Video Article

Measurement of Tactile Allodynia in a Murine Model of Bacterial Prostatitis

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Abstract

Uropathogenic *Escherichia coli* (UPEC) are pathogens that play an important role in urinary tract infections and bacterial prostatitis[1]. We have recently shown that UPEC have an important role in the initiation of chronic pelvic pain[2], a feature of Chronic prostatitis/Chronic pelvic pain syndrome (CP/CPPS)[3,4]. Infection of the prostate by clinically relevant UPEC can initiate and establish chronic pain through mechanisms that may involve tissue damage and the initiation of mechanisms of autoimmunity[5].

A challenge to understanding the pathogenesis of UPEC in the prostate is the relative inaccessibility of the prostate gland to manipulation. We utilized a previously described intraurethral infection method[6] to deliver a clinical strain of UPEC into male mice thereby establishing an ascending infection of the prostate. Here, we describe our protocols for standardizing the bacterial inoculum[7] as well as the procedure for catheterizing anesthetized male mice for instillation of bacteria.

CP/CPPS is primarily characterized by the presence of tactile allodynia[4]. Behavior testing was based on the concept of cutaneous hyperalgesia resulting from referred visceral pain[8-10]. An irritable focus in visceral tissues reduces cutaneous pain thresholds allowing for an exaggerated response to normally non-painful stimuli (allodynia). Application of normal force to the skin result in abnormal responses that tend to increase with the intensity of the underlying visceral pain. We describe methodology in NOD/ShiLtJ mice that utilize von Frey fibers to quantify tactile allodynia over time in response to a single infection with UPEC bacteria.

Video Link

The video component of this article can be found at https://www.jove.com/video/50158/

Protocol

1. Bacteria Preparation for Mouse Inoculation

The following must be done under aseptic conditions.

1. Take one 17 x 100 mm polypropylene tubes with caps and add 3 ml of fresh Luria broth (LB) media. Using autoclaved tips, take some frozen bacteria glycerol stock of strain CP1[2] and transfer a small quantity of the culture into the tube containing the LB media. Replace the tube cap so that oxygen is still able to enter the tube - the culture needs to grow under aerobic conditions. Place tube at 37 °C in a shaking incubator at 220 rpm overnight.
2. The next day, transfer 5 µl of overnight culture to a fresh tube with 3 ml LB media and grow under static conditions in an incubator overnight at 37 °C.
3. On day 3 transfer 40 µl of culture into a a 50 ml tube containing 40 ml LB media each. Place in a 37 °C static incubator overnight.
4. Turn on and set centrifuge to 4 °C. Once centrifuge has reached final temperature, transfer each 40 ml culture into a 40 ml Nalgene centrifuge tube.
5. Weigh tubes to balance centrifuge - adjust volume with LB media as needed. Spin tubes at 6,000 rpm for 20 min.
6. Carefully remove supernatant and gently suspend bacterial pellet in 40 ml sterile PBS.
7. Repeat step 1.5 with PBS.
8. Aspirate off supernatant and suspend bacteria in 500 µl sterile PBS. Transfer the solution into a 1.5 ml microfuge tube. Take 10 µl of suspension and dilute in 990 µl of ice-cold PBS. Use a spectrophotometer to read the $OD_{420\ nm}$ to determine the volume needed for inoculation. To determine the appropriate volume of ice-cold PBS to suspend pellet in: Take the $OD_{420\ nm}$ number (in ml) and subtract the estimated volume of the bacterial pellet [ex. $OD_{420\ nm}$ = 0.545, pellet approximately 0.075 ml. 0.545 - 0.075 = 0.470].
9. Remove 10 µl of bacterial suspension and dilute in 990 µl ice-cold PBS. Read the $OD_{420\ nm}$. The goal is to achieve an $OD_{420\ nm}$ value for the diluted suspension of 1.000 ± .010. If the number of your dilution is above this target, add more volume to the suspension and take another reading. If the number is below the .990 then spin down the suspension and repeat $OD_{420\ nm}$ reading until obtaining desired reading.

2. Animal Infection

Mice (5 to 7 weeks old, ten per group) were purchased from Jackson Laboratory (Bar Harbor, ME).

