Gene Expression Profiling of Mouse Bladder Inflammatory Responses to LPS, Substance P, and Antigen-Stimulation

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Inflammatory bladder disorders such as interstitial cystitis (IC) deserve attention since a major problem of the disease is diagnosis. IC affects millions of women and is characterized by severe pain, increased frequency of micturition, and chronic inflammation. Characterizing the molecular fingerprint (gene profile) of IC will help elucidate the mechanisms involved and suggest further approaches for therapeutic intervention. Therefore, in the present study we used established animal models of cystitis to determine the time course of bladder inflammatory responses to antigen, *Escherichia coli* lipopolysaccharide (LPS), and substance P (SP) by morphological analysis and cDNA microarrays. The specific aim of the present study was to compare bladder inflammatory responses to antigen, LPS, and SP by morphological analysis and cDNA microarray profiling to determine whether bladder responses to inflammation elicit a specific universal gene expression response regardless of the stimulating agent. During acute bladder inflammation, there was a predominant infiltrate of polymorphonuclear neutrophils into the bladder. Time-course studies identified early, intermediate, and late genes that were commonly up-regulated by all three stimuli. These genes included: phosphodiesterase 1C, cAMP-dependent protein kinase, iNOS, β -NGF, proenkephalin B and orphanin, corticotrophin-releasing factor (CRF) R, estrogen R, PAI2, and protease inhibitor 17, NF κ B p105, c-fos, fos-B, basic transcription factors, and cytoskeleton and motility proteins. Another cluster indicated genes that were commonly down-regulated by all three stimuli and included HSF2, NF- κ B p65, ICE, IGF-II and FGF-7, MMP2, MMP14, and presenilin 2. Furthermore, we determined gene profiles that identify the transition between acute and chronic inflammation. During chronic inflammation, the urinary bladder presented a predominance of monocyte/macrophage infiltrate and a concomitant increase in the expression of the following genes: 5-HT 1c, 5-HT α 7, β 2 adrenergic receptor, c-Fgr, collagen 10 α 1, mast cell factor, melanocyte-spe-

cific gene 2, neural cell adhesion molecule 2, potassium inwardly-rectifying channel, prostaglandin F receptor, and RXR- β cis-11-retinoic acid receptor. We conclude that microarray analysis of genes expressed in the bladder during experimental inflammation may be predictive of outcome. Further characterization of the inflammation-induced gene expression profiles obtained here may identify novel biomarkers and shed light into the etiology of cystitis. (*Am J Pathol* 2002, 160:2095–2110)

Clinical and animal models of acute and chronic urinary bladder inflammation have provided several lines of evidence suggesting a central role of mast cells, sensory nerves, and neurokinin (NK)-1 receptors. Fundamental work regarding the participation of sensory nerves and mast cells in cystitis provides indirect evidence, such as increased numbers of mast cells in the detrusor and submucosa, and morphological evidence of mast cell activation and degranulation.^{1–4} In addition, the extensive tissue remodeling seen in some clinical forms of bladder inflammation such as interstitial cystitis,⁴ along with increased urinary levels of histamine and tryptase⁵ suggest a role for mast cells. Because both mast cells^{6–8} and NK-1 receptors are increased in bladder biopsies of interstitial cystitis (IC) patients,^{9,10} it is tempting to propose a role for sensory peptides-mast cell communication in the pathogenesis of this disorder.

Experimentally, we have used classical morphometric analysis and microarray technology to determine the role of mast cells, NK-1 receptors, and bacterial toxins in animal models of cystitis. Time-course experiments indicated early and late genes involved in bladder inflammatory responses.¹¹ The availability of mice genetically deficient in neurokinin-1 receptor (NK-1R^{-/-}) allowed us to propose a mandatory role of SP receptors in cystitis.¹² We also determined genes that depend on the presence of tissue mast cells for their expression by comparing inflammatory responses in mast cell-deficient (*Kit^{W/W}*)

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Kit^{W^{-v}}), congenic normal (+/+), and *Kit*^W/*Kit*^{W^{-v}} mice that were reconstituted with +/+ bone marrow stem cells (BMR) to restore mast cells.¹³ Bladder inflammation occurred in +/+ and BMR, but not in *Kit*^W/*Kit*^{W^{-v}} mice. These results demonstrate an important role for mast cells in allergic cystitis and indicate that mast cells can alter their environment by regulating tissue gene expression.¹³ An interesting hypothesis for increased pain observed in cystitis is that bacterial products can exacerbate the activity of sensory peptides. Among the possible mechanisms of LPS-peptide interaction, we found that LPS induced a time-dependent gene up-regulation in the bladder.¹¹ We also reported that intravesical inoculation of mice with LPS induced up-regulation of peptide receptors such as bradykinin-1 (BK1)¹⁴ and NK1.¹⁵ Moreover, we observed that LPS-induced cystitis was associated with activation of nuclear transcription factor κ B (NF- κ B).¹⁵ Inhibition of NF- κ B with lactacystin blocked LPS-induced inflammation and NK1 receptor up-regulation.¹⁵

It is not clear from available data which responses of the urinary bladder are inflammatory stimulus-specific, and/or whether there are some central universal patterns of response. Defining the universal and stimulus-specific patterns of response to inflammatory stimuli is critical to begin to understand why some acute inflammatory responses resolve and some transition to the chronic inflammatory process. New methods of cDNA microarray analysis of gene expression provide fresh tools to address these issues definitively.

Our overall hypothesis is that bladder responses to inflammation elicit a specific universal gene expression response regardless of the stimulating agent. Therefore, we sought to compare bladder inflammatory responses to antigen, LPS, and SP by morphological analysis and cDNA microarray profiling. In addition, we determined gene profiles that identify the transition between acute and chronic inflammation. Such studies may provide important insight into human bladder disorders such as IC, as obtaining large-scale gene expression profiles of inflammation may allow for the future identification of subsets of genes that function as prognostic disease markers or biological predictors of a therapeutic response.

Materials and Methods

Animals

All animal experimentation described here was performed in conformity with the "Guiding Principles for Research Involving Animals and Human Beings" (OUHSC Animal Care and Use Committee protocol #00-109).

Three groups of 10- to 12-week-old female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in these experiments. Animals were maintained in housing facilities and allowed food and water *ad libitum*.

Antigen Sensitization Protocol

All mice in this study were sensitized intraperitoneally (i.p.) with 1 μ g DNP₄-human serum albumin (HSA; Mo-

lecular Probes, Eugene, OR) in 1 mg alum on days 0, 7, 14, and 21. This protocol induces sustained levels of IgE antibodies up to 56 days postsensitization.¹⁶ One week after the last sensitization, cystitis was induced.

Induction of Cystitis

Acute cystitis was induced as we described previously.^{11-13,17} Briefly, sensitized female C57BL/6J mice were anesthetized (ketamine 40 mg/kg and xylazine 2.5 mg/kg, i.p.), then transurethrally catheterized (24-gauge; 3/4 inch; Angiocath, Becton Dickinson, Sandy, UT), and the urine was drained by applying slight digital pressure to the lower abdomen. The urinary bladders were instilled with 150 μ l of one of the following substances: pyrogen-free saline, SP (10 μ mol/L), *Escherichia coli* LPS strain 055:B5 (Sigma, St. Louis, MO; 100 μ g/ml), or antigen DNP₄-OVA (1 μ g/ml). Substances were infused at a slow rate to avoid trauma and vesicoureteral reflux.¹⁸ To ensure consistent contact of substances with the bladder, infusion was repeated twice within a 30-minute interval and a 1-ml Tb syringe was maintained on the catheter end retained intravesical solution for at least for 1 hour. After that the catheter was removed and mice were allowed to void normally. One, four, and twenty-four hours after instillation, mice were sacrificed with pentobarbital (20 mg/kg, i.p.) and bladders were removed rapidly.

Chronic cystitis was induced by LPS (100 μ g/ml) instillations performed every 24 hours for 4 days. Mice were sacrificed 24 hours after the last instillation. Control mice for this group received the same volume of pyrogen-free saline at the same time points and will be denoted as saline chronic hereafter. Bladders from all experimental treatments were randomly distributed into the following groups: RNA extraction ($n = 3$), replicate of RNA extraction ($n = 3$), and morphological analysis ($n = 6$).

