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Mucosal Surface Molecules of the Urinary Bladder

by

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ABSTRACT

It is well established that carbohydrate-rich networks bathe and protect the mucosal surfaces of the body. It has been suggested that a loss of integrity of this barrier could result in or from the submucosal inflammation seen in interstitial cystitis. Thus, this study set out to compare the distribution of mucosal surface molecules in healthy and inflamed human urinary bladders using immunohistochemical techniques. Heparan sulfate, chondroitin-4-sulfate, mucin and placental lactose galectin were detected at the mucosal surface of healthy uninflamed regions. However, increasing levels of submucosal inflammation were accompanied by denudation of the uroepithelium, significant decreases in surface-cell-staining for all markers, and increased levels of lectin and heparan sulfate in the basal lamina. Therefore, a loss of mucosal integrity accompanies interstitial cystitis. Furthermore, an animal model of interstitial cystitis was initiated to demonstrate that these mucosal surface molecules contribute to the prevention of submucosal inflammation.

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DEDICATION

This thesis is dedicated to Elesha, my best friend and the love of my life.

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LIST OF ABBREVIATIONS

μm	micrometer
BL	basal lamina
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
°C	degree Celsius
cc	cubic centimetre
C-4-S	chondroitin-4-sulfate
CIP	chronic idiopathic prostatitis
CRD	carbohydrate recognition domain
CRP	complement regulatory protein
CT	connective tissue
DMSO	dimethyl sulfoxide
DNBS	2,4-dinitrobenzenesulfonic acid
EGF	epidermal growth factor
g	gram
G	globular domain
GAG	glycosaminoglycan
Gal	galactose
GalNAc	N-acetylgalactosamine
GalUA	galuronic acid

GlcNAc	N-acetylglucosamine
GlcUA	glucuronic acid
Gly	glycine
H&E	hematoxylin and eosin
HA	hyaluronic acid
HS	heparan sulfate
IC	interstitial cystitis
kDa	kilodalton
L	litre
LDL	low density lipopolysaccharide
LPS	lipopolysaccharide
M	molar
mL	millilitre
N/A	not applicable
NSAIDS	non-steroidal anti-inflammatory drugs
P	probability
PBS	phosphate buffered saline
PE	polyethylene
PG	proteoglycan
PLL	placental lactose lectin
PS	protamine sulfate
Ser	serine
SEM	standard error of the mean

SD	standard deviation
SI	submucosal inflammation
SLRP	small leucine rich proteoglycan
TENS	transcutaneous electrical nerve stimulation
TGF-β	transforming growth factor-beta
UC	umbrella cell
UE	uroepithelium
Xyl	xylose

INTRODUCTION

The mucosal surfaces of the respiratory, gastrointestinal and urogenital tracts are bathed in a layer of mucous which essentially consists of complex carbohydrates and the proteins that bind them. These are known as mucosal surface molecules and often include lectins, mucins and glycosaminoglycans. This layer protects the underlying mucosal membranes because even though they are internal to the body, they are exposed to the outside world. Thus, without this barrier the underlying tissue could be exposed to harmful environmental agents and suffer from tissue damage and inflammation. Mucosal inflammatory diseases have been known to occur at virtually every mucosal membrane of the body. For example, they may manifest themselves in the urogenital tract as interstitial cystitis (IC) or prostatitis; and in the gastrointestinal tract as inflammatory bowel disease. It is estimated that 450,000 Americans have IC, half of all males are affected by prostatitis at least once in their lifetime and at least 250,000 Americans have ulcerative colitis (Interstitial Cystitis Association, 1998; Berger and Hanno, 1990; Horgan *et al.*, 1997). In many cases, the causes of these diseases are unknown. When an infection *is* identified as the cause and treated with antibiotics or anti-fungal drugs, the question still remains; why was this person susceptible to the infection in the first place? The general hypothesis of our laboratory is that a lack of mucosal integrity can predispose an individual to mucosal inflammatory diseases. This thesis will focus on the research that I have done in relation to interstitial cystitis, a mucosal inflammatory disease of the urinary bladder. The following sections of the introduction will;

1) define some of the components of mucous; namely lectins, mucins and GAGs.

2) discuss some of their properties and functions.

3) discuss mucosal inflammatory diseases of the urogenital tract.

4) state the hypotheses and objectives of the thesis.

A. LECTINS

1. Definition

Lectins are multivalent proteins which bind specific carbohydrates (Sharon & Lis, 1989). They are therefore capable of crosskicking the complex carbohydrates found at mucosal surfaces, thereby contributing to the stability of the mucosal barrier.

2. Structure and Specificity

Most lectins contain a short amino acid sequence which is known as the carbohydrate recognition domain (CRD) (Drickamer, 1988). This is where the lectin-carbohydrate interaction occurs. Lectins are divided into four families based on the homology of this sequence; the C-type lectins, P-type lectins, pentraxins and galectins (Barondes *et al.*, 1994). The C-type lectins are so named because their binding activity is Ca^{2+} dependent. Most of the C-type lectins are membrane bound

(Sharon & Lis, 1989). The P-type lectins are specific for mannose-6-phosphate (P) and may be cation-dependent or independent (Kornfeld, 1992). There are two types of pentraxins, both of which are serum proteins. They include the C-reactive protein and serum amyloid P (Andersen *et al.*, 1992). Finally, the galectins are ß-galactoside-specific. They are found extracellularly and are a focus of this study. The placental lactose lectin (PLL) is a 14.5 kDa galectin isolated from the human placenta. Galectins have been found at the mucosal surfaces of the intestine, lung and prostate (Fang & Ceri, 1990; Ceri *et al.*, 1991; Oke, 1996), and it is believed they may be involved in the organization of the urogenital mucosal barrier.

B. MUCINS

1. Definition

Mucous glycoproteins (otherwise known as mucins) comprise a large portion of the mucous which lubricates and protects the mucosal membranes of the body. These large molecules interact non-covalently with lectins and each other to form the protective gel-like substance.