1. Mice are anesthetized by inhalation of 1-4% isoflurane in a Plexiglas chamber and are monitored until recumbent.
2. One mouse at a time is taken for instillation of bacteria by catheterization with a polyethylene (PE10) catheter attached to a modified 30 G needle of a glass Hamilton syringe (length of 1.5-2.0 cm). The catheter is inserted to the hub of the needle. The mouse is placed on its dorsal surface under anesthesia maintained using a nose cone.
3. The penis of the mouse is extruded by gentle pressure and liberal amounts of lubricant on cotton swabs is used for lubrication of the entrance to the penile urethra.
4. 10 µl of phosphate-buffered saline containing 1 x 10^8 bacteria is introduced into the urethra of anesthetized mice following catheterization.
5. Mice are maintained in an anesthetized state for 15 min in the Plexiglas chamber after bacterial introduction to allow bacterial attachment and to prevent immediate urination.
6. Mice are placed back in their cages and monitored for the next 24 hr.

3. Behavior Testing

Mice were tested prior to infection (baseline) and on days 3, 7, 14, 21 and 28 after infection. Referred hyperalgesia and tactile allodynia was tested using von Frey filaments applied to the abdomen[11,12] and the plantar region of the hind paw[13]. Testing was perfomed at a fixed time-of-day, standard methodology and single experimenter testing of all animals were employed. Blinded testing of groups was utilized to combat the limitations of behavior-based pain testing in animal models. Five individual von Frey filaments with forces of 0.04, 0.16, 0.4, 1.0 and 4.0 g (Stoelting, USA) were applied to the abdomen and frequency of withdrawal responses was calculated.

1. After a 30 min period of acclimatization, mice are placed in individual Plexiglas chambers (6 x 10 x12 cm) made in-house with a stainless steel wire grid floor.
2. Referred hyperalgesia and tactile allodynia were tested using the five von Frey filaments. Each filament was applied for 1-2 sec with an inter-stimulus interval of 5 sec for a total 10 times, and the hairs were tested in ascending order of force.
3. Each filament is applied to the lower abdominal area in the general vicinity of the prostate and care was taken to stimulate different areas within this region to avoid desensitization or "wind up" effects. Filaments were applied in increasing order of force for 1-2 sec with at least 5 sec intervals between stimulations for a total of 10 times.
4. Three types of behavior are considered a positive response to filament stimulation: 1) sharp retraction of the abdomen; 2) immediate licking or scratching of the area of filament stimulation; 3) jumping.
5. Response frequency was calculated as the percentage of positive response (out of 10, *e.g.* 5 responses of 10 = 50%) and data was reported as the mean percentage of response frequency ± SE
6. Animals with more than 25 positive total baseline responses are excluded from the study.
7. Tactile allodynia was tested on the plantar region of the hind paw using von Frey filaments with forces of 0.04, 0.16, 0.4, 1.0 and 4.0 g. The median 50% withdrawal threshold (5) was assessed using the up-down method where testing was started with 0.04 g filament applied perpendicularly to the plantar surface of the hind paw until the filament bent slightly. Filaments were tested in ascending order until a positive response was observed. A positive response to the filament was defined as either a sharp withdrawal of the paw or licking of the test paw. When a positive response was recorded the next weaker filament was applied, and if a negative response was observed, then the next stronger filament was applied.
8. The experiments and methodology described have been reviewed and approved by the Northwestern University animal care and use committee.

Representative Results

We examined NOD/ShiLtj and C57BL/6J male mice for the presence of chronic pain upon bacterial infection. Male mice (5 to 7 weeks old) were instilled with saline or bacteria into the urethra (**Figure 1**). Mechanical stimulation of the pelvic area with von Frey filaments of saline-instilled C57BL/6J mice and UPEC-infected C57BL/6J mice resulted in a response frequency that did not change during the 28-day course of the experiment (**Figure 1**). In contrast, UPEC-infected NOD mice exhibited responses to pelvic stimulation that were significantly greater and remained significantly elevated until day 28 ($P < 0.05$; **Figure 3**). Bacterial infection resulted in no changes in tactile sensitivity of the plantar region of the hind paw (data not shown).