Alterations at Histological Level

The urinary bladder was evaluated for inflammatory cell infiltrates, mast cell numbers, and the presence of interstitial edema. A semiquantitative score using defined criteria of inflammation severity was used to evaluate cystitis (11-13,17). A cross-section of bladder wall was fixed in formalin, dehydrated in graded alcohol and xylene, embedded in paraffin, and cut serially into four 5- μ m sections (8 μ m apart) to be stained with hematoxylin and eosin (H&E) and Giemsa. Tissues that received chronic stimulation with saline or LPS underwent immunohistochemistry with rat anti-mouse macrophage monoclonal antibody (MCA519G, Serotec LTD, Oxford, UK) and secondary antibody anti-mouse Fab-HRP. This antibody reacts with the 110-kd Mac-3 antigen expressed on the mouse mononuclear phagocytes.¹⁹ Histology slides were scanned using a Nikon digital camera (DXM1200; Nikon, Japan) mounted on a Nikon microscope (Eclipse E600, Nikon). Image analysis was performed using a MetaMorph Imaging System (Universal Imaging Corporation, West Chester, PA). The severity of lesions in the urinary bladder was graded^{11-13,17} as follows: 1+, mild (infiltrate

tion of a 0–10 neutrophils/cross-section in the lamina propria, and little or no interstitial edema); 2+, moderate (infiltration of 10–20 neutrophils/cross-section in the lamina propria, and moderate interstitial edema); 3+, severe (diffuse infiltration of >20 neutrophils/cross-section in the lamina propria and severe interstitial edema). Identification of mast cells and quantification of their degree of degranulation was performed in Giemsa-stained sections. Degree of degranulation is presented as the percentage of mast cells per cross-section that exhibited degranulation.

Sample Preparation for cDNA Expression Arrays

We used the same sample preparation technology as described previously.^{11,13} Briefly, three bladders from each group were homogenized together in Ultraspec RNA solution (Biotech, Houston, TX) for isolation and purification of total RNA. Mouse bladders were pooled to ensure enough RNA for gene array analysis. The justification for this approach is that there is not enough RNA in a single mouse bladder for performing cDNA array experiments and the purification step reduces the yield of total RNA. RNA was DNase-treated according to manufacturer's instructions (Clontech Laboratories, Palo Alto, CA), and 10 μ g of RNA was evaluated by denaturing formaldehyde/agarose gel electrophoresis. This procedure was repeated using an additional three bladders in each experimental group. Therefore, two pools of RNA were generated per experimental group for a total of six mice and two separate hybridizations per group.

Mouse cDNA Expression Arrays

cDNA probes prepared from DNase-treated RNAs obtained from each of the experimental groups were hybridized simultaneously to membranes containing Atlas Mouse 1.2 Arrays (Clontech, Cat. #7853–1). A complete list of genes present in this array can be found at <http://www.clontech.com/atlas/genelists/index.html>. Briefly, 5 μ g of DNase-treated RNA was reverse-transcribed and labeled with [α -³²P]dATP, according to the manufacturer's protocol (Clontech). The radioactively labeled complex cDNA probes were hybridized overnight to mouse cDNA expression arrays (Clontech) using ExpressHyb hybridization solution with continuous agitation at 68°C. After high- and low-stringency washes, the hybridized membranes were exposed overnight at room temperature to a ST Cyclone phosphor screen (Packard BioScience Company, Downers Grove, IL).

Quantification of Gene Expression

The phosphorimaging screen contains phosphor crystals that absorb the energy emitted by the radioactivity of the sample and re-emits that energy as a blue light when excited by a red laser. Results are presented as digital light units (DLU). Spots on the arrays were quantified using grid analysis provided by OptiQuant Image Analysis Software (Packard BioScience Company, Downers

Grove, IL). Quantification of each detectable spot was performed by measuring the digital light units generated by OptiQuant. The results were placed into an Excel spreadsheet (Microsoft Corporation) and the background was subtracted. Previous experiments indicate that inflammation did not alter β -actin expression.^{11,13} As described before,¹³ within each membrane, expression was calculated as percentage of β -actin. Genes whose expression was lower than 0.2% of β -actin were considered too close to the background and discarded.

To determine the reproducibility of the arrays, we performed regression analysis using an Excel spreadsheet. This test compared the results obtained with two pools of RNA isolated from mice that were challenged with saline. cDNA probes were prepared from DNase-treated RNAs and two separate hybridizations were performed. The same analysis was performed using RNA isolated from mice that were challenged with antigen, LPS, and SP.

Cluster Analysis

Genes presenting a similar expression profile were identified based on a Euclidean distance metric, and normalized within experiments to a mean of zero and a standard deviation (SD) of 1. Cluster analysis was performed using self-organizing maps (SOMs) as described by Tamayo et al.²⁰ SOM is an unsupervised network learning algorithm which has been successfully used for the analysis and organization of large data files.²¹ We applied SOM to analyze the time course of gene regulation during LPS-induced cystitis¹¹ and others have shown that SOM is an excellent tool for the analysis and visualization of gene expression profiles.^{20–22} SOMs are a type of mathematical cluster analysis that is particularly well suited for recognizing and classifying features in complex, multidimensional data.²⁰ The method has been implemented in the GeneCluster software, which performs the analytical calculations and provides easy data visualization (http://www_genome.wi.mit.edu/cancer/software/software.html). This focuses attention on the “shape” of expression patterns rather than on absolute levels of expression. The advantage of this approach is that large data sets can be clustered much faster than by using hierarchical clustering because a lower number of clusters are assigned.

Bladder Genes Regulated in Common by LPS-, SP-, and Ag-Induced Inflammation

To determine which genes had their expression altered during inflammation regardless of the initiating stimulus, we set the arbitrary criteria that the gene should be up-regulated at least threefold in response to antigen-, LPS-, or substance P-challenge when compared to saline-treated bladder tissue. For this purpose, Venn diagram analysis was performed using GeneSpring software (Silicon Genetics, Redwood City, CA) using raw data and filtering genes that were at least threefold up-regulated when comparisons were made within each animal group between two conditions, antigen- and saline-challenge. Another set of analyses was performed on the same

group of mice by comparing gene up-regulation in response to SP- and saline-challenge and between LPS- and saline-challenge. Finally, we determined genes that satisfied all three conditions in response to antigen, LPS, and substance P. To determine gene down-regulation, we determined the ratio of expression between saline-treated and the respective stimulus.

Bladder Genes Expression Specifically Regulated by Each Stimulus

To determine which genes had their expression specifically regulated by each stimulus, we set the arbitrary criteria that the gene should be up-regulated at least threefold in response to one stimulus and the same gene should not be up-regulated in response to the other two stimuli. For this purpose, Venn diagram analysis was performed using GeneSpring software (Silicon Genetics) using raw data and filtering genes that were at least threefold up-regulated in a single group.

LPS-Induced Acute and Chronic Alteration in Gene Expression

Results obtained with acute LPS stimulation (1, 4, and 24 hours) were compared to chronic LPS stimulation. For this purpose, genes that were up- or down-regulated at least threefold regarding their respective control were sorted by SOM as described above.

Statistical Analysis

For each group, 1200 genes were analyzed by two different hybridizations using two different pools of RNA isolated from sensitized mice that were challenged with saline, antigen, LPS, and SP. Mice were sacrificed following three different time points (1, 4, and 24 hours after acute stimulation). Therefore, a total of 28,800 data points were analyzed using GeneCluster software. Comparison between acute (1, 4, and 24 hours) and chronic LPS- and saline-stimulation were performed using 19,200 data points and were also analyzed by GeneCluster software. For analysis of acute and chronic results, SOMs were constructed by choosing a 6×4 grid that generated 24 clusters. As this type of analysis assumes that the data can be divided into a certain number of clusters and that they are well separated, it was also our concern to present the fewest clusters possible that would still give a clear picture of antigen- and substance P-induced gene expression. Increasing the number of clusters by increasing the grid did not produce additional correlation between genes. The GeneCluster software provides a list of genes present in each cluster and a centroid. However, to permit comparisons, in each cluster, gene expression was averaged and the SEM calculated. In each cluster, comparisons between gene regulation in response to antigen-, LPS, and SP-challenge were obtained by the ratio of gene expression in relation to the correspondent

Table 1. Histological Severity of Antigen-, SP-, and LPS-Induced Cystitis

Stimulus	Edema	PMNs	Mast cells*
Saline	0.10 $\pm$ 0.14	0.18 $\pm$ 0.14	8.0 $\pm$ 0.7
LPS	2.70 $\pm$ 0.10 [†]	2.30 $\pm$ 0.10 [†]	10.1 $\pm$ 1.7
SP	2.10 $\pm$ 0.20 [†]	2.20 $\pm$ 0.20 [†]	8.4 $\pm$ 2.6
Antigen	2.88 $\pm$ 0.06 [†]	2.42 $\pm$ 0.11 [†]	7.5 $\pm$ 0.4 [‡]

The histologic severity of cystitis was graded by a score of 0–3.

