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# Experimental autoimmune cystitis in the Lewis rat: a potential animal model for interstitial cystitis

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**Abstract** To develop an autoimmune animal model for interstitial cystitis (IC), we injected rats with Freund's adjuvant (CFA) containing bladder homogenate (experimentals) or CFA alone (shams). We observed a doubling of urinary frequency in the experimental animals over the shams ($P = 0.004$) and histopathologic changes (venular congestion) consistent with IC. Statistically significant bladder capacity changes were not found. Mast cell (MC) number was not statistically different between experimentals and controls but the number of MCs from section to adjacent section within the same animal's bladder did vary markedly, indicating that MC counts are not a reliable measure of disease in the rat bladder. Splenocytes cultured from the experimental animals and transferred to naive syngeneic recipients were capable of transferring the urinary frequency changes and vascular congestion while splenocytes from animals which did not develop the condition were without effect. In summary, we have developed an autoimmune model for IC consistent with the clinical features of IC. The features of this model can be transferred to naive syngeneic recipients via adoptive splenocyte transfer. The model will permit us to ask and answer important questions about the pathogenesis and treatment of the human disease.

**Key words** Interstitial cystitis · Animal model · Urinary frequency · Venular congestion

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## Introduction

Interstitial cystitis (IC) is a painful, disruptive, debilitating disease of the human urinary bladder most commonly diagnosed in young to middle-aged women. While the NIH has established guidelines for defining IC for research purposes [4], the actual clinical definition of IC is highly variable. As early as 1978 Messing and Stamey [9] recognized two forms of IC – a classical form characterized by Hunner's ulcers and reduced bladder capacity and a nonclassical form which can only be identified by pain, increased urgency and frequency, and the presence of glomerulations (petechial hemorrhages) upon bladder distension. Even these glomerulations are not diagnostic as they may appear in bladder cancer as well. In addition to the lack of a precise definition, and because of it, the etiology of IC is completely unknown.

Several authors have suggested the possibility of an autoimmune etiology for IC based on findings of (1) Igs in the bladder wall of patients with IC [5], (2) autoantibodies directed at bladder antigens found in patient sera (although the low titers found are not common for classical autoimmune diseases) [6, 10, 12], (3) increased incidence of other autoimmune diseases such as systemic lupus erythematosis in IC patients [11], (4) abnormal expression of class II major histocompatibility complex (MHC) antigens in urothelial cells in IC bladders with Hunner's ulcer [3] and (5) the abnormal percentage of gamma/delta T-cells in biopsies from these patients [3]. Little conclusive data are available,

however, due to several problems involving appropriate experimental design.

The first problem is the lack of a suitable animal model for IC. This is compounded by lack of a precise pathologic histologic definition (except for the 20% of cases with Hunner's ulcer). At present, only clinical criteria define this disease [4] and these are very difficult to identify in animal species; for example, what characterizes increased urgency in a rat?

Based on the autoimmune hypothesis, we have used methods common for inducing other experimental autoimmune diseases and which have recently been used to induce experimental autoimmune cystitis (EAC) in mice [1], to induce EAC in female Lewis rats. In this paper we describe investigations aimed at characterizing this disease as "IC-like," both behaviorally and histologically.

## Materials and methods

### Induction of cystitis

Fifty female Lewis rats (VAF, Harlan Sprague-Dawley) weighing 180–200 g were anesthetized with ketamine/xylazine (50 mg ketamine/10 mg xylazine per kilogram). Two hundred microliters of bladder homogenate in complete Freund's adjuvant (CFA) (29 animals) or CFA plus phosphate-buffered saline (PBS) for sham (21 animals) was injected subcutaneously at the base of the tail. Lewis rats were chosen due to their genetic predisposition to autoimmunity (they are used as the animal of choice for many other autoimmune animal models such as experimental autoimmune encephalomyelitis) [14]. Four separate experiments were conducted. In the first experiment, four of the experimental animals plus five shams had their bladders distended under anesthesia to measure bladder capacity (experiment 1a). The remaining animals (four in each group) were not distended (experiment 1b). All were analyzed for histologic changes. In experiments 2 and 3 both histology and urinary frequency were analyzed. In experiment 4, four experimental animals with elevated urinary frequency and four sham animals with normal frequency were studied for their bladder capacity only.