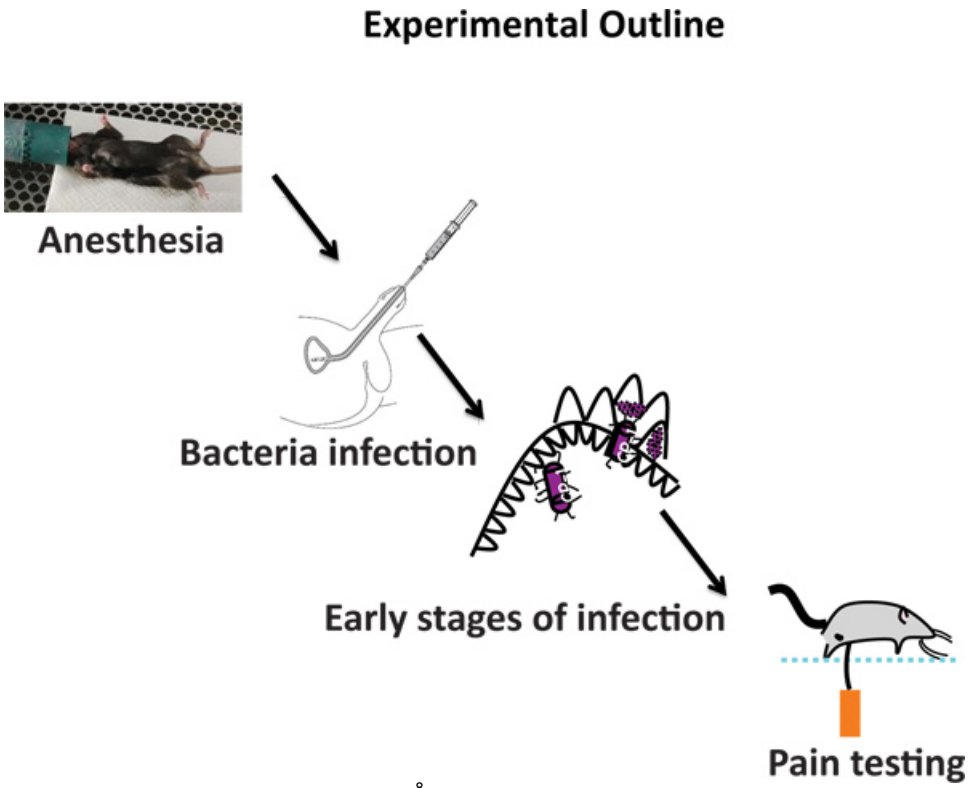

Figure 1. Experimental flow chart. 1 x 10^8 bacteria prepared by a three-day culture was suspended in PBS and instilled into the urethra of anesthetized mice by catheterization. Resulting tactile allodynia was quantified using von Frey filaments with forces of .04, .16, .4, 1, and 4 g.

