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Experimental autoimmune cystitis: further characterization and serum autoantibodies

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Abstract Previously, we described an animal model for interstitial cystitis (IC), experimental autoimmune cystitis (EAC) [Luber-Narod et al. Urol Res 24:367]. Further characterization of animals with EAC indicates that peak and mean urinary frequency are elevated compared with sham-injected controls and that the disease progresses with at least two cycles of exacerbations and remissions. We had shown evidence suggesting EAC to be autoimmune in nature. In this paper, we identify serum autoantibodies from 9/10 EAC animals which bind to a protein specific to rat bladder with a relative molecular weight of 12-kDa. Such autoantibodies are absent in 12/13 normal and sham-injected animals as well as animals which fail to develop EAC despite disease induction. These findings suggest that EAC is a reproducible model of cyclical increases of urinary frequency, and that a 12-kDa antigen is the target of autoantibodies which correlate with those elevations. Identification of this target antigen may explain the pathogenesis of increased urinary frequency in these animals and potentially in IC as well.

Key words Interstitial cystitis · Animal models · Autoantibodies · Urinary frequency · Autoimmunity

Introduction

Interstitial cystitis (IC) is a debilitating human condition characterized by pain, increased urinary urgency and frequency, and petechial hemorrhages (''glomerulations'') upon bladder hydrodistension, in the absence of positive bacterial urine cultures or bladder cancer. The etiology of IC is currently unknown. Several authors have suggested an autoimmune mechanism for this condition due to (1) the presence of immune complexes in bladders from these patients [3], (2) the presence of autoantibodies in sera and urine from these patients [4, 11, 10], (3) the association of the occurrence of IC with other immunologic diseases such as lupus erythematosus [5], and (4) the reported decrease in symptom severity when patients are treated with cyclosporine [2].

We recently described an animal model of IC [8] which we have called experimental autoimmune cystitis (EAC). We have shown that the animals exhibit pathology (a statistically insignificant increase in degranulated mast cells, vascular congestion and edema) and behavioral traits similar to those seen in the human condition. Changes in bladder capacity were not observed; however these changes are also not observed in many IC patients [10]. Further, we checked the urine of our animals post-distension for signs of hemorrhage as is often observed for IC patients. All animals, experimentals and shams, exhibited blood in their urine post-distension due most likely to trauma from the catheterization; thus no conclusion could be reached. We have also shown that EAC is autoimmune in nature. Most relevantly, the animals exhibit increased urinary frequency, which is transferable by splenocytes from animals with EAC to naïve syngeneic recipients. In this paper, we further characterize this model and identify serum autoantibodies found only in those animals exhibiting enhanced urinary frequency. The target antigen of these autoantibodies is a 12-kDa bladder antigen.

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Materials and methods

EAC induction

The principles of laboratory animal care of the NIH were compiled with. Rats were authorized by anesthetic overdose. EAC was induced in female Lewis rats (Harlan-Sprague-Dawley, India) by injecting bladder homogenates from male rats in complete Freund's

adjuvant (Sigma, Mo) subcutaneously at the tail base as previously described [8]. Sham animals were injected with complete Freund's adjuvant alone. The data presented represent the summation of three separate experiments.

Urinary frequency

Urinary frequency was measured using ultraviolet (UV) light to detect spots of urine captured on filter paper, as previously described [8, 9].

Western blots

Blood samples were collected under anesthesia via heart puncture 13–15 weeks after bladder antigen injection and the whole blood was kept refrigerated for 24 hours to allow clotting. Animals which underwent heart puncture were killed immediately afterwards. Sera were separated from the cells via centrifugation and stored frozen at −20°C until use. Tissue homogenates from normal Lewis rats (Charles River, MA) were prepared and proteins (~600 μg per lane) separated via electrophoresis under reducing conditions according to the method of Laemmli [6] on 15% polyacrylamide gels. Only one preparation per tissue type was used for each blot, which was then probed with multiple sera. Thus differences in staining from lane to lane can not be due to variations in the tissue preparation. Proteins were transferred to nitrocellulose and nonspecific binding to the blot was blocked using 3% nonfat dry milk solids in phosphate buffered saline (PBS; pH 7.4). Sera were incubated with the blot in a Decaprobe apparatus (Hoeffer, a division of LKB-pharmacia, CA) at a 1:200 dilution in 1% nonfat dry milk/PBS overnight. After extensive washing, goat anti-rat antiserum coupled to peroxidase (Boehringer Mannheim, CT) was used at a dilution of 1:250 as a secondary antibody. Color was developed using diaminobenzidine according to the manufacturer's directions (Vector, CA).