2. Structure

Mucins consist of a protein core to which hundreds of oligosaccharide side chains are covalently attached. The protein core is rich in serine, threonine and proline (Mantle & Allen, 1989). The oligosaccharide side chains are branched and are composed of non-repeating sequences of N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), galactose, fucose and sialic acid (Reid & Clamp, 1978). The side chains attach to the serine and threonine residues through GalNAc-O-alpha bonds (Reid & Clamp, 1978). It is well established that mucins play a protective role at mucosal surfaces throughout the body.

C. PROTEOGLYCANS AND GLYCOSAMINOGLYCANS

1. Definition

Proteoglycans (PGs) are glycosylated proteins which are found in all connective tissues, in all extracellular matrices and on the surface of many cells, particularly epithelial cells (Jolles, 1994; Darnell *et al.*, 1990). They are most recognized for their ability to resist compression in articular cartilage. However, they have also been detected at mucosal surfaces where they are thought to participate in the structure of the protective mucosal barrier.

2. Structure and Nomenclature

Proteoglycans consist of a protein core to which one or more glycosaminoglycan (GAG) chains are covalently attached (Erlinger, 1995; Kurth, 1994). Glycosaminoglycans are long chains of repeating carboxylated and/or sulfated disaccharides (usually both). The structure of the disaccharide dictates the name the GAG chain (Darnell *et al.*, 1990). These disaccharides may include combinations of GalNAc, GlcNAc, galactose and hexuronic acids such as glucuronic acid (GlcUA) and galuronic acid (GalUA). Chondroitin-4-sulfate (C-4-S) is composed of GlcUA and GalNAc disaccharides and heparan sulfate (HS) is a GAG composed of GlcUA and GlcNAc disaccharides. Proteoglycans may be classified according to their structures and/or functions, but for the purposes of this study the most practical way is to name them based on the GAGs which they contain.

Proteoglycans are highly negatively charged due to the carboxyl and sulfate groups of the GAGs. This property is crucial for many of their biological functions (Jolles, 1994; Kurth, 1994; Hurst, 1994). At the reducing end of most GAGs of PGs is a trisaccharide (-Gal-Gal-Xyl-) which covalently attaches it to a serine residue in the protein core through a xylose-O-alpha bond (Reid & Clamp, 1978). The consensus sequence for the attachment site has been derived: (acidic)-(acidic)-X-Ser-Gly-(hydrophobic) (Neame *et al.*, 1989). After the core protein of a PG is digested extensively with proteases, the serine residue involved in the interaction remains associated with the GAG (Jolles, 1994).

D. MOLECULAR DISTRIBUTIONS

Lectins, mucins and GAGs have been identified at several mucosal surfaces in humans and non-human animals alike. For example, chondroitin sulfates have been detected in the forestomach of the sheep (Yamamoto *et al.*, 1995) and at the uroepithelium of the normal human bladder (Hurst *et al.*, 1996). A 14.5 kDa galectin has been detected in the secretory and basal cells of the glandular epithelium in normal human prostate tissue (Oke, 1996). Although they have not been localized to the mucosal surface, chondroitin sulfates have also been identified in normal and benign hyperplastic human adult prostate glands (Jolles, 1994). Furthermore, GAGs have been detected at the surface of the entire length of the cilia of the porcine airway epithelial cells (Erlinger, 1995). Ceri *et al.* have also found lectins and mucins at the mucosal surfaces of the quail intestine and the upper airways of the human lung (Fang *et al.*, 1993; Ceri *et al.*, 1991).

E. INTERMOLECULAR INTERACTIONS

1. Lectins and Mucins

There is strong evidence that lectins and mucins interact with each other in addition to mucins interacting non-covalently with themselves. It has been demonstrated that lectins and mucins co-localize in the intestine of the quail (Fang *et al.*, 1993) and in the human prostate (Oke, 1996). Furthermore, by eluting endogenous quail galectins from quail intestinal mucin using lactose, Fang *et al.* (1993) demonstrated that galectins have the capacity to bind mucins. It has also been shown that lectins play a role in the regulation of mucin secretion (Fang *et al.*, 1993; Ceri *et al.*, 1991; Ceri *et al.*, 1998).

2. Proteoglycans

Proteoglycans interact with other components of the extracellular matrix through their GAG side chains or functional domains in their protein cores.

2.1 SLRPs and Syndecans

Decorin and fibromodulin are members of the small leucine rich family of PGs (SLRPs). Decorin has 10 leucine-rich tandem repeats in the central region of the core which interact with collagen fibrils (Iozzo, 1997; Scott, 1988; Oldberg & Svensson, 1997). Similarly, fibromodulin interacts with collagen fibrils through its negatively charged GAGs at the N-terminus of the core (Iozzo, 1997; Scott, 1988; Oldberg & Svensson, 1997). Both decorin and fibromodulin play important roles in the structure and modulation of collagen fibrillogenesis (Scott, 1988). Furthermore, syndecans make up a family of cell-surface PGs whose HS chains also interact with collagen fibers (Elenius & Jalkanen, 1994). Thus, PGs have the potential to interact with other molecules at the mucosal surface of the urogenital tract.

2.2 Modular Proteoglycans

The modular family of PGs have repeated protein "modules" along their cores which show homology to other known structures such as growth factors, laminin, LDL receptors, lectins and immunoglobulins (Iozzo & Murdoch, 1996). The modular PGs consist of the hyalectins and the non-hyaluronan binding PGs. The hyalectins are so-named because they bind hyaluronic acid (HA), otherwise know as hyaluronan, and they also have lectin-like domains (Iozzo & Murdoch, 1996). Hyaluronan is a unique GAG in that it does not have a protein component. This polysaccharide is synthesized without a core protein at the plasma membrane of cells (Jolles, 1994).

Aggrecan is a chondroitin and keratan sulfate-containing hyalectin of cartilage. It has three globular domains; two at the N-terminus of the core (G1 and G2) and one at the C-terminus (G3) (Heinegard *et al.*, 1990; Heinegard & Oldberg, 1989). The G1 domain binds HA with a strong affinity (Heinegard *et al.*, 1990; Heinegard & Oldberg, 1989). The association between aggrecan and hyaluronan is strengthened by a protein called link protein which also has homology to the G1 domain and binds both hyaluronan and the G1 domain of aggrecan (Heinegard *et al.*, 1990; Heinegard & Oldberg, 1989). The G2 domain has homology with G1 but it doesn't bind HA (Heinegard *et al.*, 1990). G3 has a C-type lectin-like domain, an EGF-like domain and a CRP-like domain (Iozzo & Murdoch, 1996). The C-type lectin-like domain can bind fucose and galactose (Halberg *et al.*, 1986). It therefore has the potential to interact with other complex carbohydrates at the mucosal surface.