*Number of mast cells per bladder cross-section. Bladder tissue was fixed in formalin and then paraffin-embedded. Four 5- μ m serial sections (8 μ m apart) were stained with hematoxylin and eosin for histological analysis. Additional sections were stained with Giemsa for evaluation of mast cell numbers. Values are means $\pm$ SEM. *P* values were obtained using Wilcoxon's rank sum test.

[†]*P* < 0.0001 vs. the respective control (instilled with saline).

[‡]Not significant when compared with the respective control (instilled with saline).

saline group. Significant differences were obtained by unpaired Student's *t*-test.²³

The statistical analysis of histological data were performed using Wilcoxon's rank sum test. Results are expressed as mean $\pm$ SEM. The *n* values reported refer to the number of animals used for each experiment. In all cases, a value of *P* < 0.05 was considered indicative of significant difference.²³

Reagents

Dinitrophenyl (DNP) was conjugated to ovalbumin (OVA) and human serum albumin (HSA) (Sigma) as previously described.²⁴ Alum adjuvant was purchased from Inter-gen (Purchase, NY). *E. coli* LPS strain 055:B5 was purchased from Sigma. All drugs were prepared in pyrogen-free saline immediately before use.

Results

Morphological Consequences of Acute Antigen-, SP-, and LPS-Challenge

We examined whether the mouse bladder mounts an inflammatory response to all three stimuli by morphological analysis of tissue sections (Table 1). LPS, SP, and Ag induced slightly different degrees of acute inflammation characterized by vasodilation, edema, and intense polymorphonuclear neutrophil (PMN) infiltration in the mucosa and submucosal layers (Table 1). Confirming our results obtained with LPS,¹¹ substance P,¹⁷ and antigen stimulation,¹² both edema formation and PMN migration were observed as early as 4 hours and were stabilized 24 hours after stimulation (data not shown). In a separate study, we also examined additional time points at 48 and 72 hours after LPS stimulation to make sure the maximal PMN infiltration and edema was obtained at 24 hours.¹¹ In conclusion, our results indicate that both edema and PMN migration were observed as early as 4 hours and peaked at 24 hours. In contrast, animals catheterized and instilled with saline did not develop an inflammatory response (data not shown).

Figure 1. A-F: Acute and chronic inflammation. **A:** H&E stains of cross-sections of mouse urinary bladder responding to acute (**A** and **D**) and chronic (**B**, **C**, **E**, **F**) intravesical stimulation with LPS. Notice intense leukocyte infiltrate in the acute response (**A**, **D**) and intense perivascular infiltrate of plasma cells, lymphocytes, and monocytes in the chronic model (**B**, **C**, **E**, **F**). **D-F:** Immunohistochemistry of cross-sections obtained from chronic inflamed bladders stained with rat anti-mouse macrophage/monocyte antibody and secondary antibody anti-mouse Fab-HRP. Black **arrow** and **circle** indicate a plasma cell, red **arrows** indicate PMNs, white **arrows** indicate lymphocytes; and green **arrows** indicate macrophages/monocytes.

Morphological Alterations Secondary to Chronic LPS Stimulation

Chronic inflammation was provoked by repeated bladder instillation with LPS. During chronic inflammation, cross-sections of the mouse bladder presented a mixed inflammatory cell infiltrate containing predominantly macrophages, lymphocytes, and plasma cells, with some polymorphs as minor components (Figure 1). The most dramatic alteration of chronic bladder stimulation with LPS was the presence of macrophages/monocytes in the suburothelial and submucosa layers as determined by immunohistochemistry using a rat anti-mouse macrophage/monocyte antibody (MCA519G) and a decrease in the numbers of PMNs.

Reproducibility of Array Hybridization

We previously presented evidence of the reproducibility of gene-array methodology for the analysis of bladder inflammatory genes¹¹ and verified the results using RNase protection assay.¹³ In the present work, we determined the reproducibility of our hybridization technique by performing regression analysis from values obtained with two different pools of RNA. Table 2 represents regression analysis of RNA isolated from the bladder of sensitized mice that were challenged with saline, LPS, antigen, and SP and sacrificed 24 hours later. All results indicate good correlations between samples resulting in correlation coefficients and slopes of the regression lines not different from 1 (Table 2).

Bladder Genes Commonly Regulated by LPS-, SP-, and Ag-Induced Inflammation

We profiled gene expression across multiple time points in the bladder following stimulation with antigen, LPS, SP, or saline. Organs were pooled from at least three mice for each time point of the study, to provide enough RNA for the study and to minimize variation between animals. In all, 24 1.2K mouse cDNA microarrays were used to assess two replications of gene expression in response to four different stimuli (saline, LPS, antigen, SP) at three time points (1, 4, and 24 hours).

To determine which genes had their expression altered during inflammation regardless of the initiating stimulus, we set the arbitrary criteria that the gene should be up-regulated at least threefold in response to antigen-,

Table 2. Reproducibility of Arrays

Treatment	Regression	Correlation coefficient
Saline	1.1271x–1.280	0.9484
LPS	0.9459x–1.040	0.9715
SP	1.1363x–0.126	0.9528
Antigen	0.9245x–0.620	0.9840

Mice were treated with SP, LPS, antigen, and saline and sacrificed 24 hours after stimulation. Bladders from all experimental treatments were randomly distributed into the following groups: A) RNA extraction ($n = 3$); B) replicate of RNA extraction (see Materials and Methods). Regression lines were obtained by plotting gene expression results of group A versus B.

Figure 2. A-B: Venn diagram of ratio of gene expression obtained in bladder isolated from sensitized C57BL/6J mice that were instilled with 150 μ l of one of the following substances: pyrogen-free saline, SP (10 μ mol/L), LPS (100 μ g/ml), or antigen DNP₄-OVA (1 μ g/ml). Twenty-four hours after, bladders were removed, RNA was extracted, reverse-transcribed to cDNA, and hybridized to 1.2K mouse membranes (Clontech). Venn diagrams were obtained using raw data and filtering genes that were at least threefold up-regulated when comparisons were made within each animal group between the two conditions, antigen and saline, SP and saline, and LPS and saline. Results in **A** represent number of genes that were up-regulated and in **B** genes that were down-regulated. For each group 1200 genes were analyzed by two different hybridizations using two different pools of RNA isolated from sensitized mice that were challenged with saline, antigen, LPS, and SP. Mice were sacrificed following three different time points (1, 4, and 24 hours after acute stimulation). Therefore, a total of 28,800 data points were analyzed.

LPS-, or SP-challenge when compared to saline-treated bladder tissue. Venn diagram analysis indicates that 60 genes were up-regulated in response to antigen, SP, and LPS (Figure 2A). In contrast, 24 genes were down-regulated by all three treatments (Figure 2B).

Genes that were up-regulated in response to all three stimuli were further sorted according to peak expression at 1, 4, and 24 hours in early (Table 3A), intermediate (Table 3B), and late genes (Table 3C) and with few exceptions, commonly up-regulated genes exhibited the same time-to-peak of expression in response to all three stimuli. This finding further indicates a consistency of this response to inflammatory stimulus. Those genes included several cell surface and cytoplasmic receptors (neuropeptides, neurotransmitter, hormones, and growth factors), adhesion proteins, transcription factors, and signal transducers. In addition, genes involved in cell sur-

vival and proliferation such as nerve growth factor (NGF) and proto-oncogenes (iNOS) where co-expressed with death receptor proteins, proteases, and protease inhibitors. Moreover, inflammation also altered the expression of cytoskeleton and motility proteins.