Immunoprecipitation

Spleen, kidney, lung, liver and bladder were taken from the same female Lewis rat for this study. Tissues were homogenized in 10 volumes of ice cold 50 mM Tris HCl buffer (pH 7.5) containing 150 mM NaCl, 10 mM EDTA, 1% Triton-X100, and 1 mM each Nα-p-Toeyl-L-lysine Choloromethyl Ketone (TLCK), N-p-Tosyl-L-phenylalanine Choloromethyl Ketone (TPCK) and Phenylmethylsulfonyl Fluoride (PMSF) as proteinase inhibitors and boiled for 10 min. Insoluble material was removed by centrifugation and then 1 ml of each supernatant was incubated with 50 μl of positive antiserum from EAC animal 212 overnight at 4°C. Protein G-coupled agarose was added for 1 h at room temperature. The beads were collected by centrifugation and antigens and antibodies were eluted by boiling in Laemmli buffer. Equal volumes of eluted antigen were added per lane on a 15% precast minigel (Biorad, CA) and blotted as described above except that the substrate used was the Supersignal Ultra chemiluminescent peroxidase substrate (Pierce, IL) detected on autoradiographic film (Kodak XAR, NY).

Statistics

Data were analyzed using the Student's *t*-test and one way ANOVA (Prism 2.01, Graphpad, CA) test. An occasional animal (3/27) developed bacteriuria (as determined by positive urine cultures) and thus was discarded from the analyses.

Results

Elevated urinary frequency

In a previous paper [8] we defined animals as having EAC when the urinary frequency exceeded a threshold of 1 standard deviation above the mean frequency for sham-injected animals. We have now studied such animals for longer periods of time, up to 9 weeks post-disease induction and have two indices we can use to define EAC, "peak urinary frequency" and "mean urinary frequency".

Peak urinary frequency

We defined "peak urinary frequency" as the highest number of urinations/2 h achieved over the first 9 weeks post-disease induction. Bladder antigen injected animals had an statistically significant increase in peak urinary frequency (peak frequency = 16 ± 1 SE urinations per 2 h, $n = 18$) over the peak frequency of sham-injected controls (peak frequency = 12 ± 1 urinations per 2 h, $n = 6$, $P = 0.015$, Students *t*-test) (Fig. 1A). Further, if