F. ROLES IN DEVELOPMENT AND DISEASE

1. Lectins and Proteoglycans in Development

Lectins and PGs have tremendous impacts on fetal development. For example, PGs are arranged in a network which acts as an instructional "map" for the development of neurites. In essence, the structure of this "map" tells the neurites when, where, and how to develop by functioning either as receptors or as ligands, thereby affecting their growth, polarity and migration (Wight *et al.*, 1992). It has been demonstrated that the expression of lectins and PGs are developmentally regulated. For example, both are expressed in a regionally specific and time-dependent fashion in the rat lung during development (Sannes *et al.*, 1993; Powell & Whitney, 1980).

2. Lectins and Proteoglycans in Disease

Proteoglycans and lectins have been implicated in abnormal tissue development. Proteoglycan changes have been shown to occur in the stroma of tumors. These increased PG levels are thought to support the progression, invasion and growth of tumors. For example, increased levels of chondroitin sulfate PGs have been found in the tissue stroma of human colon cancer (Schamhart and Kurth, 1994). Lectin and mucin distributional changes have also been detected in hyperplastic and cancerous prostates. Normally, galectins are found in the secretory and basal cells of the glandular epithelium (Oke, 1996). However, galectins are only found in the basal cells of hyperplastic prostates and in both the epithelial cells and the stromal tissue of cancerous prostates.

G. FUNCTIONS AT THE MUCOSAL SURFACE

1. Lectins and Mucins at the Mucosal Surface

There is some evidence that supports the notion that lectins are involved in the regulation of mucin secretion. Fang *et al.* (1993) showed that endogenous heparin lectins and mucins were co-secreted at the mucosal surfaces of the human lung and that the distributions of these secretions were altered in cystic fibrosis (Ceri *et al.*, 1991). Furthermore, the binding of lectins to the mucosal surface of the quail intestine has been shown to regulate mucin secretion (Fang *et al.*, 1993). Phytohemagglutinin is a lectin derived from red kidney beans. Ceri *et al.* (1998) demonstrated that excess phytohemagglutinin disrupts the mucus barrier in the rat small intestine resulting in increased bacterial adherence and bacterial overgrowth. Also, excess phytohemagglutinin results in decreased mucin secretions in the small intestine of the rat *in vitro*. These results suggest that lectins may play a role in the regulation of mucin secretion at mucosal surfaces.

2. Glycosaminoglycans at the Mucosal Surface

Glycosaminoglycans are large, negatively charged molecules which attract cations (such as K^+ and Ca^{2+}) resulting in an osmotic influx of water molecules (Erlinger, 1995). They are therefore ideally suited for lubricating and protecting mucosal surfaces throughout the body (Hurst, 1994; Nickel *et al.*, 1998). It has been suggested that GAGs have an important function at the mucosal surface of the bladder. They are thought to form a "mucosal barrier" (or "glycocalyx") in the bladder to help prevent the adherence of harmful bacteria to the uroepithelium (Hurst, 1994; Parsons, 1994a; Parsons, 1994b). This barrier is also impermeable to cytodestructive solutes in the urine (Hurst and Zebrowski, 1994; Nickel *et al.*, 1998). Yamamoto *et al.* (1995) have suggested that chondroitin sulfates may serve a similar function by acting as a charge barrier in the stomach.

2.1 Experimental Evidence

Several results support the concept that complex carbohydrates such as GAGs and mucins participate in a "mucosal barrier" which protects the underlying uroepithelium from harmful urine solutes. For example, agents such as protamine sulfate (PS) or ethanol are capable of disrupting the mucosal barrier in the bladder resulting in pain and urgency in patients (Hawkins *et al.*, 1997; Parsons, 1994a; Parsons, 1994b; Stein *et al.*, 1996) and increased uroepithelial permeability in rabbits (Tzan *et al.*, 1993). Protamine sulfate is a polycation quaternary amine which neutralizes the electronegative surface polysaccharide (Niku *et al.*, 1994). These effects can be reversed by the exogenous administration of GAGs which are thought to restore this surface charge density (Parsons, 1994b). This reversibility suggests that the disrupting agents are not acting cytotoxically. However, protamine followed by urea treatment (100-200 g/L) has resulted in uroepithelial cell death in rabbit bladders (Niku *et al.*, 1994). Niku *et al.* (1994) have proposed this procedure as a method to remove uroepithelial carcinomas. Furthermore, Hurst *et al.* (1996) have demonstrated that a deficit of chondroitin sulfate PGs at the mucosal surface of the bladder occurs in correlation with IC. Additionally, there is a decrease of GAG concentrations in the urine of patients with IC compared to normal individuals (Hurst *et al.*, 1993). Hurst *et al.* (1993) suggest that this reflects the binding of excreted GAGs to the damaged uroepithelium in an attempt to compensate for the loss. Exogenously administered heparin has been used to treat IC with some success. In over half of the patients studied, the symptoms were controlled with continued improvement after one year of treatment (Parsons *et al.*, 1994). These experiments indicate that the uroepithelium becomes susceptible to damage when the GAG layer is compromised.

Prior to the development of the "mucosal barrier" concept, Schlager *et al.* (1995) suggested that a so-called "superbacterium" was the causative infectious agent in urinary tract infections. However, upon further research, they showed that bacteria comprising the normal flora were responsible for the infections and that certain individuals were simply more susceptible than others (Schlager *et al.*, 1995).

Additionally, it has been demonstrated that HS GAGs could play a role in the establishment of an infection by functioning as bacterial receptors (Van Putten *et al.*, 1995; Raymond *et al.*, 1996). Thus, the host susceptibility to infection may lie in both the integrity and composition of the mucosal barrier, in addition to the virulence of the bacteria.

