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Determination of mouse bladder inflammatory response to *E. coli* lipopolysaccharide

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Abstract Evaluation of the severity of histologic changes associated with cystitis is often subjective and inconsistent from one sample to the next. The objective of this study was to establish a consistent, reproducible method to quantify histologic changes in a mouse model of lipopolysaccharide (LPS)-induced cystitis. Either LPS ($n = 8$) or pyrogen-free saline ($n = 8$) was instilled intravesically into the bladders of female C57bk-6 J mice. Twenty-four hours later, mice in these groups as well as eight untreated controls were sacrificed and bladders were removed, fixed in formalin, and stained with hematoxylin and eosin (H&E). A bladder inflammatory index (BII) was described by reviewing tissues for edema, leukocyte infiltration, and hemorrhage. Cross-sections were evaluated by a single pathologist in a blinded manner based on the objective BII described. The BII method for objectively analyzing bladder inflammation was effective and reproducible. Bladders instilled with LPS had significantly increased inflammation scores for edema, leukocyte infiltration, and hemorrhage compared with those instilled with saline or untreated controls ($n = 8$, $P < 0.05$). These results demonstrate that LPS causes bladder inflammation when instilled intravesically and that inflammation of mouse bladders can be objectively quantified using the histological method described.

Key words Bladder · Inflammation · Histology · Lipopolysaccharide

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Introduction

Inflammation of the bladder is associated with urinary tract disorders, including urinary tract infections (UTI) and certain cases of interstitial cystitis (IC) [9, 17] Clinical symptoms often associated with bladder inflammation include increased urinary frequency, urgency, nocturia, suprapubic pressure, and pain that is generally relieved by voiding [14]. In addition evidence exists that prolonged inflammation of the bladder may lead to the development of bladder cancer, one of the most common of human cancers [4]. This may be due to the formation of highly reactive oxygen radicals in neutrophils, macrophages, and other inflammatory cells present in chronic inflammation [19]. Furthermore, chronic inflammation increases cell proliferation which is associated with malignant disease [16, 26].

Previous studies from our laboratory demonstrated that transurethral instillation of *Escherichia coli* lipopolysaccharide (LPS) into the urinary bladders of mice induces edema of the mucosa and submucosa, recruitment of polymorphonuclear (PMN) leukocytes and an increase in the release of pro-inflammatory cytokines [10]. LPS, a compound found in the outer membrane of all Gram-negative bacteria, is a complex molecule consisting of lipid A, a core polysaccharide, and a set of O-polysaccharides specific for the serotype of the bacterial cell antigen [18]. The LPS has been implicated in endotoxic shock, sepsis-induced ileus, multiple organ failure, and inflammatory bowel disease [3, 7, 12, 24]. It has also

been considered to be the causative agent in *E. coli* O157:H7 induced hemolytic uremic syndrome (HUS) and hemorrhagic colitis [21]. The LPS is also utilized in several in vivo animal models of inflammation [1, 11, 20, 25]

Stein et al. instilled LPS in conjunction with protamine sulfate into rat bladders and reported migration of neutrophils and macrophages as well as increased protease activity and permeability of the bladder urothelium [23]. Aronson et al. report bacterial-induced shedding of bladder urothelial cells believed to be associated with LPS [2]. In addition, de Man et al. report LPS-induced release of interleukin 6 from mouse bladders in vivo [6]. Despite this, the response of the mouse urinary bladder to LPS remains largely undescribed, and present methods of evaluating and quantifying bladder inflammation histologically in animal models are incomplete [5, 8, 13] . The objective of this study was to establish a standardized method of objectively analyzing bladder inflammation by histologic examination in a controlled model of LPS induced cystitis in mice.