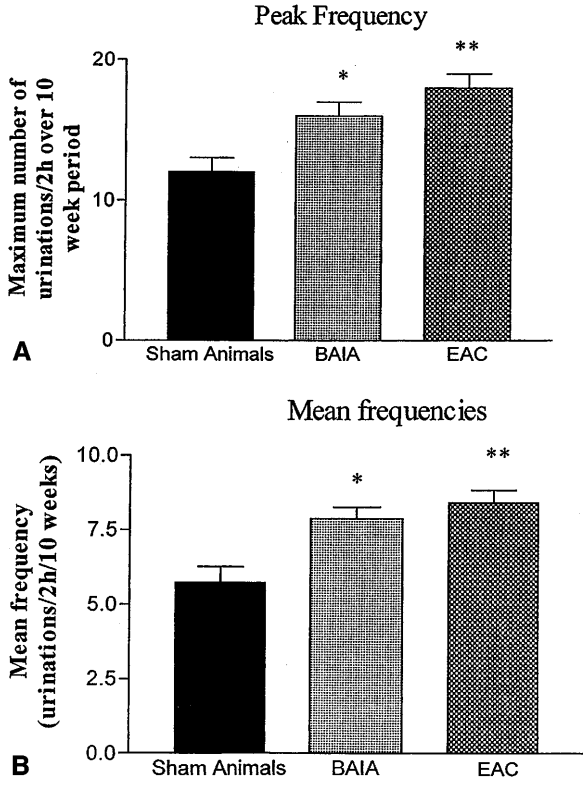

Fig. 1 A Difference in peak urinary frequency between sham, all bladder antigen-injected animals (BAIA), and experimental autoimmune cystitis (EAC) animals. EAC animals were defined as animals whose peak urinary frequency exceeded the threshold of 14 urinations per 2 h as described in the text. Frequency was measured the same day each week from 10:00 to 12:00 h using a fluorescent assay [9]. Data significant to the $P = 0.0055$ level, one way ANOVA (Prism, Graphpad). Using the Bonferroni Multiple Comparisons test, BAIA and EAC are both significantly different from shams: $^*P < 0.05$; $^{**}P < 0.01$. **B** Difference in mean urinary frequency between sham and EAC animals over the duration of the study. EAC animals were defined as above. Frequency was measured the same day each week from 10:00 to 12:00 h using a fluorescent assay [9]. Data significant to the $P = 0.0050$ level, one way ANOVA (Prism, Graphpad). Using the Bonferroni Multiple Comparisons test, BAIA and EAC are both significantly different from shams: $^*P < 0.05$; $^{**}P < 0.01$

non-responders were excluded from the analysis, i.e., those animals which received the bladder antigen injections but never passed the threshold of 14 urinations per 2 h (mean of shams +1 standard deviation), the mean peak frequency for EAC animals increased to 18 ± 1 urinations per 2 h (statistically significant difference from shams, $P = 0.0001$, Students t-test, $n = 13$; 13/18 = 72% are responders; when analyzed together, by one way ANOVA, the means are statistically different with a P value of 0.0115).

Mean urinary frequency

When urinary frequencies were averaged over 9 weeks, the EAC animals still exhibited a "mean urinary frequency" (8.1 ± 0.5 urinations per 2 h, per week, $n = 13$) significantly above that of sham controls (5.6 ± 0.6 urinations per 2 h, per week, $n = 6$, $P = 0.0041$, repeated measures ANOVA) (Fig. 1B). Thus a threshold mean urinary frequency can also be set for EAC at 6.9 urinations per 2 h (1 standard deviation above the mean for shams).

Exacerbations and remissions

Interestingly, in 8/10 of the animals meeting both of the above EAC criteria, the urinary frequency peaked above threshold (14 urinations per 2 h) twice over the 9-week period, with the peaks separated by at least 1 week of below-threshold frequency. These peaks can be seen for the entire EAC population when the data are presented as the urinary frequency per week for EAC animals, normalized to weekly sham values (Fig. 2).

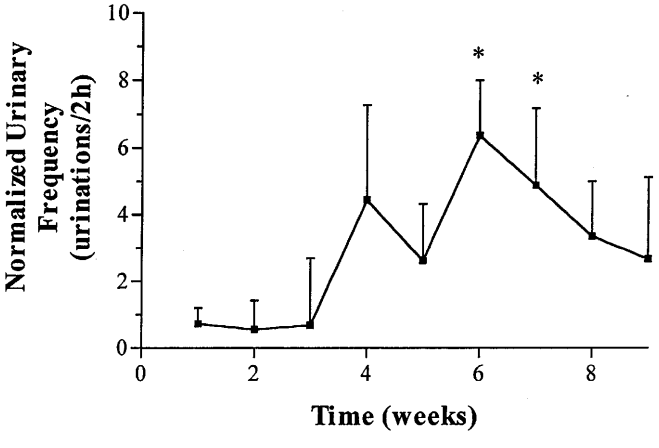

Fig. 2 Urinary frequency changes for all EAC animals over a 9-week period. Animals were injected as described in Materials and methods on day 1. Frequency was measured the same day of the week every week from 10:00 to 12:00 h using a fluorescent assay [9]. Data presented as weekly urinary frequency (over 2 h) above that observed for sham animals on the same day. * Significantly different from shams (Student's t-test)

Western blots

Our previous paper suggested that EAC was an autoimmune disease due to its transferability by adoptive transfer of splenocytes from EAC but not sham animals [8]. However, the lack of classical inflammatory infiltrates was puzzling. We suggested that these data imply that the disease might be due to a secreted product of the transferred cells, such as an antibody, rather than by direct cellular immune response. Thus, in this paper, we investigated the presence of autoantibodies in sera from EAC positive and negative animals [normals, shams, and bladder antigen-injected animals that did not meet the criteria for EAC].