H. DISEASES OF THE UROGENITAL TRACT

1. Definition of the Prostate

In men, the prostate gland is similar in size and shape to a chestnut (Canadian Cancer Society, 1994). It is a fibromuscular, glandular organ which is located at the base of the penis in front of the rectum, just below the bladder. It is composed of extensive acinar and ductal networks which are rich in secretory material and produces the fluid portion of semen (Canadian Cancer Society, 1994).

2. Diseases of the Prostate

The prostate is said to be one of the most disease prone organs of the human body (Boyle *et al.*, 1996). These diseases include acute/chronic bacterial prostatitis, chronic idiopathic prostatitis (CIP) and benign prostatic hyperplasia (BPH). In the United States, benign and malignant diseases of the prostate are responsible for more surgery than diseases of any other organ in men over the age of fifty (Pretlow *et al.*, 1995).

2.1 Symptoms

Some of the most common symptoms associated with all prostate problems are (Canadian Cancer Society, 1994):

- pain in the lower back, pelvic area or upper thighs
- frequent, difficult, or painful urination
- dribbling urine
- blood or pus in the urine
- painful ejaculation

2.2 Bacterial Prostatitis

Prostatitis is simply defined as an inflammation of the prostate (Nickel, 1989). Although up to one half of all men have symptoms of prostatitis at some point in their life, not all of them have infected prostates. In fact, only five percent of all men with symptoms of prostatitis have bacterial prostatitis which is treated with antibiotics (Berger and Hanno, 1990). However, it is often difficult to eradicate the bacteria completely which may be due to a variety of factors; the bacteria may be resistant to particular antibiotics, there may be more than one type of bacteria present, the antibiotics may not reach sufficient concentrations in the prostate or the bacteria may form biofilms. Bacteria, when growing attached to a substrate, form thin biofilms which render them increasingly resistant to certain antibiotics (Costerton *et al.*, 1995). Nickel *et al.* (1995) have suggested that these protected bacterial microcolonies grow deep within the prostate. The infection could also be passed between sexual partners. It is therefore not surprising that when bacterial prostatitis is treated with antibiotics, the infection often recurs with the same bacteria being cultured out of the urine time after time (Berger and Hanno, 1990).

2.3 Chronic Idiopathic Prostatitis and Benign Prostatic Hyperplasia

The remaining ninety-five percent of the men with symptoms of prostatitis either have CIP or BPH (Berger and Hanno, 1990). The causes of both of these syndromes are unclear (Meares, 1991; Nickel, 1989). There is little or no evidence that they are caused by chronic infections or are sexually, or otherwise transmissible (Berger and Hanno, 1990).

a) Chronic Idiopathic Prostatitis

Chronic idiopathic prostatitis involves the inflammation of the prostate in the absence of any detectable bacterial infection (Meares, 1991). Some scientists have suggested that this disease is an autoimmune process (Keetch *et al.*, 1994; Rivero *et al.*, 1995; Seethalakshmi *et al.*, 1996). Animal models have been developed in which a prostate-specific autoimmune response is induced (Keetch *et al.*, 1994; Rivero *et al.*, 1995; Seethalakshmi *et al.*, 1996). These animals experience an inflammation of the prostate which is similar to clinical CIP (Keetch *et al.*, 1994).

b) Benign Prostatic Hyperplasia

Benign prostatic hyperplasia is an age-related, non-malignant enlargement of the prostate which commonly occurs in men over 40 (O'Leary, 1995). This enlargement puts pressure on the urethra making urination difficult. Autopsy studies have revealed that 88% of men over the age of 80 had BPH. It accounts for 2 million physician office visits annually in the United States. Benign prostatic hyperplasia is the most common cause of major surgery in men over the age of 55 (Boyle *et al.*, 1996).

There is some evidence that hormones are involved in the development of BPH (Srinivasan *et al.*, 1995). The accumulation of dihydrotestosterone with age, the reawakening of the inductive potential of the prostatic stroma, the imbalance between net cell proliferation and net cell death rates and the age-related changes in the ratio of testosterone to estrogen have all been implicated (Sensibar, 1995; Srinivasan *et al.*, 1995). In some cases, there is a regression of preexisting BPH following castration or anti-androgen therapy (Srinivasan *et al.*, 1995; Winter *et al.*, 1995). Interestingly, estrogen and androgen have been shown to influence the levels and locations of chondroitin sulfates in the guinea pig prostate (Horsfall *et al.*, 1994).

2.4 Treatments

Many of the modern day treatments for BPH and CIP are mainly symptomatic. There are no cures. Amitriptyline is often prescribed to treat the pain associated with BPH and CIP (Berger and Hanno, 1990). This drug was originally used as an antidepressant but it also has analgesic properties. These analgesic properties may be due to its mood altering effects (Montogomery, 1996). Chronic idiopathic prostatitis has also been treated with drugs such as hydroxyzine and adrenergic alpha-antagonists (Berger and Hanno, 1990). Hydroxyzine acts as an antihistamine which reduces the inflammation and adrenergic alpha-antagonists reduce the inflammation by blocking the sympathetic nervous system. This suggests that there may be a neurogenic component to the chronic inflammation. Chronic idiopathic prostatitis may respond to antibiotics temporarily, suggesting that there may be a bacterial component which has not yet been identified (Meares, 1991; Nickel, 1989). Finasteride is a drug used to shrink the hyperplastic prostate (Berger and Hanno, 1990). The growth of the prostate gland is under hormonal control. Finasteride reduces its size by inhibiting 2,5-alpha-reductase, the enzyme which converts testosterone to dihydroxytestosterone.

3. Definition of the Urinary Bladder

The urogenital tract includes the urethra, bladder and the prostate gland. The bladder is a triangular-shaped chamber in the lower abdomen which stores urine. Like a balloon, the bladder's elastic walls relax and expand to store urine and contract and flatten when urine is emptied through the urethra. The adult bladder typically stores about 1-1/2 cups of urine (Harris, 1997).