### Bladder antigens

Bladders from allogeneic Sprague-Dawley VAF (virus antigen free) rats (Charles River) were prepared according to the method of Bullock et al. [1] with some modification. The bladders were minced with scissors and placed in sterile PBS, 0.1 M, pH 7.2, containing 0.5% Triton X-100. The tissues were homogenized using a Polytron homogenizer which had been previously sterilized. The material was centrifuged for 10 min at 1000 *g*, and then the supernatant was assayed for protein content via the BCA protein microassay (Pierce). The homogenate was diluted in PBS to a concentration of 10 mg/ml protein and then further diluted 1:2 in CFA.

### Histopathology

Immediately upon animal sacrifice (at 4 weeks post-injection for experiment 1b, 12 weeks for experiment 2 and 10 weeks for experiment 3), the bladders were excised, the whole bladder was fixed in 2% paraformaldehyde for 24 h followed by fixation in buffered formalin for 1–7 days, and stained with hematoxylin and eosin (H&E) and/or toluidine blue to identify mast cells. All slides were reviewed blind to treatment modality for mast cell counts, inflammation, edema, congestion and lymphocytic infiltrates (see below). In experiment 3, brains and spinal cords were also removed, fixed in formalin and stained with H&E.

### Behavioral tests – urinary frequency, bladder capacity

#### *Urinary frequency*

We initially attempted to measure urinary frequency using a modification of classical metabolic cages. The cages had electronic devices attached to measure the number of drops of urine. While accurate for normal female rats, having a volume per urination of 500–1000 µl urine, this apparatus gave erroneous results for the bladder antigen-injected animals due to the very small volumes of urination from these animals (often $<100$ µl per urination). These small volumes evaporated before registering electronically or collecting in collecting tubes, thus causing frequency measurements and total volumes recorded to be less than actual.

Urinary frequency was therefore determined by lining empty rat cages with gel dryer filter paper (model 583, Bio-Rad, CA) separated from the rats by stainless steel screening to prevent gnawing. Animals were placed in the cages at 0930 hours and removed at 1530 hours (frequency abnormalities are perhaps most problematic during sleep times). The papers were carefully removed and the number of urinations determined by counting the number of spots observed under UV light ($\lambda = 244/356$ nm). Spot counts were performed blind to the animal treatment. Overlapping spots were easily identified. Average frequency was determined at 7 weeks post-induction (experiments 2 and 3) (Fig. 3). Evaporation, and hence loss of data, could not occur under this system as the filter paper was placed directly below the screening. Thus capillary action alone would guarantee that even tiny amounts of urine would be captured. (We measured volumes as small as 4 µl.)

Four normal animals were first observed over a 4-day period in order to determine if there was any significant effect of the estrus cycle on urinary frequency and in order to determine normal frequency values. No changes correlated with the estrus cycle were identified. Seven normal male animals were also analyzed using this method to test the reproducibility of the method.

In experiment 3, a study of the time course of onset of EAC, animals were regarded as having EAC if the urinary frequency was elevated (+) for 2 consecutive weeks in order to insure that a single elevation was not a random occurrence. Frequency was measured once per week, for 10 consecutive weeks.

#### *Bladder capacity*

Bladder capacity was determined according to the method of Bullock et al. [1] at 4 weeks post disease induction for experiment 1a and 10 weeks post induction for experiment 4 with slight modification. Animals (nine experimentals, eight shams) were anesthetized and the urethra was catheterized with a 24-gauge angiocatheter and the residual urine was withdrawn. Using a 1-ml pipette as a water column, capacity was determined under 10, 15, 20, 30 and 40 cm $H_2O$ pressure.

### Adoptive transfer of cystitis

This procedure was performed according to the procedure described by Bullock's group [1], with slight modification. Spleens from the donor animals (experiment 2) were removed aseptically and the cells within were dispersed through a screen and then collected in

RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), non-essential amino acids, L-glutamine and penicillin/streptomycin. Red cells were removed via centrifugation through Hypaque 1083. The cells were then cultured at 37 °C, 5% $CO_2$ in the same medium plus concanavalin A (con-A, 3 μg/ml) ($1.5 \times 10^6$ cells/ml) for 5 days. After 5 days, naive syngeneic recipients were injected intraperitoneally with $\sim 2.5 \times 10^7$ washed cells in 0.5 ml sterile PBS.