SDS-soluble proteins from a normal bladder were separated by electrophoresis and blotted onto nitrocellulose. Each lane was then incubated with an individual animal's serum. All blots yielded several immunoreactive bands, as would be expected for whole sera containing multiple antibodies. However, only a single immunoreactive protein band was identified in bladder tissue with specificity for EAC (Fig. 3A, B), i.e., the autoantibody was absent from the sera of sham-injected animals, normals, and bladder antigen-injected animals that did not meet the criteria for EAC (Fig. 3A, B, Table 1). The target protein thus identified was determined to have a molecular weight, under reducing conditions, of 12-kDa. In total (summarized in Table 1), 11/12 female animals which were normal, sham-injected or bladder antigen-injected animals but negative for EAC by our criteria, were found to lack the autoantibody directed at the 12-kDa protein, while 9/10 EAC positive animals were found to express this antibody. These data thus indicate a nearly perfect correlation between elevated peak and mean urinary frequency (and, hence, EAC) and expression of this antibody. Of further interest was the finding that with 3 of the 10 EAC positive sera, staining of a prostate antigen of the same molecular weight occurred.

Immunoprecipitation

Using antiserum from EAC animal 212 which was positive for the 12-kDa protein (see Fig. 3C), the presence of the antigen in different tissues was studied by immunoprecipitation followed by Western blotting. Immunoprecipitation was chosen for this experiment in order to increase the assay sensitivity. The 12-kDa protein was precipitated (along with several others) and further identified by the same antiserum. The 12-kDa protein was present, as before, in bladder but was not found in lung, spleen, and kidney, or liver.

Discussion

This paper expands upon an animal model which we have called experimental autoimmune cystitis (EAC).