4. Interstitial Cystitis

Interstitial cystitis is a chronic inflammation of the bladder wall which can lead to scarring and stiffening of the bladder, decreased bladder capacity and, in rare cases, ulcerations. It is estimated that 450,000 Americans have IC (Interstitial Cystitis Association, 1998). Ninety percent of them are women and it most commonly strikes between the ages of 20 and 50. There is no evidence that IC is hereditary or that it leads to cancer (Harris, 1997). However, certain drugs such as cyclophosphamide can cause IC and lead to cancer of the bladder (Gossmann, 1997; Tanaka *et al.*, 1995). Cyclophosphamide is an immunosuppressive and antineoplastic drug used in the treatment of lymphomas and some leukemias. In most cases of IC the etiology is unknown (Harris, 1997).

4.1 Symptoms

The clinical symptoms of IC include the following in the absence of any detectable bacterial infection (Harris, 1997):

- decreased bladder capacity
- painful, urgent and frequent urination
- pain around the bladder, pelvis and perineum which increases as the bladder fills
- painful sexual intercourse
- in men, discomfort or pain in the penis and scrotum

4.2 Diagnosis

Histology plays a supportive role in the diagnosis of IC. Lynes *et al.* (1990) conducted a histological study with 17 IC patients and 7 control patients using H&E staining and found that there were only two consistent histological markers of IC. The IC patients had a statistically increased incidence of submucosal lymphocytic infiltrate and associated denuded epithelium.

4.3 Treatments

Many of the current medications and medical procedures treat the symptoms. They include oral treatments with hydroxyzine, an antihistamine; or acetylsalicylic acid and ibuprofen, which are both analgesics and non-steroidal anti-inflammatory drugs

(NSAIDS). Other treatments involve bladder washes wherein the bladder is filled with heparin or dimethyl sulfoxide (DMSO) for 15 minutes before it is expelled through a catheter. Some urologists believe that DMSO passes into the bladder wall and reduces the inflammation, blocks pain, and prevents painful muscle contractions. The heparin, in contrast, is thought to restore the negative charge density at the uroepithelial surface. Transcutaneous electrical nerve stimulation (TENS) has also been used to treat the pain associated with IC. In the case of IC, special devices which deliver mild electrical pulses are inserted into the vagina of women or into the rectum of men. Although this is an unproven method, some patients experience relief. It has been suggested that the electrical pulses trigger the release of hormones that block the pain (Harris, 1997).

I. RATIONALE

The current trend of thought is that GAGs play a protective role at the mucosal surface of the bladder. The negatively charged sulfate and carboxylate groups which are characteristic of GAGs are thought to contribute to a charged barrier which prevents the passage of harmful urine solutes into the bladder wall. For example, the passage of potassium into the bladder wall could potentially depolarize sensory nerves resulting in urinary urgency, frequency and pain in the absence of any bacterial infection, all of which are symptoms of IC (Parsons *et al.*, 1994). Levin *et al.* (1995) conducted a study that looked at the contractile response of the feline bladder to KCl and the effect of physically removing the mucosa. They discovered that the mucosa has a significant inhibitory effect on the contractile response. Additionally, the mucosal barrier is thought to keep potentially toxic urine solutes out and water in the bladder wall. Therefore, a breakdown of this barrier could result in many of the very symptoms which are characteristic of IC.

It is well documented that GAGs such as HA and heparin have been used to treat IC with some success in relieving the symptoms (Porru *et al.*, 1997; Parsons *et al.*, 1994). This suggests that there may be a deficit of GAGs at the mucosal surface in IC patients. The exogenously administered GAGs are thought to restore the barrier at the naked bladder mucosa. In cases of IC (or feline urologic syndrome in cats), GAG levels which are found normally in the urine decrease (Erickson *et al.*, 1997; Buffington *et al.*, 1996). Furthermore, Gill *et al.* (1982) discovered that heparin binds chemically injured uroepithelia more readily than normal urepithelia resulting in a restorative effect.

Protamine sulfate is an arginine-rich polypeptide of size 5000MW which, when instilled into the bladder, is thought to neutralize the negative charge density at the mucosal surface. Treatment of human and rabbit bladders with PS increases the permeability of urea across the bladder wall (Nickel *et al.*, 1998; Parsons *et al.*, 1990), resulting in IC symptoms which are alleviated with heparin treatment (Lilly & Parsons, 1990). The heparin is thought to restore the negative charge density.

Protamine sulfate treatment has also been shown to increase bacterial adherence which suggests that GAGs have an anti-microbial effect at the uroepithelium as well (Parsons *et al.*, 1988). In fact, Mostafavi *et al.* (1995) proposed that E. coli produces a soluble virulence factor which has similar effects on the bladder wall as the experimentally used PS. They found that E.coli grown in human urine resulted in a bacteria-free supernatant which contained a cationic protein of molecular weight 3.5kD capable of binding to a heparin agarose affinity column. Rabbit bladders pretreated with the supernatant followed by E.coli inoculation resulted in a more persistent infection. In addition, studies have been done in the rat and rabbit wherein the bladders are flushed with PS, to presumably disrupt the mucosa, followed by an irritant such as urea or LPS. This combination results in a denudation of the uroepithelium and an inflammation of the bladder wall (Stein *et al.*, 1996; Niku *et al.*, 1994). Similar experiments have been done in animal models of colitis whereby ethanol is used as the so-called "barrier breaker" followed by a chemical irritant such as 2,4-dinitrobenzenesulfonic acid (DNBS) to induce inflammation (Hawkins *et al.*, 1997). Only the combination of these two chemicals results in an inflammatory response in the gut. Thus, these experiments punctuate the importance of the negative charge density for a healthy bladder and the usefulness of PS and ethanol in my study. They suggest that when the integrity of any mucosa is compromised, the underlying tissue becomes susceptible to irritation and inflammation.

J. HYPOTHESIS

The hypotheses of this project were the following:

1) HS, C-4-S, PLL and human mucin would be found at the mucosal surface of the uninflamed human urinary bladder.

2) As the degree of submucosal inflammation and denudation increased, there would be significant decreases in the levels of these markers at the mucosal surface.

3)These mucosal surface molecules would contribute to the protection of the underlying tissue which would otherwise become susceptible to irritation and inflammation.