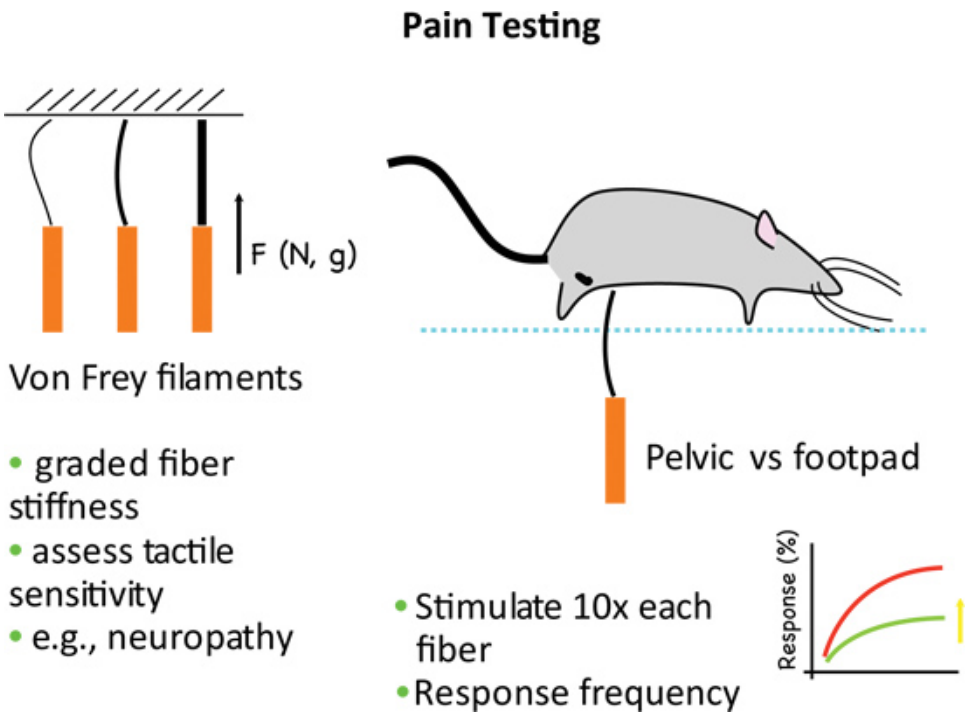

Figure 2. Methodology for testing tactile allodynia using von Frey fibers. Mice were tested before bacterial infection and at post-infection days (PIDs) 7, 14, 21, and 28. Three different types of behavior were considered as positive responses to filament stimulation: 1) sharp retraction of the abdomen; 2) immediate licking or scratching of the area of filament stimulation; or 3) jumping. Response frequently was calculated as the percentage of positive response (out of 10) and data were reported as the mean percentage of response frequency ± SE.

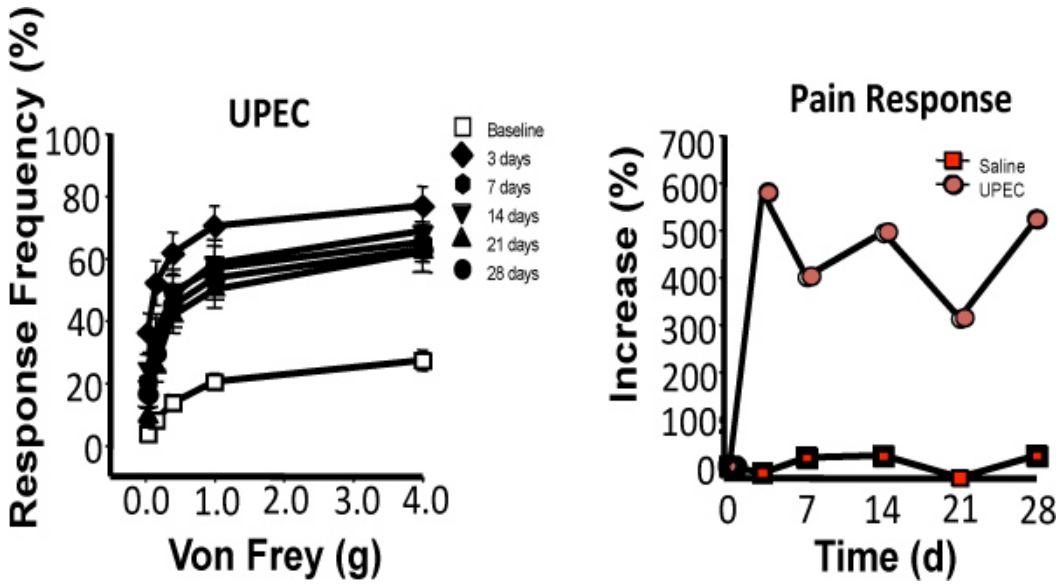

Figure 3. Tactile allodynia induced by UPEC bacteria. Referred visceral hyperalgesia was measured as responses to mechanical stimulation of the pelvic region using von Frey filaments of five calibrated forces. Data are reported as the mean percentages of positive responses ± SE before instillation of bacteria (baseline) and at PIDs 7, 14, 21, and 28. ANOVA indicated a significant increase in response frequency at PIDs 7 to 28 compared with that at the baseline for all filaments tested in UPEC-infected NOD mice ($P < 0.05$). The percent response at each PID was calculated as total responses to all fibers relative to baseline responses.

