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CNS INDUCED NEUROGENIC CYSTITIS IS ASSOCIATED WITH BLADDER MAST CELL DEGRANULATION IN THE RAT

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ABSTRACT

Purpose: To determine if bladder mast cell degranulation is involved in the genesis of neurogenic cystitis induced by pseudorabies virus (PRV) invasion of the central nervous system (CNS).

Materials and Methods: Rats received a total of 4×10^6 plaque forming units (pfu) of PRV-Bartha in the abductor caudalis dorsalis (ACD) muscle. Granulated bladder mast cells per mm^2 of bladder tissue and urine histamine content were monitored as the cystitis developed over the next few days. In a subgroup of rats, intravesical resiniferatoxin was used to remove capsaicin-sensitive sensory bladder afferents, while another subgroup was pretreated with a mast cell degranulator.

Results: PRV injection into the ACD muscle leads to neurogenic cystitis. Histamine levels were elevated in the urine of virus injected rats before any behavioral or microscopical signs of cystitis were present. When the cystitis became clinically manifest, urine histamine returned to control levels, and the number of granulated mast cells dropped significantly. Rats in which capsaicin-sensitive afferents had been removed did not show any signs of cystitis, or increase in urine histamine, or change in the number of granulated mast cells. Pretreatment of animals with a mast cell degranulator completely prevented the appearance of cystitis without altering the CNS disease.

Conclusion: These results provide further evidence that mast cells are involved in neurogenic cystitis induced by changes in CNS activity.

KEY WORDS: histamine, urine, Pseudorabies virus, capsaicin, denervation

A subset of patients suffering from interstitial cystitis have increased urinary levels of histamine and its metabolites,[1] and an associated proliferation and degranulation of bladder mast cells.[2,3] Given that release of neuropeptides induces mast cell degranulation, and the close proximity of mast cells and peptidergic nerve terminals in the bladder,[4] it has been suggested that the nervous system is involved in the pathophysiology of interstitial cystitis. Release of neuropeptides in the bladder would lead to increased extracellular histamine,[4,5] intraparenchymal migration of leukocytes,[5] enhanced production of, and response to, pro-inflammatory cytokines,[4,6,7] and pain. Furthermore, mast cell activity in the bladder is influenced by the CNS since emotional stress worsens the symptoms of interstitial cystitis in humans, and immobilization stress in rats causes mast cells to degranulate.[8]

We have previously developed a rat model of neurogenic cystitis in which activation of bladder-related circuits in the CNS occurs in response to invasion of neighboring circuits by a neurotropic virus, PRV.[9] While many features of this model are reminiscent of interstitial cystitis, the involvement of bladder mast cells in its pathophysiology remains to be shown. The present study therefore measured the number of non-degranulated mast cells in the bladder wall as well as the amount of histamine in the urine of animals at different stages of the disease. The same parameters were also assessed in animals in which peptidergic bladder afferents had been removed. Finally, to further examine the possible contribution of mast cells to this bladder inflammation, mast cells were degranulated prior to induction of the cystitis.

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MATERIALS AND METHODS

Animals. Sixty-one male Sprague Dawley rats (270–300 gm.; Harlan Sprague Dawley, Indianapolis, IN) were used in the present study (table). Cystitis was induced by injection of the Bartha strain of PRV (4×10^6 pfu) into the abductor caudalis dorsalis (ACD) tail muscle as previously described.[9] Experiments were done in accordance with the regulations of the Georgetown Biosafety and Animal Research Committees. All animals were exposed to light 12 hrs per day; food and water were available ad libidum. For all surgeries, rats were anesthetized using 1–2% halothane (Solvay, Mendota Height, MN) and oxygen delivered through a facial mask.

Primary afferent denervation. Resiniferatoxin was used to remove capsaicin-sensitive C-fiber bladder primary sensory afferents, without affecting sympathetic innervation[10] or the majority of myelinated primary afferents.[11] This treatment was carried out 24 hr. after ACD inoculation to ensure that it would not interfere with viral uptake from the injection site.[9] The bladder was exposed and, using a 30 gauge needle, 1.0 ml. of resiniferatoxin (10 nmol. in 1% Tween 80 and 0.5% ethanol) was injected into its lumen and removed 20 minutes later. The presence of a blink reflex in response to corneal stimulation was confirmed in all rats at the time of perfusion to exclude possible systemic effects of resiniferatoxin.