### Statistical analyses

Statistical analyses were performed using the Graphpad "Inplot" program (version 4.02) and "T-ease," a Student's *t*-test program, on an NEC IBM-compatible personal computer.

## Results

### Histologic evaluation

It has been reported that the number of mast cells per field is an indicator of IC [2]. In our animal model, we counted mast cells (both intact and degranulated) from toluidine-blue-stained sections from normal rat bladder, bladders from sham-injected animals (experiment 2) and bladders from bladder-antigen-injected animals (experiment 2) in longitudinal cross-sections of the entire bladder. No statistically significant difference ($P > 0.05$) was found between groups for total mast cell number or degranulated mast cells (Table 1). However, there was a tendency for the number of degranulated mast cells to be increased in the bladder-antigen-injected animals. The significance of this findings is further dampened, however, by the lack of correlation in the number of mast cells from section to section *within the same animal.*

Human IC biopsies (from patients conforming to the NIH guidelines for IC [4] without Hunner's ulcers) exhibited no inflammatory infiltrates (Fig. 1) in agreement with the findings of Johansson and Fall [7]. There were also no inflammatory cuffing or inflammatory infiltrates in bladders from either animal group (Fig. 2a, c). What we did find in the bladder antigen-injected animals (Fig. 2c) was an abundance of microvessel and small venule congestion as compared with the sham-injected animals (Fig. 2a) and with four normal, untreated animals. The small vessel congestion in the absence of any inflammatory infiltrates or perivascular cuffing was specific for the bladder-antigen-injected animals and clearly identifiable in adjacent sections. In chemically induced cystitis and bacterial cystitis (data not shown) venule congestion may or may not be present but *inflammatory infiltrates always are.* When assessed blindly, and rated "+" for very congested vs "−" for very little, if any, congestion, a clear correlation between sections with high levels of venule and microvessel congestion and the bladder-antigen-injected treatment was found (Table 2). Such congestion is clearly evident in the human IC biopsies as well (Fig. 1).

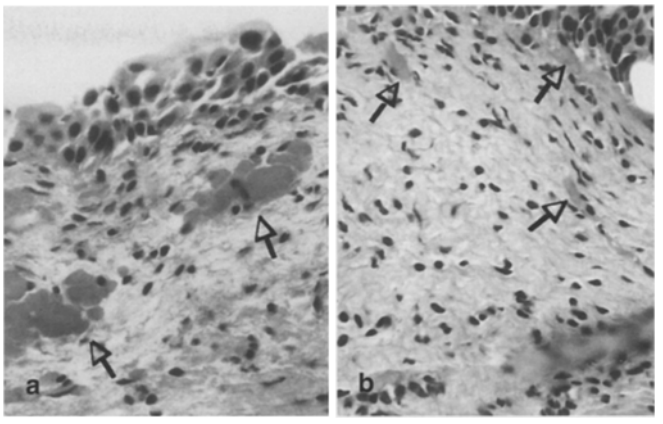


**Fig. 1a, b** H&E stain of bladder biopsies from two IC patients. Tissues were prepared as described in "Methods." Note (*arrows*) the congested vessels, red cell extravasation and lack of mononuclear cells, ×630

**Table 1** Lack of correlation between mast cell counts between adjacent bladder sections and between treatment groups. Counts were performed blind to treatment. There is no statistical difference between groups for each category

| Animal | Mast cell count per bladder cross-section | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Total MCs | | | | Degranulated MCs | | | |
| | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 (section #) |
| 1 | 12 | 22 | 15 | 14 | 0 | 3 | 7 | 4 |
| 2 | 1 | 45 | 18 | | 0 | 6 | 2 | |
| 6 | 62 | 48 | 36 | | 2 | 0 | 2 | |
| Sham-injected average: | | | | 27 ± 18 | | | | 3 ± 2 |
| 3 | 46 | 35 | 27 | | 15 | 16 | 3 | |
| 4 | 15 | 45 | 17 | | 0 | 20 | 4 | |
| 5 | 69 | | | | 16 | | | |
| 7 | 13 | 33 | 23 | | 1 | 20 | 2 | |
| 8 | 23 | 17 | | | 6 | 5 | | |
| 9 | 10 | 46 | 29 | 35 | 0 | 22 | 10 | 17 |
| 10 | 35 | 16 | | | 0 | 2 | | |
| Bladder-antigen-injected average | | | | 30 ± 15 | | | | 9 ± 8 |