Discussion

Infection of the mouse prostate with UPEC allows for the *in vivo* modeling of events that may be involved in the pathogenesis of bacterial prostatitis, CP/CPPS or as a predisposing event in chronic inflammation. The methods described for bacterial preparation and instillation draw upon a large body of literature on UPEC models in female urinary tract infection[7,14]. The model has wide applicability for studying pathogenesis, testing potential vaccine candidates and mechanisms of immune modulation. The ability to follow pain behavior in a quantifiable manner allows for the study of infection-induced pain pathogenesis and potentially as a preclinical model for testing pain therapeutics.

There are several critical steps that determine the success of the mouse infection model. The three-day culture method with shaking and static cultures is performed for optimal expression of pili that are known to be important for UPEC attachment to epithelial cells. It represents a critical step for successful organ colonization by UPEC. The techniques for bacterial growth have been designed specifically for UPEC growth with the OD_{420} step optimized for obtaining 10 µl of phosphate-buffered saline containing 1 x 10^8 bacteria[7]. Other species or strains of bacteria potentially grow at different rates and standardization would be important to derive the appropriate O.D that gives 1 x 10^8 bacteria. Different strains of bacteria adhere to cells in the bladder and prostate in unique ways that are largely dependent on the virulence factors expressed by the strain[14]. Successful infection of the murine prostate would therefore be dependent on utilization of an UPEC strain with the appropriate complement of virulence factors. Immediately following the infection, maintaining animals in a lightly anesthetized state for a minimum of 15 min is important to ensure that instilled bacteria have time to attach and initiate pathogenesis before the normal mechanisms of urination clear the instilled volume from the urethra.

Tactile allodynia in mice is a consequence of the underlying visceral pathology and as such is characterized by exquisite host strain specificity. We have previously described that a pathogenic strain of UPEC in male NOD/ShiLtJ mice induces allodynia that peaks in 3 days and is sustained chronically whereas the same strain is incapable of inducing allodynia in C57BL/67 mice[2]. Our studies suggest UPEC-induced acceleration of autoimmune processes in NOD/ShiLtJ mice as the underlying cause. Thus the host genetic background is important in the development of tactile allodynia. Other important steps that are key to an unbiased and successful measurement of allodynia are ensuring that the tester is always blinded to the identity of the treatment, the same tester is responsible for measurement of all time-points in the experiment, utilization of similar testing times during the day, housing conditions of the mice and ensuring standardized conditions both within time-points in an experiment as well as between experiments.

The murine infection model has a number of limitations that need to be carefully considered during result interpretation. Bacteria instilled intraurethrally have the capability of infecting the bladder as well as the prostate. This complicates the interpretation of any systemic or general parameters as the underlying pathology could be from multiple organs. However, utilization of clinical and prostate derived bacterial strains as well as concurrent examination of the bladder and the prostate allows for appropriate interpretations. In addition, the methodology of infection simulates the likely ascending infection modality in human males.

The infection model described here differs from those previously reported primarily in the strain of UPEC utilized, the host mouse strain and differences in culture techniques[6] and volume of bacteria instilled into the urethra[15]. The present study uses a well-characterized prostate-derived chronic prostatitis UPEC strain to mediate disease[2]. Earlier studies have used bacteria derived from bladder infections or acute prostatitis that was utilized primarily for examining inflammation associated events but not characterized in terms of tactile allodynia[6,15]. Numerous other animal models have been reported that utilize autoimmune mechanisms[5,16], hormonal manipulation[17] and neonatal thymectomies[18] to induce prostatitis. While each of these models have some positive attributes, including organ-specific disease pathology and utilization of pre-existing techniques the relevance of these methods to the actual pathogenesis of CP/CPPS is unknown. In contrast, the methodology described in this manuscript

refers to a potential mechanism that has been postulated to be an initiator of disease pathogenesis in the prostate. In addition, beyond its utility to understanding CP/CPPS pathogenesis, the techniques could well be utilized in establishing and examining chronic inflammation of the prostate and it's role in BPH (Benign prostatic hyperplasia)and prostate cancer.

Disclosures

No conflicts of interest declared.

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