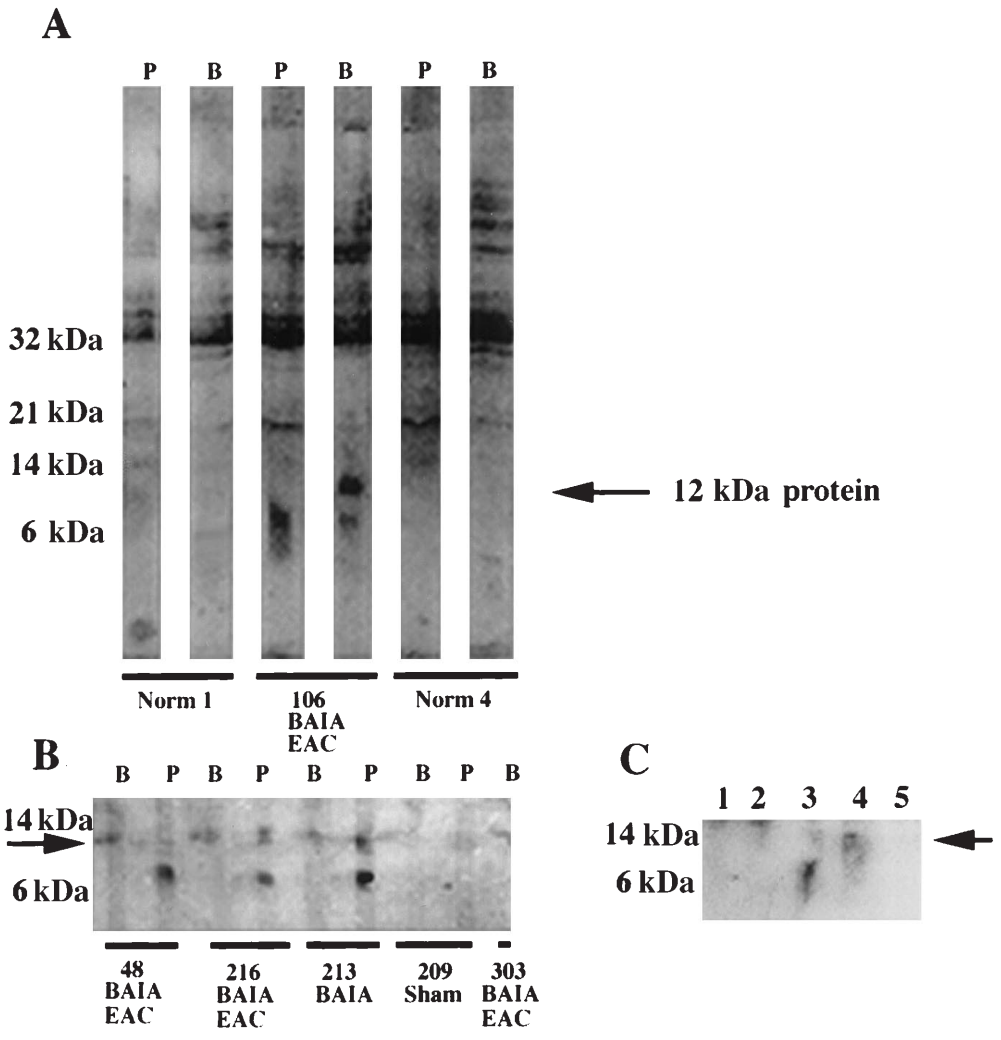

Fig. 3 Western blot analysis identifies a 12-kDa protein (*arrow*) as the target antigen for autoantibodies in EAC sera. *B* bladder homogenate proteins, *P* prostate homogenate proteins. Blot **A** Identification of the 12-kDa protein by serum antibodies: lanes 1, 2 normal sera (Norm 1); lanes 3, 4 EAC animal serum (106); lanes 5, 6 normal sera. Note that only the 12-kDa protein is specific for bladder and disease. Blot **B** Specificity of the anti-12-kDa antibodies for EAC: lanes 1, 2 EAC (48); lanes 3, 4 EAC (216); lanes 5, 6 EAC (213); lanes 7, 8 sham (209); lane 9 EAC (303). Blot **C** Specificity of the antibody for bladder. Immunoprecipitated proteins (see Materials and methods) from the indicated tissues were blotted with antisera from EAC rat No. 212. Lane 1 liver, lane 2 kidney, lane 3 spleen, lane 4 bladder, and lane 5 lung

Table 1 Presence or absence of autoantibodies in serum as detected by Western blot

Animals with normal urinary frequency		Animals with elevated urinary frequency	
Animal ID	Western	Animal ID	Western
Sham 13	–	BAIA 22	+
Sham 14	–	BAIA 27	+
Sham 18	–	BAIA 29	–
Sham 19	–	BAIA 48	+
Sham 209	–	BAIA 49	+
		BAIA 106	+
Norm C1	–	BAIA 108	+
Norm C4	–	BAIA 114	+
Norm C5	–	BAIA 216	+
		BAIA 303	+
BAIA 21	–		
BAIA 43	–		
BAIA 44	–		
BAIA 213	+		
BAIA (male)	–		
Total Controls	12/13 "–"	EAC 9/10 "+"	

BAIA bladder antigen-injected animal, *Sham* complete Freund's adjuvant without bladder antigens injected, *Norm* untreated animals, *EAC* experimental autoimmune cystitis.

We have shown previously [8] that EAC is characterized by pathology (a statistically insignificant increase in degranulated mast cells, vascular congestion and edema) and increased urinary frequency in the absence of bacteriuria as is IC. In this paper we expand upon this definition by showing that not only is the peak urinary frequency elevated in EAC but, in addition, the mean urinary frequency over 9 weeks is elevated when compared with that of sham-injected animals. We also described how the increased urinary frequency in EAC exacerbates and remits much as occurs in IC and other autoimmune diseases (e.g., multiple sclerosis). Further, we identify autoantibodies in the sera of the animals much as autoantibodies have been found in IC patient sera [11]. In other work, not reported here, we have also begun to find similarities between EAC and IC in that both have decreases in urinary glycosaminoglycans which may contribute to the pathogenesis of the condition.

