability to induce tactile allodynia, voiding dysfunction and colonize the murine prostate. Host immune responses to bacterial instillation were analyzed by flow cytometry.

Results—PI strains (*Staphylococcus haemolyticus* 2551, *Enterococcus faecalis* 427 and *Staphylococcus epidermidis* 7244) induced and maintained tactile allodynia responses (200% increase above baseline) for 28 days in NOD/ShiLtJ mice. Conversely the healthy subject derived strain (*Staphylococcus epidermidis* NPI) demonstrated no significant pelvic allodynia induction. Intra-urethral inoculation of the four bacterial strains into C57BL/6 mice did not induce significant increases in pain responses. Infected NOD/ShiLtJ displayed significant voiding dysfunction compared to their control counterparts. Colony counts of prostate tissues from both NOD/ShiLtJ and C57BL/6 mice at day 28 demonstrated that bacterial strains colonized equally well, including NPI. We also determined that mechanistically, the patient-isolates induced prostate inflammation specifically involving T-cells and monocytes.

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Conclusions—Gram-positive isolates from CPPS patients showed enhanced ability to induce tactile allodynia compared to a single taxonomically similar gram-positive strain isolated from a healthy control. Responses were shown to be dependent on host genetic background and not on colonization differences between strains.

Keywords

CPPS; Pain; Infection; Prostatitis; Gram-positive

1. Introduction

Prostatitis accounts for approximately two million outpatient visits each year in the U.S. alone(1), with a prevalence of 8.2%(2) and a cost of care of over \$4,000 each year per patient(3). Patients with prostatitis symptoms are currently stratified into one of four categories: I acute bacterial prostatitis (ABP), II chronic bacterial prostatitis (CBP), III chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), or IV asymptomatic inflammatory prostatitis (AIP)(4). CPPS is the most common form accounting for more than 90% of all diagnoses and is thought to be non-infectious, with or without appreciable numbers (10 per high powered field) of leukocytes in the expressed prostatic secretion (EPS) of patients. The syndrome clinically manifests as chronic pain in the pelvic and genital regions extending occasionally into the abdomen. Lower urinary tract dysfunction and decreased quality of life are also clinical impacts reported by patients(1).

CPPS is nominally deemed non-infectious despite the isolation of small numbers of pathogenic and non-pathogenic bacteria from EPS and urine samples of both patients and healthy men(5–7). Uro-pathogenic bacteria in prostatitis are predominantly gram-negatives such as *Escherichia coli*, with the occasional exception of pathogenic gram positives such as *Enterococcus faecalis* and *Staphylococcus aureus*. (7) Interestingly, most other gram-positive bacteria (8–14) are currently deemed asymptomatic, clinically insignificant and transient colonizers of the lower urinary tract in patients (15). However, appreciable levels of clinical benefit have been observed following fluoroquinolone antimicrobial treatment in prostatitis patients with either gram-negative or gram-positive bacteria (13). This has been assumed to be through mechanisms such as cytokine blocking, proinflammatory side effects of the class of antibiotic used rather than through bacterial clearance(16).

Our laboratory has previously demonstrated the ability of a gram-negative *E.coli* strain, termed CP-1, to recapitulate CPPS symptoms in mice(17). These symptoms remain long after bacterial clearance suggesting that gram-negative bacteria may potentiate emergence of symptoms that persist in the absence of colonization. Gram-positive bacteria are much more readily isolated from CPPS patients and healthy controls than their gram-negative counterparts. Here, we analyzed whether examples of commonly gram-positive bacterial isolates from EPS and VB3 samples of CPPS patients could recapitulate patient symptoms in mice and thus merit greater consideration in the etiology and pathogenesis of CPPS. Furthermore, we compared the effects of these “pathogenic” strains to a “commensal” strain isolated from a healthy control, termed NPI (non-pain inducing). We have previously shown that this strain does not induce pain despite colonization in a murine model(18) of CPPS.