Materials and methods

Intravesical instillation with lipopolysaccharide

Female C57bk-6J mice, 8–10 weeks old were anesthetized with ketamine HCl (40 mg/kg, i.m) and xylazine (2.5 mg/kg, i.m.). A 24-gauge polypropylene catheter was introduced transurethrally into the bladder and advanced until urine appeared. After draining urine from the bladder by applying light pressure on the abdomen, 50 µl pyrogen-free saline (0.9%), $n = 8$, or LPS (100 µg/ml) in saline, $n = 8$, was instilled into the bladder. Intravesical instillation was performed at a slow rate to avoid trauma and vesicoureteral reflux. To ensure consistent contact of substances with the bladder, infusion was repeated 30 min later. Twenty-four hours after instillation, all treated mice, in addition to eight untreated controls were euthanized with sodium pentobarbital (100 mg/kg, i.p.). The bladders were removed by a single investigator and placed in 10% formalin solution. They were then embedded in paraffin, cross-sectioned three times 40 µm apart, and the sections were stained with hematoxylin and eosin (H&E). During imbedding, bladders were oriented to ensure that cross sections were obtained perpendicular to the longitudinal axis, neck to dome.

Quantification of inflammation

Inflammation of the bladder was quantified by a single investigator in a blinded manner, using a grading scale termed the bladder inflammatory index (BII). The index evaluates bladder inflammation in regard to three criteria: edema, leukocyte infiltration, and hemorrhage.

Edema

To quantify edema, the cross-section of the bladder was divided into quadrants. The edema in each quadrant was evaluated by a scale as follows: 0 = no edema, 1 = mild edema, not expanding the width of the submucosa, 2 = moderate edema, expanding the mucosal region less than double the normal size, and 3 = severe edema, doubling the area of the mucosal region or greater. The sum of the scores of all four quadrants was divided by 12 (the maximum possible score) and multiplied by 100. This value is termed the edema score.

Leukocyte infiltrate

To quantify leukocytes, each bladder cross-section was divided into mm^2 subsections. Leukocyte infiltration [polymorphonuclear (PMN) and mononuclear cells] in each section was evaluated by a scale as follows: 0 = no leukocyte infiltrate per mm^2, 1 = mild infiltration or less than 30 leukocytes found per mm^2, 2 = moderate infiltration, i.e., between 30 and 60 leukocytes found per mm^2, and 3 = severe infiltration, or greater than 60 leukocytes present per mm^2. The sum of the scores of all subsections was divided by the total number of mm^2 subsections times 3 (maximum possible score) and multiplied by 100. This was termed the leukocyte infiltration score.

Hemorrhage

To quantify hemorrhage, each bladder cross-section was evaluated, at a magnification of 100×, for the number of visible areas of extravascular erythrocytes. Any cross-section having greater than nine areas of hemorrhage was considered confluent hemorrhage and scored 10. The number of areas of hemorrhage for each cross-section was divided by 10 (the maximum possible score) and multiplied by 100. This number was termed the hemorrhage score.

Since the bladders were cross-sectioned three times, inflammation could be quantified at three separate locations throughout the bladder. This was done because inflammation varies throughout the bladder. The individual scores for the three cross sections were averaged to obtain one score per animal. The scores of all three criteria were expressed as described in the following section.

Edema leukocyte hemorrhage scores

These three scores collectively were termed the Bladder Inflammatory Index (BII). The scores for each animal instilled with LPS were compared with scores for bladder sections from animals instilled with saline. A paired Students *t*-test was used to evaluate differences between the two groups [22]. Those *P*-values less than 0.05 were considered indicative of statistical significance.

Results

Bladder cross-sections from animals instilled with LPS had significantly greater edema scores, leukocyte scores and hemorrhage scores than those untreated or treated with saline (Fig. 1). LPS induced a BII (edema/leukocyte infiltration/ hemorrhage) average of 72/64/58 while saline induced a BII average of 15/20/10 ($P < 0.05$, $n = 8$). Untreated bladders exhibited a BII average of 15/4/4.