The etiology of IC is unknown although many authors have suggested that it might be an autoimmune disease [4, 11, 13, etc.]. This idea remains controversial, however [1, 12]. One of the contentions with IC being an

autoimmune disease is the lack of inflammatory infiltrates in most bladder specimens (only 20% of all cases, those which have so-called Hunner's ulcers, contain major inflammatory infiltrates). The lack of inflammatory infiltrates is also true for our animal model, EAC. Yet, we have shown it to have an autoimmune etiology [8] via splenocyte transfer experiments. We hypothesized that the reason for the dearth of inflammatory cells in our model as well as in IC must be the presence of a soluble agent such as an antibody or cytokine acting distally to the inflammatory cells or amplifying the effect of a small number of such cells.

We therefore investigated the presence of antibodies in the serum of EAC animals. Indeed, antibodies to a 12-kDa bladder antigen were found which were specific for the disease, i.e., the antibody(ies) was absent or in very low titer in normal and sham-injected animals, as well as bladder antigen-injected animals that did not exhibit increased urinary frequency. The antigen was apparently absent from liver, spleen, lung and kidney and was also absent from prostate in 7/10 cases.

It is intriguing to speculate from these data that there might be some resemblance of the bladder antigen to a prostate antigen. A study similar to ours of target antigens in experimental autoimmune prostatitis [7] also identified a 12-kDa protein specific to prostate. This prostate antigen was identified as a steroid-binding protein. The possibility of our 12-kDa protein being a related protein remains. As EAC occurs only in female rats (our unpublished data) and as IC is associated 10:1 with females over males, the role of steroid hormones in the condition is of extreme interest and warrants further investigation. Further, the suggestion has been made that IC and human prostatitis may be the same or similar diseases. Thus, we have begun to purify our bladder antigen for the purposes of sequencing. Once sequenced we can address the possible involvement of steroids and/or steroid-binding proteins in EAC and hence IC, as well as to address other potential pathogenic mechanisms such as the possible involvement of the protein in neural, growth or immune pathways. Also, once purified we will be able to obtain ample purified antibody with which to identify the anatomical location of the protein via immunohistochemistry.

We have described a strong positive correlation between the presence of autoantibodies in serum directed at a single bladder antigen (the 12-kDa protein identified in Fig. 3) and the disease state. This does not of course prove a causal relationship; however, it is strong evidence in support of one. Transfer of the disease via immune sera alone will determine definitively if autoantibodies are indeed the cause of the condition. We have begun such investigations with encouraging results. Further, induction of the disease with the purified 12-kDa protein will be necessary to prove its role in the disease pathogenesis.

Obviously the significance of our model to IC and hence the significance of the autoantibody against the 12-kDa bladder antigen to the pathogenesis of IC are still open questions. Other forms of cystitis may also present with urinary frequency/urgency and varying degrees of inflammation. However antibodies may be components of these conditions as well. For example in bacterial cystitis, an immune response against the bacteria must occur and antibodies are most likely produced, perhaps leading, at times, to autoimmunity via mimicry. The same could be true for eosinophilic cystitis and is clearly true for Lupus cystitis. Thus the antibodies in any or all of these conditions may be the cause of the pollakiuria observed in these conditions.

As we mentioned above, one hypothesis for the etiology of IC is autoimmune disease. However due to the lack of bladder specificity of the autoantibodies which have been identified in IC sera to date [e.g., 11], these autoantibodies have been called secondary phenomena. The only paper which has performed Western blots of IC sera [11] did not identify a 12-kDa bladder protein as a target of the autoantibodies. However, the IC sera selected for Western blot analysis in this paper were a select group that had been previously identified as positive for the clinical test for antinuclear antibodies (ANA). This assay involves the staining of a nonbladder cell line with the antisera. Thus sera containing bladder-specific antibodies may have been overlooked. Further, sera from normal patients were not compared via Western blot. As the absence of this antibody in control sera was how we identified it, our protein may have been overlooked or it may not be the target antigen in ANA positive IC patients but may be involved in other IC patient subgroups. Further, Och's study was biased to identify only high molecular weight target antigens (their study used 10% polyacrylamide gels; we used 15% gels) and hence may have overlooked a 12-kDa protein. We are currently using our Western blotting techniques to investigate the presence of autoantibodies and their target antigen(s) in IC sera, paying particular attention to the possibility that our 12-kDa protein may be the target antigen of at least some of the IC autoantibodies. We hope these investigations may shed light on the current controversy as to whether or not IC may have an autoimmune etiology.

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