Mast cell degranulation. To prevent mast cell degranulation at the time of the induction of the cystitis, we chronically degranulated mast cells by systemic administration of compound 48/80 (5 mg./kg. s.c., Sigma, St. Louis, MO) for 5 days starting two days before the viral inoculation of the ACD.

Urine collection and euthanasia. Rats were anesthetized

Summary of experimental groups

Experimental Group	Delay before Perfusion	Data Collected	n = 61
PRV-ACD injected	24 hrs	Behavior, mast cell counts, urine histamine, CNS PRV-immuno	4
	36 hrs	same	4
	48 hrs	same	4
	60 hrs	same	4
	72 hrs	same	4
	84 hrs	same	4
	96 hrs	same	4
	108 hrs	same	4
Controls	none	Bladder histology, mast cell counts, urine histamine	6
PRV-ACD injected and intravesical resiniferatoxin (10 nmol)	108 hrs	Behavior, mast cell counts, urine histamine, CNS PRV-immuno	4
Controls, intravesical resiniferatoxin (10 nmol)	108 hrs	Behavior, mast cell counts, urine histamine, CNS PRV-immuno	4
Controls, intravesical resiniferatoxin (100 nmol)	48 hrs	Mast cell counts	3
Intravesical 48/80 and PRV-ACD	108 hrs	Behavior, bladder histology, CNS PRV-immuno	6
Saline controls and PRV-ACD	108 hrs	Behavior, bladder histology, CNS PRV-immuno	6

Abductor caudalis dorsalis (ACD); Pseudorabies virus (PRV).

with pentobarbital (100 mg./kg., i.p.) and their bladders exposed with minimal manipulation through a midline lower laparotomy. Using sterile technique, urine was collected by direct puncture of the bladder dome using a 25 gauge needle and gentle aspiration, and then the bladder was refilled with 1.5 ml. sterile saline. The urine was transferred to sterile microtubes (0.5 ml. per tube), quick frozen, and kept at −80C until measurement of histamine levels was carried out. Rats were then perfused through the ascending aorta with Tyrode solution, followed by buffered 1% formalin (3.7% of a 37% solution in 0.1 M phosphate buffer, pH 7.4) and the bladder, brain, and spinal cord were removed.

Urine histamine measurement. Urinary histamine was quantified using an enzyme-linked immunosorbent assay (ELISA) with a commercial kit (IBL, Hamburg). Urine from treated and control rats and five standards with increasing concentrations of histamine were acetylated and then incubated with primary rabbit antihistamine antiserum and histamine-conjugated-peroxidase in wells coated with goat-anti-rabbit antiserum. The wells were then washed, and the concentration of residual bound histamine-conjugated-peroxidase, from binding to the primary-secondary antibody complex, was revealed by the peroxidation of the chromogen tetramethylbenzidine. The optical density of the solution in each well was then measured at 450 nm using a microplate absorbance reader (Model 550, Bio-Rad, Hercules, CA). Quantification of each sample was obtained by reporting its measured optical density on a line plotted from the values measured using the standard (concentration in abscissa [logarithmic] and corresponding optical density in ordinate [linear]). ELISAs on each sample were repeated twice.

Identification of mast cells. The whole bladder was divided in 2 to 4 portions which were stained according to the method of Csaba.[12] Cells were light red or unstained and histamine granules of granulated mast cells were densely stained blue. Basophils, also labeled blue by Csaba's stain, were easily distinguished by the bilobed appearance of their nucleus.

Estimation of the total number of mast per bladder. Counts of the total number of mast cells per bladder were done with the fractionator technique,[13] using a microscope equipped with the StereoInvestigator stereology system (MicroBright-Field Inc., Colchester, VT). The outline of the bladder was traced on the computer screen at low magnification (2×), and the computer overlaid a randomly placed grid that covered the entire piece of bladder (fig. 1). A counting frame was placed within each box of the overlaid grid and using a 40× lens, and mast cells that fell within the counting frame were counted. To be counted, the entire circumference of a mast cell had to be visible, exhibit an oval shape with an approximate diameter of 15 μm., and be densely granulated. The mast cells were counted throughout the thickness of the bladder (i.e., through multiple focal planes). The bladder was moved automatically to the next counting frame by stepping motors controlled by the computer. The size and number of counting frames was determined from a preliminary population estimate, and varied from 25 to 50 boxes depending on the size and shape of the piece of bladder.