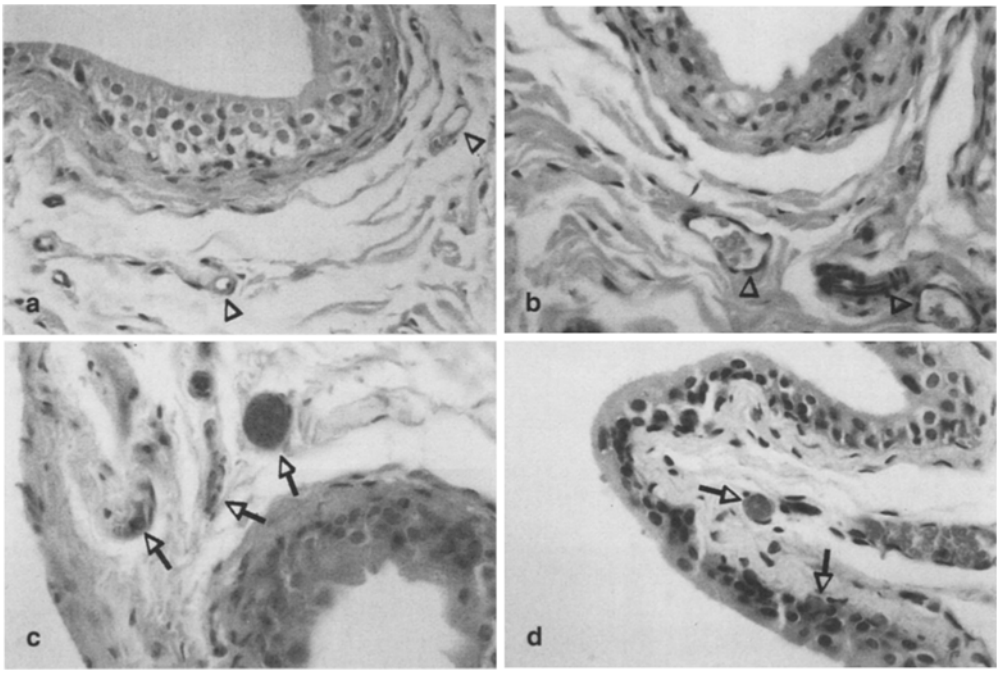


**Fig. 2a–d** H&E stain of bladders from **a** sham-injected and **c** bladder-antigen-injected animals and the recipients of their respective splenocytes (**b, d**). Tissues were prepared as described in "Methods." Note in **c, d** the congestion of small vessels (*arrows*) which is absent in **a, b** (*arrowhead*), × 630

**Table 2** Frequency and histologic changes in animals from experiments 2 and 3 (see text) (*nd* no data available)

| Experiment 2 | | | Experiment 3 | | |
|---|---|---|---|---|---|
| Animal | Frequency | Histology | Animal | Frequency | Histology |
| Sham-injected animals | | | Sham-injected animals | | |
| 1 | – | – | 10 | – | nd |
| 2 | + | + | 11 | – | nd |
| 6 | – | – | 12 | – | nd |
| | | | 13 | – | nd |
| | | | 14 | – | nd |
| | | | 15 | – | nd |
| Bladder-antigen-injected animals | | | Bladder-antigen-injected animals | | |
| 3 | + | + | 1 | – | – |
| 4 | ± | – | 2 | + | + |
| 5 | + | + | 3 | – | – |
| 7 | – | – | 4 | + | + |
| 8 | + | + | 5 | + | + |
| 9 | + | – | 6 | + | + |
| 10 | – | – | 7 | + | + |
| | | | 8 | + | + |
| | | | 9 | + | + |

In order to address the possibility of another site of autoimmunity which might affect the bladder, we examined the CNS for the presence of inflammatory infiltrates or congested venules. We reasoned that only the nervous system would affect the frequency of urination unless a gross change had occurred to another intraabdominal organ. As no such gross change was observed nor were there any signs of CNS inflammation or vascular congestion, we determined that the histologic changes observed were specific for the bladder.