2. Materials and Methods

2.1 Clinical Patient Data, Culture Collection and Bacterial Isolation

Retrospective analyses of patient information were conducted with approval of the Institutional Review Board (IRB) at Northwestern University, Northwestern Memorial hospital and the Enterprise Data Warehouse (EDW) under study number STU00046489. As the study was a retrospective analysis of patient bacterial samples a waiver of consent was approved under section 45CFR46.116d(1–4). All analyses of patient samples and data were performed in accordance with these regulations. All patients seen by a University urologist and who had EPS and VB3 cultures between 05/01/2010 and 07/21/2014 with a CPPS diagnosis were included in this study. Either the Meares-Stamey 4-glass test, with collection of pre-prostatic massage voided bladder urines (VB1 and VB2), followed by expressed prostatic secretion (EPS) with prostatic massage, and post-prostatic massage voided urine (VB3), or the 2-glass test (VB2 and VB3 only), which has strong concordance with the 4-glass test (19) were performed. Repeat patients with multiple cultures were analyzed using their original culture, unless repeat culture identified positive localization of a new strain, which was subsequently counted as a unique sample. Bacterial localizations were considered localized specifically to the prostate if bacteria were 1-log greater in the EPS/VB3 than that in the VB1 and VB2 (4-glass), or if bacteria were 1-log greater in the VB3 than the VB2 (2-glass).

2.2 Gentamicin protection assay.

Bacterial strains were grown in Luria Broth (LB) overnight at 37°C with shaking, followed by a static overnight subculture at 37°C. Bacteria were concentrated to an OD420 value of 1.0 ± 0.01 in sterile PBS giving a concentration of 2×10^{10} bacteria/ml. RWPE-1 normal human prostate epithelial cells (ATCC® CRL-11609™) were seeded at a density of 2.5×10^5 cells per well of a 6-well plate and grown overnight in K-SFM media with supplements (Gibco, 17005–042). Prepared bacteria were then added at MOI 10. Inoculated cells were incubated for 2 hours at 37°C, 5% CO₂ followed by four washes in sterile PBS. K-SFM media containing 50ug/ml of gentamicin was added and cells were returned to incubator for 30mins (Adherence) or overnight (Invasion). Following this cells were lysed in 0.5% Trypsin containing 0.1% Triton-X, serially diluted and plated on LB Agar. Cultures were grown overnight at 37°C, following which colonies were counted and colony forming units (CFU) calculated as colonies/ml.

2.3 Growth Kinetics

Bacterial strains were streaked from glycerol stocks onto LB agar and grown overnight at 37°C. One colony was picked and inoculated into 10ml of LB and grown overnight with shaking at 37°C. 5ml of this culture was inoculated into 250ml of LB and grown at 37°C with shaking. 1ml samples were measured at OD420 and OD600 every hour for 8 hours.

2.4 Animal models

5–7 week old NOD/ShiLtJ (NOD) and C57BL/6 (B6) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animal experiments and procedures were approved by the

Northwestern University Institutional Animal Care and Use Committee (IACUC). Northwestern University has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (A3283–01). Northwestern University conducts its reviews in accordance with United States Public Health Service (USPHS) regulations and applicable federal and local laws. The composition of the IACUC meets the requirements of the USPHS policy and the Animal Welfare Act Regulations. All mice procedures and protocols were performed in accordance with these regulations. Mice were randomized between groups pre-bacterial infection, and all investigators were blinded to experimental groups in all *in vivo* experiments and all subsequent assays.

2.5 Intra-urethral infections

As previously described, for *in vivo* experiments, bacteria were grown in LB overnight shaking at 37°C, followed by overnight static subculture at 37°C. The next day, bacteria were concentrated at OD420 value of 1 ± 0.01 in PBS, (2×10^{10} bacterial/ml) and 10 μ l (2×10^8 bacteria) was injected intra-urethrally into isoflurane-anesthetized mice.

2.6 Bacterial Colonization

Whole prostates, combining all lobes, and bladders were dissected under sterile conditions from euthanized mice. Tissues were minced and dissociated by shaking for 2 hours at 37°C in a 0.4 μ m filtered solution of 1 mg/ml collagenase D (Roche), 10 mM HEPES (Mediatech), and 0.01% DNase I (Sigma) in RPMI 1640 (Mediatech). Digestions were subsequently filtered through a 40 μ m nylon mesh, and single cell suspensions were plated on LB agar plates at multiple dilutions in technical duplicates overnight at 37°C.

2.7 Von Frey Behavioral Testing

Mice were placed in a Plexiglas chamber (6 $\times$ 10 $\times$ 12 cm) with a wire grid floor, as described(20). Von Frey filaments with forces of 0.04, 0.16, 0.4, 1, and 4 g (Stoelting)(21) were applied to suprapubic loci for 1–2 sec 10 times, with 5 sec between applications. Positive responses of tactile allodynia due to referred visceral hyperalgesia included jumping, retraction of the abdomen, and/or immediate licking or scratching of the stimulated area. Pain testing was performed on days 0, 7, 14, 21, and 28 post-infection.