Supplemental Table 1 (at www.amjpathol.org) lists genes that were down-regulated by all three stimuli. They include cell adhesion, transcription factors and activators, apoptosis-associated proteins, caspases, cyclins, cell cycle-regulating kinases, nucleotide metabolism, and DNA damage and repair. Together with genes involved in apoptosis and cell survival, several genes encoding growth factors, cytokines and chemokines, and metalloproteinases were also down-regulated. Moreover, inflammation also down-regulated the expression of cytoskeleton and motility proteins. The groups down-regulated by each individual stimulus are presented in Figure

Table 3A. Early Genes Up-Regulated by SP, LPS, and Antigen-Induced Bladder Inflammation

Group	Abbreviation	Gene	GenBank	Antigen			Substance P			LPS		
				1	4	24	1	4	24	1	4	24
Adhesion	semaphorin IIIB	Semaphorin IIIB	X85990	3.0	1.0	1.0	3.0	1.0	1.0	3.0	1.5	1.3
Basic Transcription Factors	CUTL2	Cut-related homeobox-2	U45665	5.5	2.2	2.7	4.0	0.5	1.1	3.4	0.8	1.0
Basic Transcription Factors	SOX13	HMG-box transcription factor	AJ000740	3.0	1.0	0.3	3.0	0.8	1.0	3.0	1.0	1.0
Cytoskeleton & Motility Proteins	vitronectin	Vitronectin precursor	X72091	3.8	1.0	1.0	3.8	1.0	1.0	3.8	1.2	1.0
Growth Factors and Cytokines	FGF 9	Fibroblast growth factor 9	D38258	3.6	1.6	0.5	3.6	1.6	1.0	3.6	1.6	1.0
Growth Factors and Cytokines	MIP2-alpha	Macrophage inflammatory protein 2 alpha	X53798	3.7	1.0	1.0	3.7	1.0	1.0	3.7	1.0	1.0
Growth Factors and Cytokines	VEGF	Vascular endothelial growth factor	M95200	3.7	1.0	0.4	3.7	1.0	1.0	8.6	1.7	1.0
Growth Factors and Cytokines	WNT5B	Wingless-related MMTV integration	M89799	3.7	1.0	1.2	3.7	1.0	1.0	3.7	0.9	1.0
Hormone Receptors	CRF R	Corticotropin releasing factor receptor	X72305	3.8	1.0	1.0	3.8	1.0	1.0	3.8	1.0	1.0
Hormone Receptors	estrogen R	Estrogen receptor	M38651	3.1	1.0	1.0	3.1	1.0	1.0	3.1	0.4	1.0
Hormone Receptors	galanin R 1	Galanin receptor 1	U90657	3.1	1.0	1.0	3.1	1.0	0.3	3.1	1.9	1.0
Hormone Receptors	melanocortin 5 R	Melanocortin 5 receptor	X76295	3.4	1.0	1.0	3.4	1.0	1.0	3.4	0.5	1.0
Intracellular Transducers	TAK1	TGF-beta-activated kinase 1	D76446	3.1	1.0	0.3	3.1	1.0	1.0	3.1	1.0	1.0
Neuropeptides	proenkephalin B	Proenkephalin B precursor	AF026537	3.1	0.5	3.6	4.1	1.1	1.8	3.1	0.9	1.7
Neurotransmitter Receptors	ACH R	Acetylcholine receptor alpha	M17640	3.1	1.0	1.0	3.1	1.0	1.0	3.1	1.0	1.0
Neurotransmitter Receptors	GABRA1	Gamma-aminobutyric-acid R alpha-1	M86566	4.6	1.0	0.6	3.0	1.0	1.0	3.0	1.0	1.0
Neurotrophins	beta-NGF	Nerve growth factor beta precursor	K01759	3.6	1.0	1.0	3.6	1.0	1.0	3.6	0.5	1.0
Oncogenes	c-fos	c-fos	V00727	3.0	0.4	0.3	3.9	0.4	1.4	5.7	2.3	2.7
Oncogenes	fos-B	Fos-B	X14897	0.9	0.9	4.4	3.0	1.0	1.0	3.0	0.8	1.0
Phospholipases & Phosphoinositol	PLC gamma	Phospholipase C gamma	X95346	1.1	0.3	3.0	5.5	1.0	1.3	17.0	3.6	2.6

Ratio of Gene Expression (Treatment/Saline) was calculated from individual expressions as % of beta-actin.
 Numbers in bold indicate peak of gene expression.

Table 3B. Intermediate Genes Up-Regulated by SP, LPS, and Antigen-Induced Bladder Inflammation

Group	Abbreviation	Gene	GenBank	Antigen			Substance P			LPS		
				1	4	24	1	4	24	1	4	24
Adenylate/Guanylate Cyclases	PDE 1C	Phosphodiesterase 1C	L76946	1.5	3.9	3.9	1.5	3.9	3.9	0.6	3.1	1.0
Apoptosis	INOS	Inducible nitric oxide synthase	M87039	1.2	3.0	1.1	1.2	3.0	1.0	1.2	3.0	1.0
Basic Transcription Factors	FOG	Friend of GATA 1	AF006492	1.0	3.8	0.3	1.0	3.8	1.0	1.0	3.8	1.0
Basic Transcription Factors	FREAC7	Forkhead-related TF7	X92498	1.7	3.4	0.5	0.5	3.4	0.4	1.7	3.4	1.0
Basic Transcription Factors	GTX	Homeobox protein GTX	L08074	1.0	3.5	0.3	1.0	3.5	1.0	1.0	3.5	1.0
Basic Transcription Factors	maf1	Transcription factor	L36435	1.0	3.0	0.7	1.0	3.0	0.3	1.0	3.5	1.0
Basic Transcription Factors	NKX-3.2	Drosophila NK3 TF	U87957	0.4	3.4	2.4	0.5	3.4	0.5	1.5	3.4	1.0
CDK Inhibitors	p57kip2	Cdk-inhibitor kip2	U20553	1.0	3.9	1.0	1.0	3.9	1.0	1.0	4.7	1.5
Cell Cycle-Related Proteins	PK cAMP	Protein kinase, cAMP dependent	X61434	4.8	2.1	4.1	1.3	0.9	3.9	3.6	1.1	1.0
Cytoskeleton & Motility Proteins	NEFM	Neurofilament triplet M protein	X05640	1.2	3.1	1.0	1.2	3.1	0.3	1.2	4.1	1.0
Intracellular Kinase Network Members	WBP6	WW domain binding protein 6 serine K	U92456	1.0	3.2	1.0	1.0	3.2	1.0	1.0	3.2	1.0
Intracellular Transducers	DIDFF	DNase inhibited by DNA fragmentation	AB009377	1.0	3.9	1.0	1.0	3.9	1.0	1.0	3.4	1.0
Intracellular Transducers	PAR4	Protease-activated receptor 4	AF080215	1.0	3.9	1.0	1.0	3.9	1.0	1.0	3.7	1.0
Neuropeptides	orphanin	Noiceptin precursor FQ	D82866	0.6	3.4	2.3	0.6	3.4	0.9	0.6	2.6	3.0
Oncogenes & Tumor Suppressors	mybL2	Myb-related protein B	X70472	1.0	3.8	1.4	1.0	3.8	0.3	1.0	4.1	1.0
Transcription Activators & Repressors	EKLf	Erythroid Kruppel-like	M97200	1.0	3.0	1.0	0.4	3.0	1.0	1.0	4.2	3.9
Transcription Activators & Repressors	IRF1	Interferon regulatory factor 1	M21065	2.9	4.4	2.7	0.8	3.1	1.5	10.1	10.8	1.0
Transcription Activators & Repressors	NF-kB p105	Nuclear factor kappaB p105	M57999	1.0	3.8	0.7	1.0	3.8	1.0	1.0	26.7	2.4
Transcription Activators & Repressors	PGDH	D-3-phosphoglycerate dehydrogenase	L21027	0.7	3.2	0.7	1.4	3.2	0.3	1.4	4.2	1.0

Ratio of Gene Expression (Treatment/Saline) was calculated from individual expressions as % of beta-actin.
 Numbers in bold indicate peak of gene expression.