## Behavioral studies

### *Urinary bladder capacity*

Our first attempt to characterize the behavior of the bladder-antigen-injected animals as "IC-like" was to investigate their bladder capacity under anesthesia (experiments 1a, 4). We were unable to identify any significant ($P > 0.05$) bladder capacity changes in the rats at 4 weeks post-induction (Fig. 3) and 10 weeks post-induction.

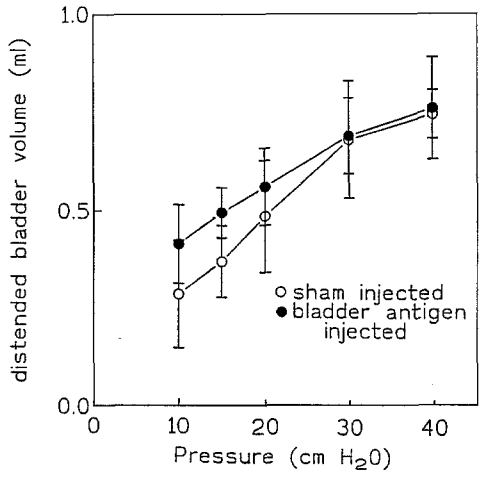


**Fig. 3** Bladder capacity of sham-injected and bladder-antigen-injected animals at various pressures. Animals were anesthetized and then a column of water was allowed to enter the bladders by gravity until equilibrated with bladder pressure. There is no statistical difference between the two groups at any pressure

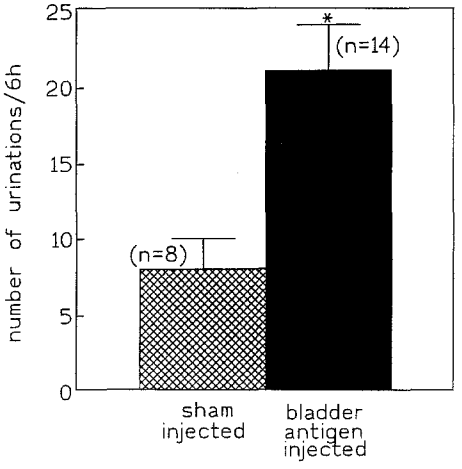


**Fig. 4** Average frequency of urination per 6 h for sham-injected and bladder-antigen-injected animals. Animals were housed in filter-paper-lined cages for 6 h and the number of fluorescent (urine) spots was counted under UV illumination. Difference between sham and experimental significant to the $P = 0.004$ level, Student's $t$-test (*). Range (1 SD from mean): shams, 4–12; bladder-injected animals, 11–31

*Urinary frequency*

We next measured changes in urinary frequency (experiments 2, 3). The sham animals exhibited an average frequency of $8 \pm 2$ SE ($n = 8$) urinations/6 h. Rats which received bladder antigens, however, exhibited a significantly elevated frequency of $21 \pm 3$ SE urinations/6 h ($n = 14$, $P = 0.004$, Student's $t$-test) (Fig. 4). Using a range of one standard deviation plus or minus from the mean, we then rated an animal's frequency as "−" if the frequency was less than or equal to ten urinations in 6 h and "+" if greater than or equal to 13 urinations in 6 h. Animals with frequencies between 10 and 13 were indeterminate ("+/−"). Total urinary output over the 6 h was determined by comparing dot diameter with a standard curve. No statistical difference was found between the two groups. ($1.5 \pm 0.5$ ml for sham animals, $2.1 \pm 0.3$ ml, respectively, for bladder-antigen-injected animals over the 6-h period).

Animals exhibiting positive histology correlated well with the animals which had elevated urinary frequency (data in Table 2). The three animals in experiment 2 which received bladder antigen injections but did not exhibit positive histology also exhibited low (+/−) urinary frequency. (One additional animal which exhibited negative histology did exhibit increased urinary frequency.) Thus using either or both of these criteria, frequency or histology, the incidence of primary EAC 8 weeks post-injection was 5/8 animals which were injected with bladder antigens in experiment 1, 4/7 in experiment 2 and 7/9 in experiment 3, or a total of 16/24 bladder-antigen-injected animals (67%). Of the sham-injected animals, 3/4 animals in experiment 1, 2/3 animals in experiment 2 and 5/5 in experiment 3, or a total of 10/12 animals studied, were "normal" (negative for histology and/or frequency, if measured) (83%).