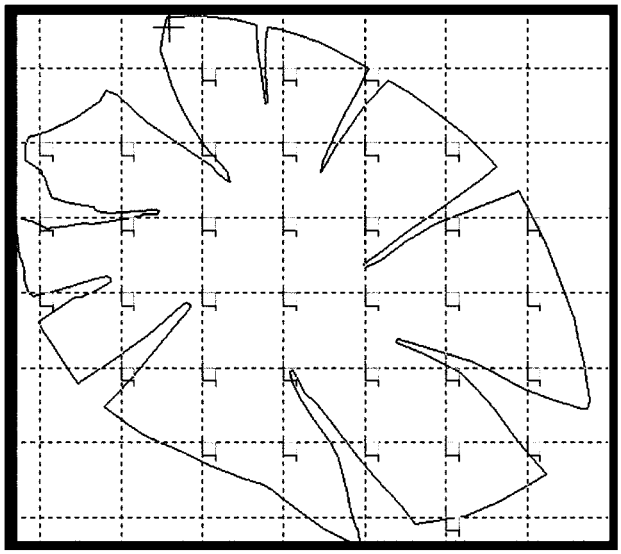

FIG. 1. Outline of bladder section, as seen on computer screen, on which grid and counting frames (small box in left upper corner of each square of grid) are superimposed. Counts of mast cells are made in counting frames and automatically positioned at each frame with motorized stage. Estimated total number of mast cells for given section is obtained through algorithm (see Materials and Methods).

The software provided the estimated total number of cells in the counting grid by dividing the area of the counting grid by the area of the counting frames and multiplying this value by the number of cells counted in the counting frames. To determine the total number of cells in the bladder, the total number of cells in the counting grid area was divided by the actual area of the bladder and then multiplied by the number of cells in the counting grid. Finally, the total number of mast cells was divided by the total surface area of the bladder to obtain the density of mast cells per area of bladder (number of mast cells per mm.^{2}). The density of bladder mast cells was then averaged for all rats in each treatment group. Values are expressed as the mean +/− SE of the mean.

Statistical analysis. An Analysis of Variance (ANOVA) was used for statistical analyses. Post-hoc testing was done using Fisher's test. Differences between two groups were considered statistically significant for $p < 0.05$.

RESULTS

Behavioral and histological signs of cystitis appeared at 84 hours post-viral inoculation and followed the same temporal course as previously reported.[9] Rats presented piloerection, rounded-back posture, and excessive and repeated grooming of the lower abdomen. These signs became more consistent and grooming more frequent during the following 24 hrs. Immunocytochemical staining of the spinal cord and brainstem using a polyclonal antiserum specific to PRV confirmed that the virus had invaded the CNS circuits neighboring those of the bladder in the same distribution as previously reported.[9]

Bladder histology. Examination of the bladder revealed an abundance of blue stained mast cells lining blood vessels in normal controls (fig. 2, *C*). The average diameter of twelve randomly selected mast cells was 15.1 +/− 0.9 μm., and their average area was 169 +/− 20.9 μm.^{2} Counting revealed that in naïve rats there was an average of 34.9 +/− 3.3 mast cells per mm.^{2} of bladder. In PRV inoculated rats, the bladder appeared normal (fig. 2, *A*) and the number of mast cells did not significantly differ from control rats until the end of the third day (fig. 3, *A*), when there was a decrease in the number of mast cells which persisted until the end of the examination period (108 hours). During the early stage (84–96 hours) of the leukocytic-hemorrhagic bladder infiltrate, mast cells were aligned along vascular profiles and aggregates of mast cells were seen in non-vascular areas (fig. 2, *D*). In contrast, at later stages (≥96–108 hours) of the disease (fig. 2, *B*), the few residual mast cells were often isolated and not associated with blood vessels (fig. 2, *E*). Counts of mast cells in resiniferatoxin treated rats showed no change in the total number of bladder mast cells at 108 hrs (fig. 3, *A*).