Table 3C. Late Genes Up-Regulated by SP, LPS, and Antigen-Induced Bladder Inflammation

Group	Abbreviation	Gene	GenBank	Antigen			Substance P			LPS		
				1	4	24	1	4	24	1	4	24
Adhesion	occludin	Occludin	U49185	3.3	1.7	4.1	0.5	3.0	3.1	1.8	2.2	3.8
Basic Transcription Factors	CLIM1B	Lim homeobox	U89487	1.0	1.0	3.4	1.0	1.0	3.6	1.0	1.0	3.3
Basic Transcription Factors	PTC1	Patched homolog 1	U46155	0.3	1.0	3.4	1.0	1.0	3.4	1.0	1.0	3.8
Death Receptors	CD 30L	CD 30L receptor	U25416	0.1	0.5	6.1	0.4	0.9	3.6	1.0	1.0	3.6
Growth Factor & Chemokine Receptors	CD 40L R	CD 40L receptor	M83312	0.4	1.0	3.0	1.0	1.0	3.9	1.0	1.2	3.4
Growth Factors and Cytokines	Lymphotoxin	Tumor necrosis factor beta	M16819	1.0	1.0	3.8	0.3	1.0	3.8	1.0	1.2	3.8
Growth Factors and Cytokines	prepro-ET-3	Prepro-endothelin-3	U32330	2.5	1.4	3.9	2.5	1.4	3.9	2.5	0.7	3.3
Growth Factors and Cytokines	THPO	Thrombopoietin precursor	L34169	1.0	1.0	3.8	1.0	1.0	3.8	1.0	0.5	3.7
Intracellular Transducers	FZD9	Frizzled homolog 9	AF033585	1.0	1.0	4.0	1.0	1.0	4.0	1.0	1.0	3.5
Intracellular Transducers	IFNGR2	Interferon-gamma receptor	S69336	1.0	1.2	3.5	1.0	3.3	1.5	1.0	3.3	3.9
Oncogenes & Tumor Suppressors	TSG101	Tumor susceptibility protein 101	U52945	2.5	1.3	7.9	0.7	0.8	3.0	1.7	2.1	4.0
Protease Inhibitors	PAI2	plasminogen activator inhibitor 2	X16490	0.8	1.2	3.8	0.8	0.9	3.8	0.8	1.3	4.0
Protease Inhibitors	PI 17	Protease inhibitor 17	AJ001700	1.7	0.4	3.8	1.7	1.0	3.8	1.7	1.0	2.7
Proteases	Kininogen	Plasma kallikrein	M58588	1.0	1.0	4.0	1.0	0.6	4.0	1.0	0.7	3.9
Recombination Proteins	DMC1	Meiotic recombination protein	D64107	2.4	0.4	3.7	2.4	1.0	3.7	6.5	0.5	3.6
Unclassified Proteins	formin 4	Formin 4	X62379	0.8	1.3	3.1	0.8	2.2	3.1	2.6	4.0	3.6
Unclassified Proteins	huntingtin-AP	Huntingtin-associated	AJ000262	1.1	0.8	3.5	1.4	2.2	3.5	7.4	5.1	3.1
Unclassified Proteins	PRESENILIN-1	Presenilin-1	AF007560	1.1	0.5	3.6	1.1	1.6	3.6	6.5	1.9	3.8

Ratio of Gene Expression (Treatment/Saline) was calculated from individual expressions as % of beta-actin. Numbers in bold indicate peak of gene expression.

2B and listed on Supplemental Table 2 that can be found on the AJP website: <http://www.amjpathol.org>.

Bladder Genes Uniquely Regulated by LPS-, SP-, and Ag-Induced Inflammation

Venn diagram analysis (Figure 2) also indicates that 82 genes were up-regulated in response to antigen alone, six genes were up-regulated by SP alone, and 39 genes were up-regulated specifically by LPS. (Supplemental Table 3A and Tables 4A and 4B)

Antigen-Induced Gene Regulation

In addition to genes that were commonly up-regulated by all three stimuli, we selected a group of genes that were up-regulated specifically by antigen challenge (Supplemental Table 3A at www.amjpathol.org). Those genes include several receptors for neurotransmitters and sensory peptides such as endothelin B, glutamate, CGRP, GABA-A transporter, and NMDA2B. Interestingly, antigen stimulation also up-regulated cytokines and growth factors, including insulin-like growth factor. In addition, genes with complementary functions were simultaneously up-regulated; this was the case of genes involved in inflammatory cell migration through the endothelium. These included GLYCAM 1, VEGFR1, VE-cadherin, and cadherin 5. Moreover, several genes related to the expression of heat shock proteins (HSP) were concomitantly up-regulated. These included HSP transcription factor 1, HSP27, and HSP105. Finally, antigen also altered the expression of proteases inhibitors, as well as glutathione S-transferase GST μ and θ 1.

SP-Induced Gene Regulation

In addition to NGF up-regulation that occurred in response to all stimuli, SP also induced the expression of glial cell line-derived neurotrophic factor (Supplemental Table 3B). Another gene uniquely altered by SP was ICAM1. It remains to be determined whether the differential expression of ICAM1 by SP and GLYCAM 1, VEGFR1, VE-cadherin, and cadherin 5 by antigen may be responsible for a differential recruitment of mast cells and immune cells to the site of inflammation.

LPS-Induced Gene Regulation

LPS specifically up-regulated several genes listed on Supplemental Table 3C. These genes included integrin- α 7 and CD14, a cell surface protein involved in LPS binding. Interestingly, in addition to the up-regulation of NGF, LPS was the only stimulus that also up-regulated NGF-inducible protein. Moreover, in addition to NF- κ B that was up-regulated by all three stimuli, LPS was the only stimulus to induce c-rel, transcription factor AP-1, and transcription factor E2F3.

Gene Up-Regulation by Chronic LPS Stimulation

Chronic stimulation with LPS led to a predominant increase of macrophages and monocytes in the bladder submucosa. Bladders obtained after chronic LPS stimulation presented a different profile of both up-regulated and down-regulated genes (Table 4, A and B, respectively).

Genes that were uniquely up-regulated during chronic inflammation included those involved in tissue remodel-

Table 4A. Genes Up-Regulated by Chronic LPS-Challenge

Group	Abbreviation	Name	GenBank	LPS/ Saline
Adhesion	NCAM2	neural cell adhesion molecule 2 precursor	AF001287	3.1
Adhesion	semaphorin F	semaphorin F	X97817	3.0
Basic Transcription Factors	DE 1	dermis expressed 1	U36384	4.3
Basic Transcription Factors	MRG1	MSG-related protein 1	Y15163	3.8
Basic Transcription Factors	MYF-6	myogenic factor	M30499	3.3
Basic Transcription Factors	PBX2	pre-B-cell leukemia transcription factor 2	AF020198	3.0
Cell Cycle-Regulating Kinases	p58/GTA	p58/GTA	M58633	3.9
Channels (Membrane)	k J12	potassium inwardly-rectifying channel	X80417	3.3
Cytoskeleton & Motility	CK4	basic keratin complex 2 gene 4	X03491	3.5
Cytoskeleton & Motility	COL10A1	collagen 10 alpha 1	X67348	3.3
Death Receptors	RXR-beta c	RXR-beta cis-11-retinoic acid R	X66224	4.2
Growth Factors and Cytokines	HGF	hepatocyte growth factor	X72307	5.4
Growth Factors and Cytokines	mast cell factor	mast cell factor	U44725	3.1
Growth Factors and Cytokines	SARP1	secreted apoptosis-related protein 1	AF017989	4.0
GTP/GDP Exchangers	Rab GDI alpha	Rab GDI alpha	U07950	4.6
Hormone Receptors	adrenergic Rb2	adrenergic receptor, beta 2	X15643	4.7
Hormone Receptors	prostaglandin F R	prostaglandin F R	D17433	4.7
Intracellular Transducers	TSC2	Tuberin	U37775	3.0
Intracellular Transducers	CISH7	cytokine inducible SH2-containing protein 7	U88325	4.4
Intracellular Transducers	Eph3	tyrosine-protein kinase receptor	L25890	3.9
Intracellular Transducers	notch3	neurogenic locus notch homolog 3 precursor	X74760	3.0
Kinase Activators & Inhibitors	Mac-Marcks	Marcks-related protein	X61399	3.3
Neurotransmitter Receptors	5-HT1c	5-hydroxytryptamine R 1c	X72230	3.1
Neurotransmitter Receptors	5-HT7	5-hydroxytryptamine R 7	Z23107	3.5
Neurotransmitter Receptors	GABA-A beta 2	gamma-aminobutyric acid	U14419	4.7
Oncogenes & Tumor Suppressors	c-Fgr	c-Fgr	X52191	3.6
Other Cell Cycle Proteins	myeloblastin	trypsin-chymotrypsin serine protease	U43525	3.7
Protease Inhibitors	ALP	antileukoproteinase 1 precursor	U73004	3.1
Receptors (by Ligands)	LDLR	low-density lipoprotein receptor precursor	Z19521	3.2
Transcription Activators & Repressors	PAX-8	paired box protein PAX 8	X57487	4.0

ing such as mast cell factor, melanocyte-specific gene 2, neural cell adhesion molecule 2, and collagen 10 α 1. Moreover, chronic stimulation with LPS up-regulated cellular and cytoplasmatic receptors such as serotonergic 1c and 7, β adrenergic 2, prostaglandin F receptor, and RXR- β cis-11-retinoic acid.