Saline-treated bladders exhibited no significant alterations in edema, leukocyte infiltration, or hemorrhage compared to untreated bladders. (Fig. 2a and b). None of the eight bladders instilled with saline exhibited moderate or severe edema. In addition, no bladders exhibited more than mild leukocyte infiltration in any mm^2 section and no bladders exhibited more than slight hemorrhage in two areas of a cross-section.

In contrast, severe disruption of submucosal structure was observed in bladders treated with LPS indicating severe edema (Fig. 3a and b). All bladders exhibited at least two quadrants of moderate edema, and six of the eight exhibited severe edema, as indicated by doubling of mucosal or submucosal width, in at least one quadrant. Furthermore, LPS-treated bladders

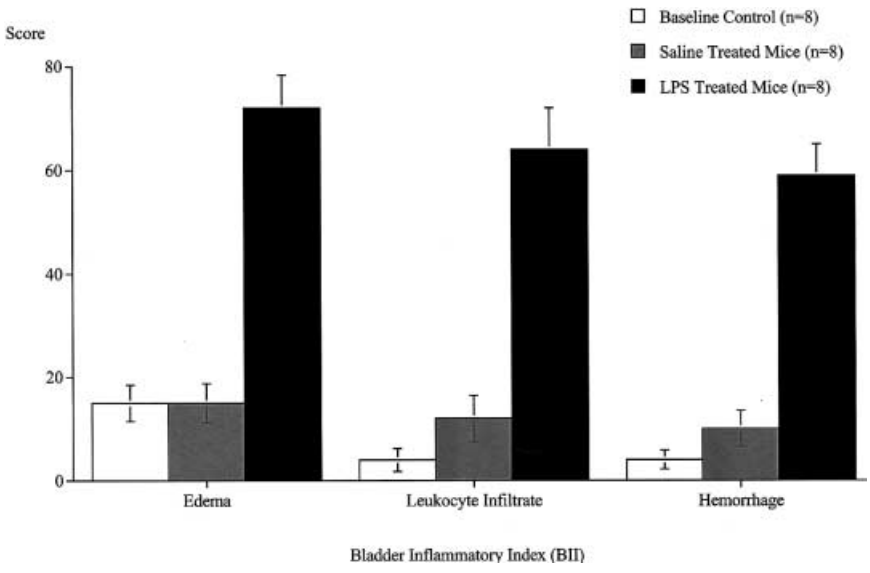

Fig. 1 Bar graph illustrating the bladder inflammatory index (BII) scores of bladders instilled with saline and lipopolysaccharide (*LPS*). The LPS induced a BII (edema/leukocyte infiltration/hemorrhage) average of 72 (±6.4)/64 (±8.5)/58 (±5.7) while saline induced a BII average of 15 (±3.8)/20 (±4.5)/10 (±3.8) ($P < 0.05$, $n = 8$). Untreated bladders exhibited a BII of 15 (±3.5)/4 (±2.2)/4 (±1.8)

exhibited significant recruitment of leukocytes (Fig. 3a and b). All bladders contained moderate infiltration in at least two mm^2 and four of the eight bladders contained at least one mm^2 of severe infiltration in which greater than 45 leukocytes were present. Both mononuclear and PMN leukocytes were present. In addition, hemorrhage was observed in all bladders instilled with LPS (Fig. 3a and b). Extravascular erythrocytes could be observed in 84% of the cross-sections in LPS-treated bladders compared to 13% for saline-treated bladders. There were no significant differences in inflammation between the three cross-sections taken within each bladder in any of the groups.

Discussion

Our results show that inflammation, induced by LPS or other agents, can be quantified objectively using the BII described. The index includes the three most quantifiable morphologic indicators of acute inflammation: edema, leukocyte infiltration, and hemorrhage. Other indicators of inflammation which are not accounted for in this index include blood vessel dilation, increase in visible capillaries, and shedding of the urothelium. Blood vessel dilation was not included because blood vessel size varies considerably from one cross-section to another, even in untreated mouse bladders, and the angle at which the vessels are sectioned can significantly affect the appearance of vessel size. Similarly, shedding of the urothelium, though often indicative of inflammation, may be biased by the angle and quality of the section.