K. OBJECTIVES

The objectives of this study were the following:

1) To map out the distributions of HS, C-4-S, PLL and human mucin at the mucosal surface of normal and inflamed (IC) bladders using immunohistochemical techniques.

2) A rat model was used to determine whether the integrity of the bladder mucosa is important in protecting the underlying tissue from irritation and inflammation.

METHODS AND MATERIALS

A. OVERVIEW

1. Human Studies

Human IC bladder tissues were consecutively sectioned, mounted on slides and immunostained for the following molecular markers using avidin-biotin systems: C-4-S, HS, PLL and human mucin. The slides were blindly scored for submucosal inflammation, uroepithelial denudation, and staining of the umbrella cells and the basal lamina. The submucosal inflammation was based primarily on the number of lymphocytes. However, other cell types such as neutrophils also contributed to the score. The scoring was done in increments of 100μm along the entire uroepithelium by Dr. Howard Ceri of the University of Calgary, Calgary, Alberta.

2. Rat Studies

Sprague-Dawley rats were supplied by the Animal Health Unit at the University of Calgary. Following treatment with a barrier-breaker and/or an irritant, the rat bladders were fixed, embedded, sectioned, mounted on slides and immunostained for the same human markers. These slides were observed by Dr. Howard Ceri for staining at the uroepithelial surface and submucosal inflammation. The following paragraphs describe the protocols in detail.

B. HUMAN TISSUES

1. Introduction

A total of 7 human urinary bladder surgical biopsies from patients who were later diagnosed with IC were examined in this study. Their ages and sexes are summarized in Table 1 below. These tissues were supplied by Drs. Nickel and Boag of Queen's University, Kingston, Ontario.

Table 1: A summary of the sexes and ages of the IC patients from which the urinary bladder biopsies used in this study were removed.

Case Number	Sex (M/F)	Age
93-11321	F	20
HC3	F	55
HC4	F	60
93-7177	F	77
93-9768	M	68
93-10421	M	69
93-10307	M	73

2. Preparation of Human Bladders for Immunostaining

The human tissues were supplied in paraffin blocks which were heated and re-embedded on VWRbrand™ embedding rings to fit the microtome chuck (VWR Canlab, Edmonton, AB). The tissues were previously fixed in 10% neutral buffered formalin. The blocks were hardened, sectioned at 8 μm with a microtome and mounted on 3-aminopropyltriethoxysilane-coated slides (Sigma, St. Louis, MO). These slides were dried overnight at 37°C, deparaffinized and rehydrated by manually dipping the slides for 3 minutes in the following solutions respectively: three times in americlear, twice in 100% ethanol and once in 95% ethanol followed by PBS or Tris buffer as per the immunostaining protocol.

C. RAT MODEL OF INTERSTITIAL CYSTITIS

1. Introduction

Two types of rat models were used to test the hypothesis. One used PS as the so-called barrier breaker and the other used ethanol. Dinitrobenzene sulfonic acid was used as the chemical irritant in both models.

2. Ethanol/DNBS Model

These methods were based on a common animal model of ulcerative colitis (Hawkins *et al.*, 1997). In the ethanol/DNBS model of IC, 13 male Sprague-Dawley rats of weight 350-450g and age 60-65 days were supplied by the Animal Health

Unit. The rats were anesthetized with 4-5% halothane for at least 10 minutes while their breathing was closely monitored. Following anesthesia, their penises were catheterized with PE10 tubing (Becton Dickinson, Sparks, MD) which was sterilized in 95% ethanol, lubricated with K-Y Lubricant (Johnson & Johnson, Montreal, PQ) and threaded onto 30G 1/2 needles. Five of the thirteen rats were injected with 0.2 mL of 100 mg/mL DNBS (IN, Aurora, OH) in 50% ethanol. This was the test group. Three rats were injected with 0.2 mL of 50% ethanol and three more were injected with 100 mg/mL DNBS in PBS. Two rats were left untreated. These 8 rats comprised the control groups. After 12 hours, the rats were sacrificed with CO_2, their bladders removed and placed in scintillation vials containing 10% neutral buffered formalin.

3. PS/DNBS Model

These methods were based on the experiments done by Stein *et al.* (1996), Niku *et. al.* (1994) and Hawkins *et al.* (1997). In the PS/DNBS model of IC, 11 male Sprague-Dawley rats of weight 350-450g and age 60-65 days were supplied by the Animal Health Unit. The rats were anesthetized with 4-5% halothane followed by catamenia hydrochloride:xylazine (85:15) at 0.05 cc per 100 g body weight intramuscularly. Following anesthesia, their bladders were emptied with gentle pressure on the abdomen and their penises were catheterized with PE10 tubing which was sterilized in 95% ethanol, lubricated with K-Y Lubricant (Johnson & Johnson, Montreal, PQ) and threaded onto 30 G1/2 needles. Seven rats were injected with 1.0 mL of 100 mg/mL PS (Sigma, St. Louis, MO) in PBS and 4 rats were injected with 1.0 mL of PBS. After 45 minutes, the bladders were emptied and washed with 1.0 mL of PBS. Four of the PS-treated rats and the 4 PBS-treated rats were then injected with 1.0 mL of 100 mg/mL DNBS in PBS. The other three PS-treated rats were injected with 1.0 mL of PBS. Table 2 summarizes these treatment groups. The rats would start to recover in 30 minutes after which the catheters were removed. After 12 hours, the rats were sacrificed with CO_2, their bladders removed and placed in scintillation vials containing 10% neutral buffered formalin.

Table 2: A summary of the treatment groups in the PS/DNBS model of IC.

Treatment Group	Number of Rats	First Injection	Second Injection
Test	4	PS	DNBS
Control 1	4	PBS	DNBS
Control 2	3	PS	PBS

4. Preparation of Rat Bladders for Immunostaining

In preparation for immunostaining, the rat bladders were fixed overnight in 10% neutral buffered formalin and embedded in paraffin with an autotechnicon. The autotechnicon would dip and agitate the tissues overnight for one hour in each of the following solutions respectively: 50% ethanol, 70% ethanol, 90% ethanol, 95% ethanol, 3 times in 100% ethanol, twice in americlear and finally, twice in hot paraffin. All blocks were hardened, sectioned at 8 μm with a microtome and mounted on 3-aminopropyltriethoxysilane-coated slides (Sigma, St. Louis, MO). These slides were dried overnight at 37°C, deparaffinized and rehydrated by manually dipping the slides for 3 minutes in the following solutions respectively: three times in americlear, twice in 100% ethanol and once in 95% ethanol followed by PBS or Tris buffer as per the immunostaining protocol.