Chronic stimulation with LPS resulted in down-regulation of several genes (Table 4B). Interestingly, some of genes down-regulated during chronic inflammation had their expression specifically up-regulated by acute LPS administration. Those include CD14, integrin α 7, and matrix metalloproteinase 11. It remains to be determined whether this down-regulation is part of a compensatory mechanism or a consequence of LPS-induced death and shedding of urothelial cells.¹⁷ As the urothelium works with underlying afferent nerves to detect the presence of irritant stimuli,²⁵ it will be of value to determine whether CD14, integrin α 7, and MMP11 are part of the unique set of urothelial genes contrasting with those found in the bladder detrusor muscle. Ongoing experiments in this laboratory are determining which genes are uniquely expressed by each of the urinary bladder layers.

Discussion

Response to Injury

The urinary bladder responds to injury with an acute inflammatory response that may be followed by chronic inflammation, repair, fibrosis,²⁶ or loss of function as indicated by a reduced bladder capacity in patients with

chronic cystitis.²⁷ This study expands understanding of two elements of this process: it characterizes both the sets of genes which respond uniformly as well as uniquely to diverse stimuli, and the differences between acute and chronic inflammatory responses.

To facilitate these studies of bladder inflammation, we used our previously developed multiple animal models of cystitis. We have also provided evidence of LPS-induced cystitis¹⁷ and the time-dependent gene expression in response to LPS,¹¹ but could not define common responses from a single model system. Morphological changes confirm the presence of the cells pathognomonic of acute and chronic inflammation in these models. Immunohistochemistry indicates that during the transition between acute and chronic stimulation, the predominant cell type that infiltrates into the bladder changed from polymorphonuclear leukocytes to monocytes/macrophages.

Genes Up-Regulated by All Stimuli

In the present work, we sought to define common pathways involved in acute forms of bladder inflammation. For this purpose, we investigated the time course of morphological and genomic alterations in responses to antigen-, SP-, and LPS-stimulation. We selected genes that were up-regulated in response to all stimuli (SP, antigen, or LPS) and therefore, represent a relatively universal bladder response to inflammation. One of the most interesting

Table 4B. Genes Down-Regulated by Chronic LPS-Challenge

Group	Abbreviation	Name	GenBank	Saline/ LPS
Apoptosis	CLU	clusterin precursor	L08235	5.0
Apoptosis	CPR	NADPH-cytochrome P450 reductase	D17571	3.7
Basic Transcription Factors	NKX-2.6	Drosophila NK2 transcription factor-related locus 6	AF030113	4.3
Basic Transcription Factors	HFH-4	hepatocyte nuclear factor 3	L13204	3.4
Basic Transcription Factors	SOX13	HMG-box transcription factor	AJ000740	4.0
Basic Transcription Factors	TF II D	transcription initiation factor TF II D	D01034	3.0
Basic Transcription Factors	GLI2	zinc finger protein	X99104	4.6
Cell-Cell Adhesion Receptors	ADAM8	cell surface antigen MS2	D10911	4.1
Cell-Cell Adhesion Receptors	EPHA6	ephrin type A R 6	U58332	5.3
Cell-Cell Adhesion Receptors	CD14	CD14	M34510	4.3
Cell-Cell Adhesion Receptors	integrin alpha 7	integrin alpha 7	L23423	4.4
Cyclins	cyclin T1	cyclin T1	AF109179	3.7
Cyclins	CCNE2	G1/S-specific cyclin E2	AF091432	3.1
Cytoskeleton & Motility	LAMC2	laminin gamma 2 subunit precursor	U43327	4.4
Cytoskeleton & Motility	talin	talin	X56123	3.5
DNA Damage/Repair	CTCBF	ATP-dependent DNA helicase II 70-kDa subunit	M38700	3.1
Growth Factor & Chemokine Receptors	PDGF	pre-platelet-derived growth factor R	X04367	6.0
Growth Factors and Cytokines	VGR1	bone morphogenetic protein 6 precursor	X80992	4.0
Growth Factors and Cytokines	CERR1	cerberus-related protein1	AF031896	4.1
Growth Factors and Cytokines	Mdkk1	dickkopf homolog 1	AF030433	3.8
Growth Factors and Cytokines	GCSF	granulocyte colony-stimulating factor	M13926	5.1
Growth Factors and Cytokines	GDF9	growth differentiation factor 9	X77113	3.0
Growth Factors and Cytokines	HBGF5	heparin-binding growth factor 5 precursor	M30643	4.1
Growth Factors and Cytokines	LIF	leukemia inhibitory factor	X06381	3.7
Growth Factors and Cytokines	SHH	sonic hedgehog homolog	X76290	3.4
Intracellular Adaptors	PTPN11	non-receptor type 11 protein tyrosine phosphatase	D84372	4.2
Intracellular Adaptors	Sik	Src-related intestinal kinase	U16805	4.6
Intracellular Kinase	S6KII-alpha	ribosomal protein S6 kinase II alpha 1	M28489	3.4
Intracellular Transducers	B-50	neuromodulin	J02809	3.7
Intracellular Transducers	TLL	tollid-like protein	U34042	3.6
Ion Channels (Voltage-Gated)	SODIUM CH	voltage-gated sodium channel	L36179	4.6
Oncogenes & Tumor Suppressors	c-fos	c-fos	V00727	5.5
Oncogenes & Tumor Suppressors	c-Src	c-Src	M17031	3.4
Oncogenes & Tumor Suppressors	ERP	ets-domain protein elk3	Z32815	4.8
Oncogenes & Tumor Suppressors	G-protein R 27	G-protein coupled receptor 27	U79525	3.9
Oncogenes & Tumor Suppressors	R-ras	R-ras protein	M21019	4.8
Oncogenes & Tumor Suppressors	MMP11	matrix metalloproteinase 11	Z12604	3.5
Oncogenes & Tumor Suppressors	TSG101	tumor susceptibility protein 101	U52945	3.4
Oncogenes & Tumor Suppressors	Vav	GDP-GTP exchange factor	X64361	7.0
Recombination Proteins	RAG2	V(D)J recombination activating protein	M64796	3.6
Transcription Activators & Repressors	ATOH2	atonal protein homolog 2	U29086	3.4
Transcription Activators & Repressors	IRF1	interferon regulatory factor 1	M21065	3.0
Transcription Activators & Repressors	mf 5	myogenic factor 5	X56182	3.9
Transcription Activators & Repressors	PSD-95	PSD-95/SAP90A	D50621	3.0
Transcription Activators & Repressors	STAT1	STAT1	U06924	4.2
Unclassified Proteins	FMR2	fragile X mental retardation syndrome	AJ001549	3.5
Unclassified Proteins	HUD	Huntington disease homolog	U24233	7.6
Unclassified Proteins	HAP1	huntingtin-associated protein 1	AJ000262	4.6
Unclassified Proteins	petaxin-related	petaxin-related C-reactive protein	X13588	3.4
Unclassified Proteins	PS1	presenilin-1	AF007560	4.6
Unclassified Proteins	tubby	tubby	U54643	3.6
Unclassified Proteins	WSB1	WSB1 protein	AF033186	4.2

aspects of this study was the finding that genes commonly up-regulated by any given stimulus have virtually identical time courses of expression (Table 3, A–C). Together these results strongly suggest the existence of recursive molecular pathways supporting bladder inflammatory responses. This pathway should protect the blad-

der from noxious stimulus, and once the stimulus ceases, gene expression should down-regulate to avoid tissue damage and chronic inflammation. The latter hypothesis is under investigation by our laboratory and preliminary results indicate that the responses of wild-type and mice deficient in anti-inflammatory cytokines (interleukin

(IL)-10 knockout mice) to LPS present a different time course of gene regulation (Nguyen N-B, Saban MR, Hammond TG, Saban R. Time course of LPS-induced gene regulation in the bladder of wild-type and IL-10 knockout mice. Genomics and Proteomics in Kidney and Urologic Diseases Conference, National Institutes of Health, July, 2001).

In addition, we found genes that are up-regulated uniquely by a single stimulus. The reason to present these results is that some readers may be interested in a particular gene or pathway, and therefore, this information indicates which stimulus should be used to alter that particular gene expression. Except for the completeness of the Venn diagrams themselves, we are not sure about the relevance of gene changes common to two stimuli when comparing unique gene alterations in response to one stimulus alone or to all three stimuli.

In terms of time response, morphological results indicate absence of inflammatory cells at early time points and therefore, gene regulation observed at 1 hour may reflect the effect of the stimulus on resident cells. In contrast, at late time points such as 24 hours, a considerable number of migrating inflammatory cells were observed and gene regulation has the potential to be a combined response from both resident and migrating cells.