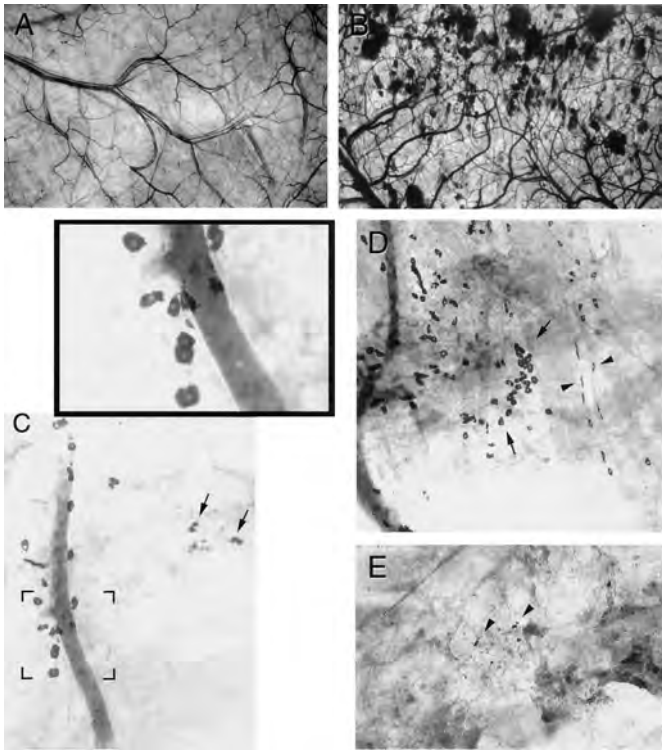

FIG. 2. Photomicrograph of Csaba stained urinary bladders at different times post viral inoculation. *A*, low magnification of bladder wall at 24 hrs., with no visible anomalies. *B*, low magnification of bladder wall at 108 hrs. showing presence of punctate diffuse hemorrhages. *C*, high magnification of bladder at 48 hrs. showing that most mast cells are found aligned along blood vessel, and only rare few are found at distance (arrows). Inset (higher magnification of boxed area in *C*) shows characteristic granulated appearance of mast cells. *D*, at 84 hrs., many mast cells are found distant to blood vessels (arrows pointing to group of mast cells), while others are still clearly associated with blood vessels (arrowheads). *E*, at 108 hrs., few mast cells (arrowhead) are found, most of which are dispersed amongst hemorrhagic exudate, even when examining large field. Original magnifications: *A* and *B*, 20×; *C*, 200×; *C* inset, 400×; *D*, 150×; *E*, 100×.

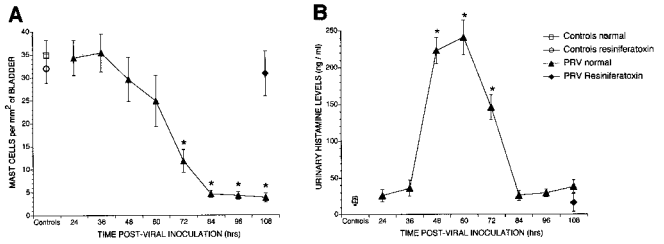

FIG. 3. Change over time in density of granulated mast cells in bladder wall and urine histamine levels after pseudorabies virus (PRV) inoculation in abductor caudalis dorsalis (ACD) tail muscle. *A*, trend towards decreased density of mast cells is observed starting at 48 hrs.; however, this change only reaches significance at 72 hours, after which it persists until end of observation period. *B*, histamine significantly increases above control values 48 hrs. post-inoculation, at time when virus is detected in preganglionic spinal sympathetic neurons and in motoneurons innervating tail muscle as previously reported.[9] Urine histamine, however, returns to normal levels by time cystitis becomes behaviorally and histologically manifest. Note similarity in latter half of two time courses suggesting that decreasing levels of urine histamine are related to decreasing numbers of granulated mast cells. * $p < 0.01$. Error bars denote SEM. Number of rats for each group is in table.

Urine histamine. In control rats, urine histamine levels were consistently low (fig. 3, *B*). In contrast, in PRV injected rats, histamine levels were significantly elevated in the urine as early as 48 hours post-injections (fig. 3, *B*), before behavioral and microscopical signs of cystitis were present. Interestingly, at 84 hours post-treatment, when the clinical signs of cystitis were first recognizable, levels of urine histamine dropped to concentrations which were no different than those of normal controls. These low levels persisted until the end of the observation period (fig. 3, *B*).