*Time course of frequency changes*

We next investigated the onset of urinary frequency elevation in order to determine the time course of onset of the disease (Fig. 5). Most animals (4) did not have elevated frequency until 6 weeks post-induction (scored as occurring on the 7th week because of our decision to count the elevation as a positive indication only if the elevation occurred in two consecutive weeks – see "Methods"). One animal, however, had increased frequency after only 1 week, remaining elevated for one additional week (thus confirming EAC) and then returning to normal for the 3rd week. By week 4 the elevation in frequency had returned, indicating that the syndrome may have relapses and remissions similar to human IC and other autoimmune diseases. No sham-injected animals (0/5) exhibited increased frequency for two consecutive weeks.

*Adoptive transfer studies*

Bullock's group [1] has shown that EAC in mice is transferable to naive animals via adoptive transfer of their splenocytes, indicating that the condition is of an autoimmune nature. We were puzzled by this result as unlike the histology observed in their animals, which also had ulcers similar to Hunner's ulcers in human IC, we did not see any signs of inflammatory infiltrates (other than an occasional polymorphonuclear cell) or ulcers in our bladder-antigen-injected rats as perhaps

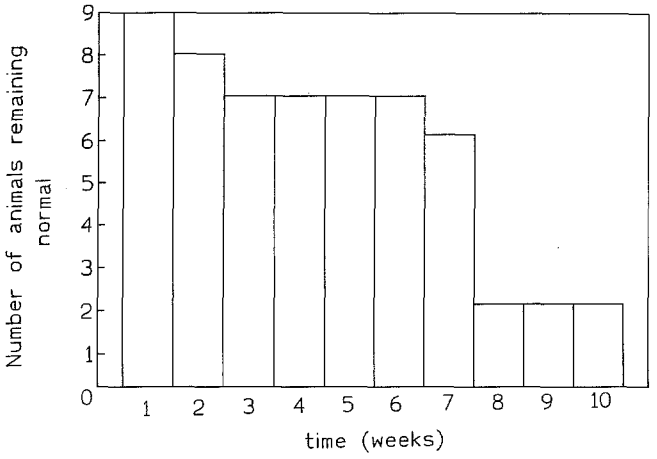


**Fig. 5** Time course of disease onset as determined by increased urinary frequency. Animals were housed in filter-paper-lined cages for 6 h and the number of fluorescent (urine) spots was collected and counted once per week under UV illumination. Positive identification of EAC was made after two consecutive weeks of elevated urinary frequency

would be expected of a classical autoimmune disease. Thus we repeated their experiment with the Lewis rats. We cultured splenocytes from both groups of animals in experiment 2, and transferred them to naive female Lewis rats. These animals were then evaluated for urinary frequency changes and the bladders were evaluated histologically as described above.

The frequency grades (Table 3) from all but one of these animals (animal #1) correlated with the donor animals' frequency, i.e. if " – " frequency in the donor, then " – " frequency in the recipient and vice versa. The histology score was also transferable in most cases (seven out of ten animals). [Two animals which had received spleens from "negative" donors exhibited positive histology (see "Discussion" below)]. Thus the behavioral scores as well as the histologic scores were transferable to the recipient animals (Table 3).

Perhaps surprisingly, however, there was no evidence of immune cell infiltration in the recipient animals from either group.

## Discussion

Interstitial cystitis has been a difficult disease(s) to investigate due to the lack of an appropriate and inexpensive animal model and due to the difficulty in defining the condition based on other than clinical criteria. In this paper we have developed an animal model based on the hypothesis that IC is an autoimmune disease and have characterized this model with behavioral criteria related to the human clinical findings. In addition we have observed histologic changes in the bladders of our animals consistent with the histology of IC bladder biopsies. Our results differ somewhat from the findings of Bullock et al. [1], who investigated EAC in the mouse. Their model included ulcers of the bladder similar to Hunner's ulcers. Our animals do not exhibit ulcers as is true of 80% of IC patients as well. Bladder capacity was also unchanged in our animals just as IC patients which do not have Hunner's ulcers may also lack changes in bladder capacity [9]. We originally thought that another reason for the lack of bladder capacity changes in our animals might be that we measured the capacity changes too early (4 weeks post-injection) as our later studies (Fig. 5) indicated that not all animals will exhibit frequency changes until 8 weeks post-injection. However, when we repeated these experiments at 10 weeks post-induction (experiment 4), in animals whose urinary frequency was clearly different, there were still no significant bladder capacity changes.