2.8 Voiding Function Testing

Utilizing the Voided Stain On Paper (VSOP) method(22), mice were placed in individual empty cages lined with filter paper for 4 hours on days –3, 5, 12, 19, and 26 post-infection. UV imaged filter papers were analyzed by ImageJ for spot counts and volumes.

2.9 Flow Cytometry

Single cell suspensions were generated from whole prostate tissues combining all prostate lobes (as above in bacterial colonization) and iliac lymph node tissues by homogenization and filtration. Cells were washed in FACS buffer (2% FCS (Hyclone) in PBS (Gibco)) and staining performed using conjugated antibodies: PerCp-CD4, Alexa-488-CD4, and PE-IL17A (Biolegend), and PC-CD11b, FITC-CD273, PE-CD274, CD3, CD8, and IL4 (eBioscience). Samples were run on an Accuri-benchtop-C6 cytometer and analyzed using

FlowJo™. For all analyses, unless otherwise stated, samples were gated on cell populations based on lymphocyte or monocyte size, as assessed by SSC and FSC, followed by gating for CD11b, CD3, or CD4. Intracellular staining was performed using IC fixation buffer (eBioscience) and Permeabilization Buffer (eBioscience). Staining was performed for 1 hour at 25°C followed by washing in FACS buffer.

2.10 Statistical analyses

Statistical analyses were performed using GraphPad Prism™. Statistical tests utilized, technical replicates, biological replicates, independent repeat experiments performed, and murine *n* values are indicated in figure legends. All Student's *t*-tests were unpaired and two-tailed, one-way Analysis of Variance (ANOVA) tests included post-hoc Tukey, and two-way ANOVA tests included post-hoc Dunnett. Data are represented as the mean ± standard deviation (SD) or mean ± standard error of the mean (S.E.M.) as appropriate, with $P < 0.05$ signifying statistical significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3. Results

3.1 Gram-positive species from CPPS patients are common and diverse

A retrospective analysis of all CPPS patient cultures from a single center identified 39 unique strains from 33 patients. Gram-positive species were the most commonly isolated strains including species of *S. epidermidis* (26%), *S. haemolyticus* (26%), and *E. faecalis* (10%) (Fig. 1a). From this group, 28 gram-positive strains were available and amenable to laboratory culture. We next attempted to model CPPS pathogenesis using these gram-positive bacterial isolates. An isolate from each of the three most common strains was selected: 7244 *S. epidermidis*, 2551 *S. haemolyticus*, and 427 *E. faecalis*. A gram-positive *S. epidermidis* strain isolated from a control subject with no history of prostate disease, termed NPI (non-pain inducing) was also included thus allowing for direct comparisons between potential pathogenic and commensal strains. A gentamicin protection assay determined that each strain can adhere to and invade RWPE-1 cells *in vitro* to an equivalent degree (Supplementary Fig. 1a) and growth kinetics also demonstrated no difference between strains (Supplementary Fig. 1b).

3.2 CPPS-derived Gram positives induce clinical symptoms in NOD but not B6 mice

Upon intra-urethral infection of NOD/ShiJ (NOD) mice with each clinical isolate, all three CPPS-derived strains induced significant tactile allodynia responses, by day 7 (Fig. 2a). Tactile allodynia was persistent for 28 days and for each Von Frey filament (Fig. 2b), mirroring the chronic nature of CPPS reported pain. NPI did not induce such responses. Spontaneous voiding spot analysis in NOD mice revealed that each CPPS isolate, but not NPI, also significantly increased the frequency of small void events (< 0.5µl) (Fig. 2c). Increased frequency of low volume voids matches the decreased capacity and increased urgency commonly reported by CPPS patients. *Ex vivo*, colony formation assays performed at day-28 post-infection from prostate and bladder tissues revealed high bacterial counts in the NOD prostate, yet no significant differences in colonization between bacteria (Fig. 2d). This indicated that these isolates are equally capable of infecting and colonizing the prostate, and that development of prostatitis symptoms was not dependent on the level of

colonization. In contrast, infection with these gram-positive pathogens in C57BL/6 (B6) mice did not induce tactile allodynia (Fig. 3a), consistent with results from the gram-negative *E. coli* murine prostatitis model(17). The NPI strain was observed to colonize B6 tissue to a greater extent than its pathogenic counterparts (Figure 3b). Decreased colonization observed for the CPPS-isolates in B6 mice compared to NOD animals (Fig. 2d, Fig. 3b), suggests that the inability of NOD mice to clear gram-positive pathogens may contribute to development of CPPS-like symptoms or that bacteria were incapable of colonizing the B6 prostate. The similar levels of NPI colonization between NOD and B6 mice highlights the strain-specific induction of CPPS in NOD animals and the commensal nature of the NPI strain.