Rats in which capsaicin-sensitive innervation of the bladder was locally destroyed by treatment with intravesical resiniferatoxin had normal levels of urine histamine (fig. 3, *B*), clear urine, and normal bladder histology at all timepoints, despite advancing CNS viral disease.

Pre-emptive mast cell degranulation. To further confirm involvement of mast cells in the appearance of bladder inflammation in the present model, we administered the mast cell degranulator compound 48/80 for five days, starting two days before the viral inoculation of the ACD. At five days post viral inoculation (i.e. two days after ceasing treatment with compound 48/80), none of the animals treated with the degranulator developed any signs of cystitis while all the vehicle injected rats developed clear signs of cystitis.

DISCUSSION

These results provide further evidence for an involvement of mast cells in the genesis of neurogenic cystitis. Three sets of data are used to support this conclusion: 1) urinary histamine is increased immediately prior to the inflammation; 2) at the time of full blown inflammation, when urine histamine levels return to normal, a large proportion of the bladder mast cells have degranulated; and 3) there is no bladder inflammation when mast cells are degranulated prior to the occurrence of the CNS disease responsible for inducing the cystitis. Although not the focus of this paper, we show that within the framework of the current study it appeared

that resiniferatoxin had no effect on mast cell number or function. This finding indicates that resiniferatoxin prevents the cystitis by acting on peptidergic primary afferent terminals, the integrity of which is necessary for activation of mast cells in neurogenic cystitis.

The increase in urine histamine in the present study is deduced to occur through neuropeptide induced degranulation of the bladder mast cells, since resiniferatoxin-induced removal of peptidergic afferents to the bladder, some of which lie in close proximity to mast cells,[14] prevented its occurrence. Significantly, no toxic effects of resiniferatoxin on mast cells was observed, the occurrence of which would have made the interpretation of these experiments more difficult. The absence of a direct toxic effect on mast cells was further verified in a subgroup of animals that were administered 100 nmol. instead of 10 nmol. of resiniferatoxin in their bladder. Despite this high dose, which caused symptoms of extravesical capsaicin receptor stimulation (bronchospasm), the number of bladder mast cells in these animals 48 hrs. later was the same as in controls.

Previous studies have indicated that peripheral nerve stimulation as well as the neurotransmitter substance P induce mast cell degranulation and release of histamine.[15–17] Substance P induced mast cell degranulation is dose dependent and blocked by the NK-1 receptor antagonist CP-96,345.[17] Mast cells can in turn directly stimulate substance P release in the periphery,[18] possibly through the release of nerve growth factor.[19] They can also increase vascular permeability and leukocyte migration,[20] inducing a positive feedback phenomenon that could explain the rapid progression of cystitis in the present model.

The present animal model thus reproduces three defining features associated with the pathophysiology of interstitial cystitis, namely the neural component, the degranulation and migration of mast cells[4,21] and increased urinary histamine.[1,22] Current evidence indicates that interstitial cystitis is a form of neurogenic inflammation in which abnormal release of neuropeptides by primary sensory afferents to the bladder induces mast cell degranulation, anomalies of the vasculature and the epithelial and subepithelial layers, and an abnormal response of the bladder to distention.[4,23] Of note, in the present study increase in urinary histamine preceded the appearance of cystitis by 48 hrs., indicating that mast cells are part of a chain of events rather than the final effector of the disease. At the stage of full blown hemorrhagic cystitis, urine histamine fell back to nearly normal levels, corresponding to a large decrease in granulated mast cell staining, suggesting that the mast cells had reached a "burn out" stage. Accordingly, when rats were observed for longer periods, we found a regression in bladder inflammation, a probable consequence of the exhaustion of local inflammatory mediators, a phenomenon that could be related to cyclic pattern of interstitial cystitis.

In conclusion, activation of the CNS, by viral infection as in the present model or as previously reported after immobilization stress,[8] can induce mast cell degranulation in the bladder. It is likely, however, that mediators other than histamine are involved in the resulting bladder inflammatory response.[4] In accord, the efficacy of the antihistamine drug hydroxyzine in alleviating symptoms of interstitial cystitis would be in part due to its ability to prevent mast cell degranulation.[24]

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