An increased number of prominant capillaries in the lamina propria is also often indicative of inflammation. However, unpublished preliminary observations indicate that this phenomenon only occurs in the very acute phases of inflammation, 2–4 h post-instillation of LPS. Increased numbers of capillaries were visable at this time, but the response was not clear enough to allow objective quantification. Furthermore, there was no increase in edema, leukocyte infiltration or hemorrhage at this time. The response observed at 24 h was much more severe than that after 2 hours and fulfilled the requirements for developing an index of inflammation.

The establishment of a mouse BII is significant because it allows comparisons to be made among models of bladder inflammation induced by LPS or other agents. Mice provide an excellent animal model because of cost, simplicity, and the availablity of specialized strains including transgenic knockouts of major mediators of inflammation.

Gray et al., Kranc et al., and Crocitto et al. have described similar methods of evaluating bladder inflammation histologically in rats [5, 8, 13]. However, these methods use subjective analysis and do not identify objectively measurable criteria for evaluating edema and leukocyte infiltration, nor do these reports dissect the bladder three times to investigate differences in inflammation throughout the bladder. In addition, these methods do not divide the cross-sections into subsections, which further differentiates severity of inflammation giving higher scores to those bladders with more general distribution of inflammation.

Additionally, our work shows that LPS causes inflammation when instilled intravesically into the mouse bladder. The concept that LPS plays a role in the pathogenesis of inflammation in urinary tract infections of Gram-negative origin is also supported.

LPS interacts with specific membrane receptors found on mononuclear phagocytic cells and neutrophils that, upon activation, initiate a signal transduction cascade that culminates in the release of inflammatory mediators, leading to inflammation [15]. These membrane receptors include: CD14 adhesion molecules found on macrophages, monocytes, and neutrophils, the LPS membrane binding protein (LPS-MBP) receptor found on macrophages, monocytes and lymphoreticular cells, the LPS scavenger receptor found on macrophages, and