Experimental bladder inflammations resulted in up-regulation of some gene families regardless of the initiating stimulus. Therefore, we focused this discussion on the commonly expressed gene families that also have been described in the urinary system, urine infection, interstitial cystitis, bladder or prostate cancer, and that participate in the inflammatory response. Those families included several cell surface and cytoplasmic receptors (neuropeptides, neurotransmitter, hormones, and growth factors), adhesion proteins, transcription factors, and signal transducers. In addition, genes involved in cell survival and proliferation such as NGF and proto-oncogenes (iNOS) were co-expressed with death receptor proteins, proteases, and protease inhibitors. Moreover, inflammation also altered the expression of cytoskeleton and motility proteins.

Genes Involved in Bladder Physiology

Several genes that were commonly up-regulated have already been described to participate in mechanisms controlling micturition. These include enkephalins, nociceptin which resembles endogenous enkephalins, GABA A receptor, and endothelins (ET). These proteins and receptors may be involved in the regulation of nociceptive processing. Indeed, systemic or central administration of nociceptin,²⁸ enkephalins (intrathecal naloxone,²⁹) and GABA,³⁰ inhibits the micturition reflex. Interestingly, peripheral inflammation induces nociceptin in primary sensory neurons³¹ and increases pro-dynorphin mRNA in dorsal horn neurons.³² Except for ET receptors which have been described in the bladder and initiate detrusor contractile activity in animals and human detrusor³³ with a potential role in bladder outlet obstruction,³⁴ the infor-

mation is scanty regarding the presence of these proteins in the urinary bladder. Therefore, our results indicate the need for tissue bath experiments to elucidate whether the local expression of these receptors also participates in the altered bladder function observed during inflammation.³⁵

Genes Encoding Proteins Already Described to Be Involved in Interstitial Cystitis

Some of the gene products found up-regulated by all three stimuli have already been associated with interstitial cystitis. These include tryptase receptors (PAR), NGF, iNOS, fibroblast growth factor (FGF), and insulin-like growth factors (IGFs). In addition, heat shock proteins were found among the genes commonly down-regulated. The latter coincides with the description that IC urine alters the production of a 72-kd stress protein by urothelial cells.³⁶

PAR and Tryptase

We presented evidence indicating that mast cell tryptase is elevated in the urine of IC patients⁵ and others presented immunohistological findings of mast cell containing tryptase in the bladder of IC patients.³⁷ Tryptase responses are modulated by proteinase-activated receptors (PAR) coupled to a G-protein. These receptors are present in bladder afferent neurons³⁸ and mediate plasma extravasation induced by tryptase and thrombin.³⁸ PA receptors are also present in normal human kidneys,³⁹ and mediate the response of human proximal tubular cells to thrombin.³⁹ Others have reported that activation of PAR induced detectable levels of IL-6 production in a dose-dependent fashion.⁴⁰ Our results indicated that PAR4 mRNA was up-regulated in response to all three stimuli. As mast cells and tryptase are common features of IC, it is tempting to suggest that tryptase generated during the mast cell degranulation along with an up-regulation of PAR4 may contribute to abnormal neurotransmission and motility in the inflamed bladder. It remains to be determined whether PAR4 are up-regulated in the bladder of IC patients.

NGF

Clinically, NGF levels are higher in samples from patients with painful bladder conditions than in samples from controls⁴¹ and NGF levels are elevated in the urine of patients with IC and bladder cancer.⁵ In addition, overexpression of NGF suggested a potential treatment for diabetic neuropathy⁴² indicating a local control of NGF in bladder motility. Furthermore, recent evidence supports the hypothesis that p75NTR is a mediator of neurotrophin dependence during the critical phase of developmental cell death and during the progression of carcinogenesis in prostate cancer.⁴³ Experimentally, we have shown that NGF is one of the early genes up-regulated by LPS-induced cystitis.¹¹ Others have shown a rapid increase in

both NGF mRNA⁴⁴ and protein⁴⁵ following experimental bladder inflammation. Such neuroplasticity may be a possible explanation for the association of bladder inflammation with long-term symptoms and pain after the inflammation subsides.⁴⁶ More recently, an intriguing hypothesis proposed that alteration in MMP activity may modify the influence of NGF on apoptosis and cell survival. The explanation for such hypothesis is that NGF is secreted in a proform (proNGF) that has high-affinity for p75NT receptor which mediates apoptosis. MMPs and plasmin cleave proNGF generating mature NGF. Mature NGF has high affinity for TrkA receptors which mediates cell differentiation and survival.⁴⁷ In this context it is interesting to notice that along with the up-regulation of NGF, all three stimuli down-regulated both MMP2 and MMP14. Further research is needed to determine the consequences of altered expression of proteinases and tissues inhibitors of proteinases on the balance of pro-NGF and NGF and its consequence on the overall inflammatory response during bladder inflammation.

iNOS

iNOS is expressed in bladder smooth muscle cells and is up-regulated during obstruction,⁴⁸ after treatment with cyclophosphamide,⁴⁹ following *E. coli* infection,⁵⁰ and LPS instillation.⁵¹ In spinal cord injury patients with a chronic indwelling bladder catheter, iNOS is found overexpressed in bladder mucosa macrophages.⁵² In interstitial cystitis patients the results are contradictory since both an increase⁵³ as well a decrease⁵⁴ in NO levels has been reported.

Insulin-Like Growth Factors

Insulin-like growth factors (IGFs), including IGF-1 and IGF-2, are mitogenic peptides for smooth muscle cells and are involved in the regulation of cell proliferation, differentiation, and apoptosis. These factors have been implicated in chronic inflammatory diseases,⁵⁵ prostate,⁵⁶ and bladder cancer.⁵⁷ Overexpression of IGF-I and underexpression of IGFBP 3 and 5 may lead to hyperplasia of the bladder smooth muscle.⁵⁸ Interestingly, IGF is decreased in the urine of IC patients⁵⁹ and an anti-proliferative factor purified from urothelial cells of IC patients has been shown to stimulate the production of IGF binding protein 3.⁶⁰

Heat Shock Transcription Factor 2

Down-regulation of this transcription factor by all three stimuli is noticeable because of the role of heat shock proteins in the balance of cell proliferation and apoptosis. Heat shock factor 1 is overexpressed in human prostate cancer cells.⁶¹ Although there is no previous information on the expression of HSF-2 in the bladder, a recent report indicates that urothelial cells can respond to urine of IC patients with a modest alteration in a 72-kd stress protein.³⁶ It has been shown that heat shock proteins and stress can reduce the inflammatory response. The pro-

posed mechanism involves induction of NF- κ B's endogenous inhibitor, I κ B α , a putative heat shock protein.⁶² Therefore, the interplay between NF κ B and heat shock proteins in cystitis should be pursued.

Cytokines and Cystitis

IL-6 was described as the major cytokine found in human with interstitial cystitis.⁶³ Experimentally however, only LPS was capable of up-regulating this cytokine. An interaction between IL-6 and M-CSF switches monocyte differentiation to macrophages rather than dendritic cells.⁶⁴ It remains to be determined whether or not the increased macrophage accumulation by chronic LPS stimulation depends on such interplay. In addition to IL-6, in experimental cystitis, all stimuli resulted in up-regulation of interferon regulatory factor-1 (IRF-1), interferon-gamma receptor, tumor necrosis factor β , and macrophage inflammatory protein (MIP). In other systems, it has been shown that LPS-induces IRF-1 function synergistically with NF κ B.⁶⁵ Recent evidences indicated that LPS/IFN γ not only induced the production of cytotoxic NO through IRF-1 but also initiated the NO-independent apoptotic pathway through the induction of caspase-11 expression.⁶⁶ In addition, MIP-2 is a major CXC chemokine involved in the migration of PMNs to sites of inflammation. Following intravesical *E. coli* infection, several C-X-C chemokines are secreted into the urine, but only MIP-2 concentrations correlate to neutrophil numbers that cross the epithelial barrier of the infected urinary tract.⁶⁷

Hormone Receptors

The group of hormone receptors (CRF R, melanocortin 5R, estrogen R, galanin R 1) deserves special attention because more than 90% of those affected with IC are women and their pain scores correlate with hormonal variations.²⁷ Indeed, in all models of acute experimental cystitis, up-regulation of several hormone receptors, including estrogen receptor β (ER β), galanin R1, and CRF-R1, was observed.