D. PREPARATION OF ANTISERA

Polyclonal antibodies specific for human mucin and human PLL were provided by Dr. M. Mantle of the University of Calgary. Both were developed in New Zealand White rabbits against purified 118 kDa glycoproteins isolated from human intestinal mucin and purified 14.5 kDa galectins from human placental tissue from the Foothills Hospital, respectively (Oke, 1996).

E. IMMUNOSTAINING OF HUMAN AND RAT TISSUES

Following treatment and preparation, both the human and rat tissues were stained for HS, C-4-S, mucin and PLL. When staining for C-4-S, the tissues were washed 3 times in 0.1 M, pH 8.0 Tris for 3 minutes then digested with chondroitinase ABC (0.01 U/mL) for 1 hour at 37°C (Sigma, St. Louis, MO) in a humidified chamber to release the antigenic unsaturated disaccharide (Hurst *et al.*, 1996). These were then washed twice in PBS and once in 1% BSA in PBS (0.01 g/mL) for 3 minutes. When staining for HS, this last step was conducted immediately following the rehydration step outlined above. When staining for PLL and mucin, the tissues were washed twice in PBS for 3 minutes and then incubated with 10% goat serum in PBS for 1 hour at room temperature in a humidified chamber immediately following the rehydration step outlined above. Excess fluid from the slides was aspirated and the primary antibodies were added to the tissues.

Heparan sulfate and C-4-S GAGs were labelled with biotin-conjugated anti-HS and anti-C-4-S mouse monoclonal antibodies (2-B-6 and F58-10E4 clones respectively; Seikagaku, Tokyo, Japan) at a dilution of 1:50 in 1% BSA in PBS (0.01 g/mL) overnight at 4°C. The anti-C-4-S antibody is specific for chondroitinase ABC digested C-4-S GAGs from any species which contain C-4-S chains that are susceptible to chondroitinase ABC digestion. Couchman *et al.* (1984) raised this antibody and using immunofluorescence, they found that C-4-S was widely distributed in the connective tissue of both the human and the rat. The epitope of the anti-HS antibody includes the N-acetylglucosamine residue of the HS chain. Heparan sulfate has also been found in the human and rat kidney (Iozzo, 1997; Kamada, 1989). The PLLs and mucins were labelled with rabbit polyclonal antibodies at a dilution of 1:100 in 1% BSA in PBS (0.01 g/mL) overnight at 4°C. This incubation was followed by 2 washes in PBS and one wash in 1% BSA in PBS (0.01 g/mL). Excess fluid was aspirated and biotin-conjugated goat anti-rabbit IgG antibodies (KPL, Burlington, ON) were added at 1:250 in 1% BSA in PBS (0.01 g/mL) for 4-6 hours at room temperature. All tissues were washed twice in PBS and once in 1% BSA in PBS (0.01 g/mL) for 3 minutes. The biotin conjugates were then labelled with alkaline phosphatase-conjugated ExtrAvidin (Sigma, St. Louis, MO) at a dilution of 1:150 (0.005 g/mL) in 1% BSA in PBS (0.01 /mL) overnight at 4°C. All tissues were aspirated and rinsed 3 times in 0.1M, pH 8.0 Tris for 3 minutes. The alkaline phosphatase activity was then visualized and developed for 5-10 minutes with Fast Red TR/Naphthol AS-MX which resulted in a red color reaction (Sigma, St. Louis, MO). This substrate also contained levamisole which blocked any endogenous alkaline phosphatase activity. The slides were rinsed in water to stop the reaction, counterstained in Gill's hematoxylin for 30 seconds and dipped in a saturated solution of Lithium Carbonate for 1 minute. The slides were rinsed in cold running tap water in between treatments and a coverslip was added using the aqueous mounting media Aquapolymount (BDH, Poole, England). Several negative control slides were generated throughout this procedure, each of which lacked the following staining components:

1) Primary Antibody
2) Secondary Antibody
3) Tertiary Antibody (for human mucin and PLL)
4) Combinations of 1, 2 and 3
5) Primary, Secondary and Tertiary (substrate only)

Otherwise, these slides were treated identically to the test slides.

F. HISTOLOGICAL EXAMINATION

After allowing the slides to dry overnight, they were examined and photographed on a Zeiss photomicroscope by the investigator. The slides used for histological scoring were chosen based on the following constraints:

1) Sections which contained healthy, uninflamed areas were included.
2) The sections displayed an identifiable uroepithelium. (It was clear that in some sections, the uroepithelium was manually disrupted by the pathologist.)
3) Only consecutive sections which were stained for all of the markers in question were used.
4) The sections were unobstructed by any artifacts such as bubbles in the mounting media and folding of the tissue.

G. HISTOPATHOLOGICAL SCORING CRITERIA

The human tissues were scored blindly at 100μm increments along the uroepithelia to determine the staining patterns and severity of the disease as indicated by denudation and submucosal lymphocyte numbers. Thus, the mucosal integrity could be studied at differing levels of submucosal inflammation along the uroepithelium.

Table 3 summarizes the histopathological scoring criteria used to make these assessments.

Table 3: The histopathological scoring criteria used to assess the severity of IC and the mucosal integrity in the human bladder tissues. The inflammatory cells in the assessment of submucosal inflammation included primarily lymphocytes and neutrophils.

Degree Assignment	Submucosal Inflammation	Uroepithelial Denudation	Staining of The Umbrella Cells	Staining of the Basal Lamina
0	No Inflammation	Intact	Complete Absence of Staining	Absent
1	Occasional Inflammatory Cells	Occasional Loss of Umbrella Cells	Occasional Light Staining	N/A*
2	Few Inflammatory Cells	Complete Loss of Umbrella Cells	Occasional Intense Staining	N/A*
3	Moderate Inflammatory Cells	Occasional Full Thickness Denudation	Continuous Intense Staining	N/A*
4	Abundant Inflammatory Cells	Complete Full Thickness Denudation		Present

*Intermediate levels of staining for the basal lamina were indiscernible.