We have found that a behavioral trait similar to that with which most patients first present, i.e. urinary frequency, can be quantified in female Lewis rats, and can be shown to differ at least twofold in the experimental animals when compared to shams. This behavioral measurement makes our study unique. As IC is a clinically defined disease, clinical criteria are the best criteria for determining if a model is "IC-like."

Secondly, we have determined that increased mast cell number per tissue section is not a reliable histologic criterion in the Lewis rat. Instead we found that mast

**Table 3** Transferability of histologic and behavioral characteristics (experiment 2) (see text) (*nd* no data available) (first two columns repeat of data from Table 2 for comparison)

| | Animal | Primary model | | After transfer | |
|---|---|---|---|---|---|
| | | Frequency | Histology | Frequency | Histology |
| Sham injected | | | | | |
| | 1 | – | – | + | ± |
| | 2 | + | + | nd | nd |
| | 6 | – | – | – | – |
| Bladder injected | | | | | |
| | 3 | + | + | + | + |
| | 4 | ± | – | – | + |
| | 5 | + | + | + | + |
| | 7 | – | – | – | + |
| | 8 | + | + | + | + |
| | 9 | + | – | + | – |
| | 10 | – | – | – | ± |

cell number appeared to vary from section to section from the same animal. We counted mast cells (both intact and degranulated) from toluidine-blue-stained sections from normal rat bladder, bladders from sham-injected animals and bladders from bladder-antigen-injected animals. We used longitudinal cross-sections of the entire bladder for these counts, as opposed to tiny fragments of the bladder wall which are taken in the case of human biopsies. Even in these cross-sections, there was no correlation in the number of mast cells from section to adjacent section within the same animal, and no statistical difference between groups (Table 1). While of course these data do not directly apply to humans, they suggest that mast cell number within a small, nonrepresentative human biopsy sample is probably not a reliable determinant of IC, in agreement with Theoharides et al. [13]. While some authors have suggested that toluidine blue is not an optimal stain for identifying mast cells in bladder [7, for example], the fact remains that in adjacent sections, stained in an identical manner at the same time, there has been little agreement.

Thirdly, in our hands (Fig. 1), and in those of others [7], the only histological criterion one could consistently find in H&E sections from IC bladder biopsies without Hunner's ulcers was the presence of congested, RBC-packed venules and/or microvessels and red cell extravasation and the lack of inflammatory infiltrates. This bladder histopathology is also found reproducibly in our animal model. The possibility exists, however, that with longer times post-induction, inflammatory infiltrates may ultimately be observed and ulcers may appear. In support of this concept is the fact that ulcers were formed in EAC mice [1] and that Hunner's ulcers in human IC have been suggested to be associated with age [8].

Fourthly, our animal model's characteristics can be transferred to recipient animals via injection of the donor animal's con-A-activated splenocytes in most cases, proving the autoimmune nature of the condition. Three animals which received spleens from "negative" bladder-antigen-injected donors exhibited positive histology. It is possible that the donor animals were in the early stages of EAC and so did not exhibit the behavioral or histologic criteria for the disease, but were capable of passing the disease on to their splenocyte recipients via some cell type. In no case did a donor animal which was positive fail to transfer this characteristic to the recipient.

Surprisingly, however, no inflammatory infiltrates were observed in the bladders of the splenocyte recipients. Most likely 1 week post-transfer is just too soon to observe such inflammatory infiltrates. However, the possibility exists that the transfer is mediated through a soluble mediator rather than the cells themselves, for example, cytokines or antibodies, or that the bladder itself is somehow abnormal in its immune response (e.g., perhaps it lacks adhesion molecules). At this time we cannot rule out any of these possibilities. We conclude from these experiments that the lack of an inflammatory infiltrate does not preclude the presence of autoimmune disease in our rats' bladders, and possibly in human bladders as well.

IC is a complicated syndrome which has not been well characterized. As better characterization of IC is accomplished, we will be better able to further evaluate this model. While no animal model is a perfect replica of human disease, we believe our animal model is consistent with important clinical features of the human condition and will permit us to ask and answer important questions about the human disease. Such models are especially useful in evaluating potential therapies. Thus the observation that an autoimmune attack against bladder antigens can lead to increased urinary frequency should make this model valuable to the field of urology, especially to the field of therapeutics.

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