3.3 Immune profiling of prostate and iliac lymph node tissues upon Gram positives infection.

The host adaptive immune response, to gram-positive infection was measured by immunophenotyping using flow cytometry. Differential cellular responses between individual bacterial strains was analyzed and posthoc analysis of pooled data between pain inducing bacterial strains and the non-pain strain was performed. We performed the analysis by comparing both quantification of the absolute number of cells between samples (Fig. 4) and also by the proportions of cell numbers per sample (Supp. Fig. 2). In whole prostate tissue single cell suspensions (Fig. 4a–i), we observed increased infiltration of CD3⁺CD8⁺, CD3⁺CD4⁺ and CD4⁺ IFN γ expressing lymphocytes in *S. haemolyticus* 2551 infected animals compared to controls, but this significant increase was not observed with the combined pain-inducing (PI) group (*E. faecalis* 427, *S. epidermidis* 7244, *S. haemolyticus* 2551) (Fig. 4b, c).

Further, CD4⁺ IL4 expressing cells trended towards an increase in all bacteria infected mice when individually (reached overall ANOVA significance) compared to controls and reached specific significance in pooled analysis for the NPI group compared to controls (Fig. 4f). This suggests that this may be a non-specific feature of bacterial colonization rather than a mechanism driving symptoms. Proportional analyses revealed an increase in CD4⁺ IFN γ expressing cells for both the PI and NPI groups compared to controls, (Supp. Fig. 2 f). Most notably, we observed increased infiltration of CD11b⁺, CD11b⁺ CD273⁺ and CD11b⁺ CD274 expressing myeloid cells in the NPI animals in both the individual and pooled analyses (Fig. 4g–i). Significant differences were also observed between the NPI and PI groups for CD11b⁺ and CD11b⁺CD273⁺ cells, these data confirm our previous observations that NPI induces expression of CD273 in the prostate to potentially mediate tissue specific immune regulation (Fig 4g–i).

In the prostate-draining (iliac) lymph nodes (Fig. 4j–r), we observed increased infiltration of total T cells, CD3⁺CD8⁺ and CD3⁺CD4⁺ lymphocytes with both pathogenic pelvic pain-inducing and commensal bacterial colonization (Fig. 4 j, k). On the contrary, CD3⁺CD4⁺ lymphocytes were significantly increased in *S. haemolyticus* 2551, *S. epidermidis* 7244, and the PI group, but not with the no pain group, compared to control in the proportional analyses, signifying a difference observed by analyses method rather than contributing to pathogenicity (Supp. Fig 2l). CD4⁺IL4⁺ expressing cells decreased in the NPI group

compared to controls (Fig. 4n), while CD11b⁺ monocytic infiltration was increased after infection with all bacteria (Fig. 4p, q) in the pooled analyses. CD11b⁺ cells expressing either CD273 or CD274 were increased only in the PI pooled group compared to controls. Gating strategies for all flow cytometry experiments are outlined in supplementary data.

4. Discussion

Gram-positive bacteria are readily found in EPS and VB3 samples from patients with CPPS, but the clinical significance of this finding is unknown. Here we demonstrated that certain strains of these gram-positives could accurately recapitulate, in mice, the symptoms reported in the patients from whom they had been isolated. Furthermore, in keeping with previous reports from our laboratory with a gram-negative *E. coli* pathogen(17), the symptoms were only observed in the specific genetic background of the NOD mouse. NOD mice have underlying genetic defects in immune response with reduced regulation and enhanced presence of autoreactive T cells(23). It is only within this context, that pathogenic bacterial instillation triggers development of CPPS-like symptoms. Interestingly, these results suggest that despite the very different etiological triggers that underlie the symptoms in the murine model, the underlying pathogenesis may indeed be generalized but may involve a bacterial initiating factor. We hypothesize that similarly diverse etiologies may underlie the heterogeneity observed in CPPS. This is further exemplified by our observation that prostatic colonization of these microbes persists up to experimental end-point, a finding that distinguishes this gram-positive induced CPPS model from our previous CP-1 model.