ER β

ER β is found in the rat urinary tract⁶⁸ and estrogen receptor- α is present in parasympathetic preganglionic neurons innervating the cat bladder.⁶⁹ Estrogen receptors are also expressed in the human prostate.⁷⁰ It is known that estrogen affects autonomic functions such as micturition. In addition, cross-talk between ER and NF κ B does occur *in vivo* and may indeed contribute significantly to effects of estrogen in inflammation.⁷¹ Estrogen receptors have been described on mast cells⁷² and may influence the outcome of cystitis.⁷³ Estrogen can enhance NGF-stimulated neurite sprouting in an ER α -dependent manner.⁷⁴ In addition, short-term estrogen replacement increases β -preprotachykinin mRNA levels in uninjured dorsal root ganglion neurons.⁷⁴ Together these observations emphasize the need of basic research to-

ward the role of estrogen receptor regulation on the bladder responses to inflammation.

Galanin R1

Galanin-immunoreactive nerve fibers have been described in the human detrusor muscle.⁷⁵ Galanin is widely distributed in enteric nerve terminals and acts to modulate intestinal motility by altering smooth muscle contraction and in the human colon, alterations in Gal1-R expression may have an important role in colitis.⁷⁶ Indeed, inflammation up-regulates this receptor by an NF- κ B-dependent pathway.⁷⁷ Interestingly, pathogenic *E. coli*, but not the nonpathogenic strain, activate NF- κ B, and increase Gal-R1 mRNA synthesis.⁷⁸

CRF

CRF is the principal hypothalamic factor governing the pituitary-adrenal axis, but the wide extra-pituitary distribution of CRF and its receptors suggest a major role for this neuropeptide in the integration of the overall physiological and behavioral responses of an organism to stress. CRF receptors have not yet been described in the bladder. However, it has been suggested that CRF from Barrington's nucleus may serve to inhibit micturition.⁷⁹ In addition, CRF receptor type 2 mRNA expression seems to be modulated by LPS and glucocorticoids.⁸⁰ Activation of mast cells seems to be a possible explanation for the pro-inflammatory actions of CRF.⁸¹ Therefore, CRF receptor-mediated effects may contribute to the link between stress and the pathogenesis of cystitis.

Signal Transduction Pathways

We have described an important role for NF- κ B in mediating experimental inflammation in response to LPS.¹⁵ The present results indicate that NF- κ B is a pathway commonly up-regulated as the bladder responds to other pro-inflammatory stimuli such as antigen and SP stimulation. In contrast to NF- κ B that was up-regulated by all three stimuli, LPS was the only stimulus to induce the transcription factor AP-1 and transcription factor E2F3. Among the signal transduction pathway, all three stimuli induced up-regulation of phosphodiesterases (PDE), TGF β -activated kinase 1 (TAK1), and cAMP-dependent protein kinase (PKA).

c-fos

Up-regulation of c-fos is part of human urothelial cell response to arsenic-induced urothelial cell proliferation.⁸² Experimental c-fos and fos-b are part of the early bladder response to LPS.¹¹ Others have shown that c-fos expression in the spinal cord is part of the immediate response of acute irritation or inflammation of the lower urinary tract⁸³ and prostate.⁸⁴ Indeed, following irritation of the lower urinary tract, c-fos gene expression is up-regulated in lumbo-sacral spinal cord neurons by a

mechanism involving activation of tachykinins and NK2 receptors.⁸⁵ As an early responder, c-fos up-regulation can be used to detect how fast a peripheral injury is relayed to the central nerve system.

PDE

Phosphodiesterase 1C was up-regulated by all stimuli. Interestingly, selective inhibitors of the phosphodiesterases have been proposed for the symptomatic treatment of detrusor instability.⁸⁶ Other have presented evidence that both the selective PDE 4 inhibitor, rolipram, and the non-selective PDE inhibitor, theophylline, markedly reduced LPS-induced pulmonary inflammation.⁸⁷ Our preliminary results using rolipram indicate that this compound reduces LPS-induced bladder inflammation (R. Saban, unpublished observations). It remains to be determined whether rolipram can block all forms of experimental cystitis. The continued investigation of PDEs and the development of potent and selective inhibitors should provide more therapeutic agents to decrease the unwanted effects of cystitis.

TGF β activated kinase 1 (TAK1), a mitogen-activated protein kinase (MAPK) is also associated with inflammation and regulates multiple protein kinase cascades activated by LPS.⁸⁸ TAK1 is implicated in TGF β , bone morphogenetic protein (BMP), and IL-1 signaling.⁸⁹ In addition, TAK1 mediates an activation signal from toll-like receptor(s) to nuclear factor- κ B in LPS-stimulated macrophages.⁹⁰ Thus TAK1 must be considered as an important component of intracellular pathways in cells involved in host responses to physiological and/or environmental stress signals during inflammation.

Acute and Chronic Inflammation

Chronic inflammation was characterized by an increased infiltration of monocytes and macrophages into the bladder wall. A distinct group of genes which characterized the transition from acute to chronic inflammation are drawn from pathways already implicated in this process. Despite the observation that the inflammatory process favors tissue degradation, during chronic inflammation, there is also evidence for an evolution toward tissue repair. The release of growth factors and cytokines capable of stimulating the proliferation and synthetic capacity of smooth muscle cells is the main driving force to this end. This process results in the deposition of extracellular matrix components, such as proteoglycans and collagens. The latter could be modulated by genes such as collagen 10 α 1 subunit precursor (COL10A1) and cyto-keratin 4 that were found to be up-regulated here specifically during chronic inflammation. In addition, the increased inflammatory infiltrate may be responsible for up-regulation of mast cell factor, melanocyte-specific gene 2, cytokine inducible SH2-containing protein 7 (CISH7), MSG-related protein 1 (MRG1), and prostaglandin F receptor.

In contrast, several genes were down-regulated during LPS-induced chronic inflammation (Table 5B). Interest-

ingly, some of the genes down-regulated during chronic inflammation had their expression up-regulated specifically by acute LPS administration. Those include CD14, integrin $\alpha 7$, and MMP 11. Both CD14 and integrin $\alpha 7$ ⁹¹ are found in the human bladder mucosa. Integrins ($\alpha 4\beta 7$ integrin) are involved in tissue homing of mast cells⁹² and CD14 plays a major role in the inflammatory response induced by LPS.⁹³ Furthermore, LPS significantly induced CD14 mRNA expression.⁹⁴

A possible explanation for the down-regulation is that during chronic inflammation, inhibitory feedback mechanisms would decrease the expression of CD14, integrin, and MMP11. Indeed, CD14 was found to be inversely correlated with severity of disease.⁹⁵ An alternative explanation is that the down-regulation is a consequence of death and shed of the urothelial cells. In support for this hypothesis, we previously described that the major morphological alteration induced by acute LPS administration in the mouse bladder was the vacuolization of urothelial cells.¹⁷ In addition, others have presented evidence that in response to infection, superficial bladder cells exfoliate and are removed with the flow of urine.⁹⁶ It remains to be determined whether the urothelium presents a unique set of genes in contrast with other regions of the bladder such as the detrusor muscle. Ongoing experiments in this laboratory are determining which genes are uniquely expressed by each of the layers of the urinary bladder.

Proteomic Correlation

To fairly interpret gene cluster analysis we must be aware of a growing body of evidence that gene and protein changes can be dissociated. For instance, increased abundance of urinary bladder nerve growth factor mRNA is not always associated with increased total urinary bladder nerve growth factor.⁴⁵ The discrepancy between two measures (mRNA and protein) may reflect retrograde axonal transport of nerve growth factor to the dorsal root ganglia.⁴⁵ Future proteomic correlation must determine how directly mRNA changes reflect translated protein levels and the physiological consequence of these proteins.⁹⁷ Despite all of the downstream regulatory steps, understanding of the pattern and timing of gene expression changes is pivotal if we are to interpret and eventually learn to intervene in the pathophysiology of cystitis.

Summary

In conclusion, the cDNA array experimental approach characterized the common pathways of inflammatory response to diverse stimuli, as well as the gene expression profiles which characterize the differences between acute and chronic inflammation. These responses in gene expression may represent a balance between the cytoprotective and degenerative processes that accompany bladder response to injury. This sets the stage for future research using gene cluster analysis techniques to begin to understand clinically relevant issues, such as how and why the transition from acute to chronic inflammation occurs only in selected circumstances, and which

pathophysiological and therapeutic strategies change the normal balance of apoptosis and necrosis in populations of bladder cells. Further characterization of the inflammation-induced gene expression profiles obtained here may identify novel biomarkers and shed light into the etiology of cystitis. By profiling the time-dependent gene and protein expression in animal model of bladder inflammation, it may soon be possible to determine potential anti-inflammatory effects of emerging therapeutics.

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