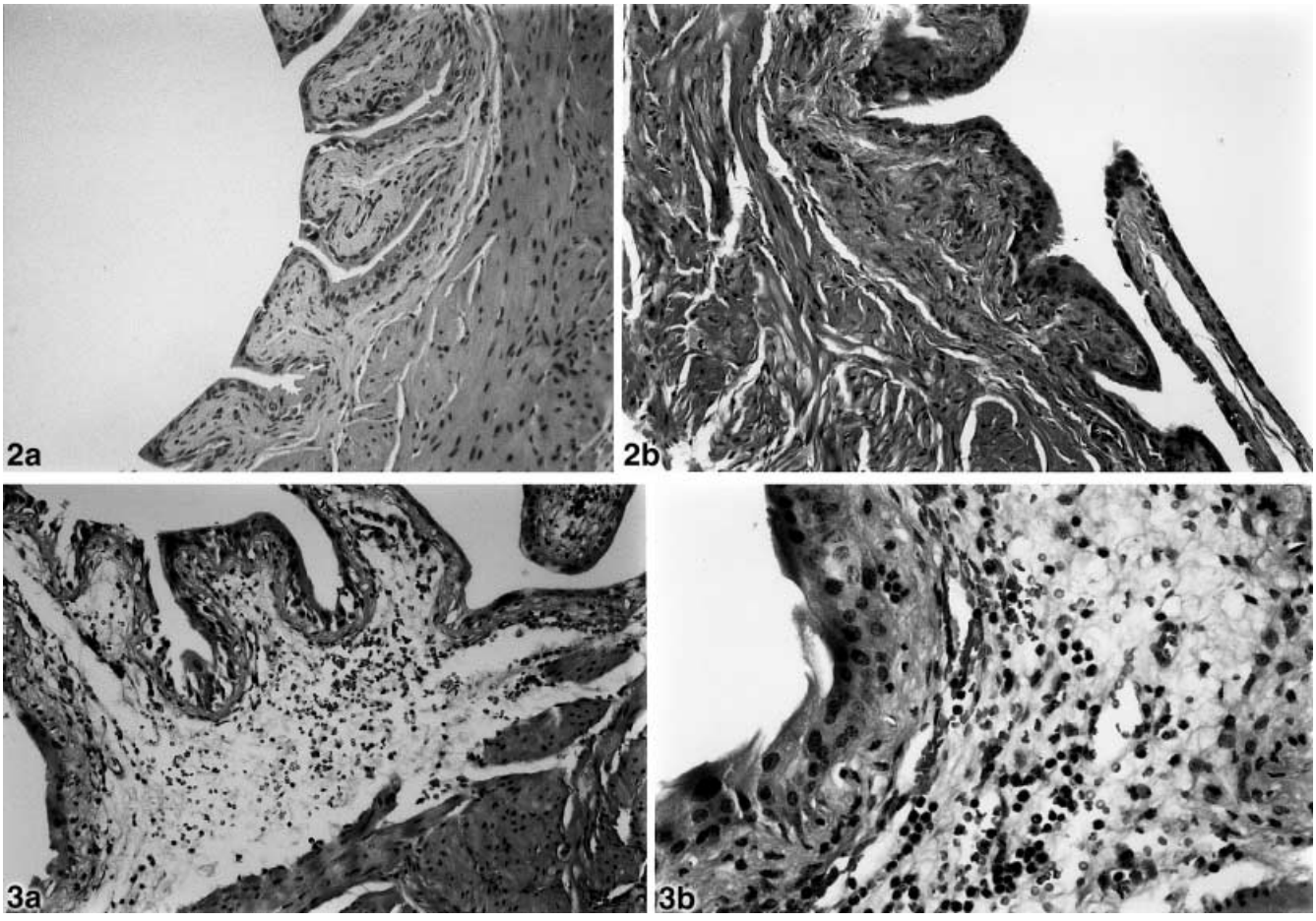

Fig. 2 **a** Normal C57bk-6 J mouse bladder urothelium, submucosa, and muscularis from an untreated mouse (H & E × 200). Note the intact nature of the structures, lack of inflammatory cell infiltrate, and lack of hemorrhage. **b** C57bk-6 J mouse bladder urothelium, submucosa, and muscularis from mouse instilled with 0.9% pyrogen free saline. (H & E ×100). Note the intact nature of the structures, lack of inflammatory cell infiltrate, and lack of hemorrhage

Fig. 3 **a** C57bk-6 J mouse bladder showing severe submucosal edema, hemorrhage and leukocyte infiltrate from a mouse instilled with 100 μg/ml *E. coli* LPS. (H & E × 100). **b** C57bk-6 J mouse bladder urothelium and submucosa from a mouse instilled with 100 μg/ml *E. coli* LPS showing severe edema, hemorrhage, and infiltrating leukocytes, most of which are polymorphonuclear (PMN) leukocytes (H & E × 200)

the CD18 glycoprotein adhesion receptor complex found on neutrophils [15]. Inflammatory mediators released or synthesized from released precursors include tumor necrosis factor-α (TNFα), interleukin-1 (IL-1), prostaglandins, leukotrienes, and platelet activating factor (PAF) [15]. These mediators, when released, cause inflammation directly through leukocyte recruitment and increased vascular permeability or by interaction with other molecules to induce inflammation indirectly [15]. Using the BII described in this study, future studies can be directed toward investigating the severity of LPS-induced cystitis by supressing various mechanisms through which LPS acts.

Conclusion

The development of the bladder inflammatory index (BII) allows assessment of bladder inflammation histologically. Subsequently, objective evaluation of bladder inflammatory mediators using LPS or other inducers of inflammation is possible. In addition, bacterial lipopolysaccharide endotoxin induces bladder inflammation when instilled intravesically into the bladder. This finding has direct implications in the study of diseases of the urinary tract in which infection and inflammation are present.

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