In probing for immune mechanisms that may specifically underlie the ability to elicit symptoms in the murine model, our results did not reveal any specific CD3⁺ T-cell subset or cytokine expression pattern that was significantly associated with pain pathogenesis. Rather, an overall increase in lymphocytes in the lymph nodes and monocytes in the prostate tissue was observed for both PI and NPI strains. The higher numbers of CD273⁺ cells in the prostate tissue of NPI infected mice suggests that this bacterial strain also induces a compensatory measure to regulate expanded T-cells numbers locally. This result is consistent with our previously reported data analyzing the role of NPI instillation in the experimental autoimmune prostatitis (EAP) murine model. It was surprising that we did not identify any significant differences in CD4 T cell mechanisms previously identified as important for the induction of prostate autoimmunity in the EAP (18) and *E. coli*-induced(17) murine CPPS models. We are currently investigating additional mechanisms for the emergence of pain upon gram +ve bacterial infection, including direct bacterial: neural cell interaction. The data does suggest however that commensal isolates (NPI) may be both beneficial to prostate health and useful as tools to restore immune balance(18). The study is limited by the use of only a single isolate from a healthy control and this aspect will be expanded to support these findings and increase potential impact.

5. Conclusions

Taken together this limited study demonstrates a potential direct causal role for gram-positive bacteria in the pathogenesis of CPPS. These bacterial strains are commonly isolated from the men with CPPS, yet their clinical role remains largely unknown. Recapitulation of

clinical CPPS symptoms in mice upon prostate infection, suggest that these strains merit consideration for their contribution to the pathogenesis and etiology of CPPS. The results also serve to strengthen the hypothesis that the etiology of CPPS is due at least in part to dysbiosis of the prostate microflora and an underlying immunologic defect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Proportions of bacterial species from CPPS culture samples.

Proportion of all CPPS patient-isolated bacteria ($n = 39$ bacteria, 33 CPPS patients). Other gram⁺ group consists of *Staphylococcus aureus*, *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Streptococcus mitis*, *Diphtheroids*, and coagulase-negative *staphylococci* species. GBS = Group B β -hemolytic *streptococcus*.

Figure 2. Patient-derived immunogenic gram-positive bacteria induce tactile allodynia and voiding dysfunction in NOD mice.

(a, b) Pelvic tactile allodynia response frequencies, as assessed by Von Frey filament testing, in naïve (control), NPI instilled, and 7244, 2551, and 427 infected NOD mice. Pain response is depicted as (a) increased percentage response frequency above baseline every 7 days for 28 days and as (b) individual Von Frey filament responses. $n = 4-5$ mice per group, repeated with 4 independent experiments, representative experiment shown. (c) Voiding dysfunction as assessed by urine spot analysis, displayed as fold change in the frequency of small void spots ($0.5\mu\text{l}$) above baseline (day -3 post-infection), performed 3 days prior to infection/instillation and repeated every 7 days. $n = 4-5$ mice per group. (d) *In vivo* colonization of prostate and bladder tissue homogenates from naïve, NPI instilled, and 7244, 2551, and 427 infected NOD mice 28 days post-infection. (a, d) Data represented as mean $\pm$ S.E.M. Statistical significance was assessed by (a, c) two-way ANOVA and (d) one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

Figure 3. Patient-isolated gram-positive bacteria do not induce tactile allodynia in B6 mice.

(a) Pelvic tactile allodynia response frequencies, as assessed by Von Frey filament testing, in naïve, NPI instilled, and 7244, 2551, and 427 infected B6 mice. Pain response is depicted as increased percentage response frequency above baseline every 7 days for 28 days. $n = 4-5$ mice per group, repeated with 2 independent experiments, representative experiment shown. (b) *In vivo* colonization of prostate and bladder tissue homogenates from naïve, NPI instilled, and 7244, 2551, and 427 infected B6 mice 28 days post-infection. Data represented as mean $\pm$ S.E.M. Statistical significance was assessed by (a) two-way ANOVA and (b) one-way ANOVA.

and CD4⁺ cells. **(p-r)** Gated on monocyte population and CD11b⁺ cells. Data represented as mean $\pm$ S.D. Statistical significance was assessed by one-way ANOVA. ** $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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