H. STATISTICAL ANALYSIS

The results from the histological assessment of the human tissues were sorted by the degree of submucosal inflammation. First, a frequency distribution was created which compared the degree of submucosal inflammation to the total length of uroepithelium encountered at that degree. Based on the shape of this curve, a non-parametric comparison of medians test known as the Kruskal-Wallis test was chosen for the statistical analysis of the data, followed by Dunn's Post test for multiple comparisons. The raw data are summarized in Appendix 1.

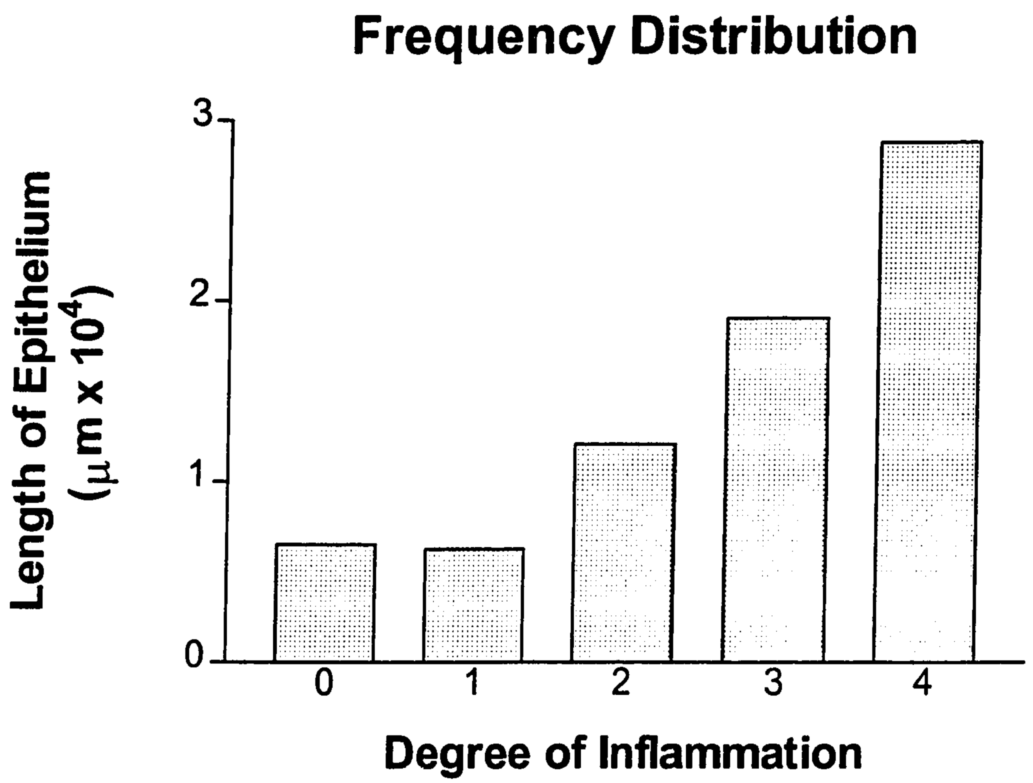
For each degree of submucosal inflammation, the mean levels of denudation and staining were graphed with standard error as the measure of variability. The median levels of denudation or staining were compared for each degree of submucosal inflammation to determine the probability that the data were sampled from populations with equal medians. The null hypothesis was that all of the column medians were equal. Thus, if the calculated probability (P) was less than 0.05, the column medians were considered significantly different.

RESULTS

A. FREQUENCY DISTRIBUTION

Under microscopic examination, all of the human bladder tissue biopsies had both healthy and diseased areas. Although, areas of submucosal inflammation were far more common than uninflamed areas. The submucosal infiltrate consisted primarily of lymphocytes. The total length of uroepithelium at each degree of submucosal inflammation is shown in Figure 1. Since the graph is not bell-shaped, the data is considered non-Gaussian or non-parametric. Thus, non-parametric tests were used in the statistical analysis.

Figure 1: A frequency distribution chart. This chart relates the total length of uroepithelium to each level of submucosal inflammation. This chart illustrates the skewed nature of the human data.


Frequency Distribution
Length of Epithelium
(μm x 10⁴)
3
2
1
0
0
1
2
3
4
Degree of Inflammation

B. NEGATIVE CONTROL SLIDES

Human and rat negative control slides were generated by omitting one or more of the antibodies used in the immunostaining procedure. These slides resulted in no red precipitate formation. Figure 2 shows light micrographs of negative control slides of healthy human and rat bladders. The Gill's hematoxylin counterstain results in the blue background and nuclei.

C. POSITIVE CONTROLS

The interstitial tissues of the bladder consistently stained positive for the markers. Thus, these were used as internal positive controls. Additionally, the arterioles underlying the uroepithelium of the human bladders stained consistently and strongly for PLL. This was not surprising since Kayser *et al.* (1993) has found a 14 kDa β-galactoside-specific galectin in the arterial walls of the right and left coronary arteries, the carotid artery, the abdominal and thoracic aortas, the pulmonary artery, and the left femoral artery. This lectin had the same specificity and a similar molecular weight as PLL. This staining pattern has also been seen in other tissue types using antibody to PLL (Oke, 1996).

D. HISTOLOGY OF THE BLADDER WALL

The uroepithelium of the healthy urinary bladder consists of three to six layers of cells (see Figure 2). The basal layers are compact cuboidal, the intermediate layers are polygonal and the surface cells are tall columnar with large round nuclei and prominent nucleoli (Burkitt *et al.*, 1993). These cells are often referred to as umbrella cells. The uroepithelium rests upon a thin matrix called the basal lamina, otherwise known as the basement membrane. Underlying the basement membrane are smooth muscles, elastic fibers and other connective tissues which contract during micturition (Burkitt *et al.*, 1993).

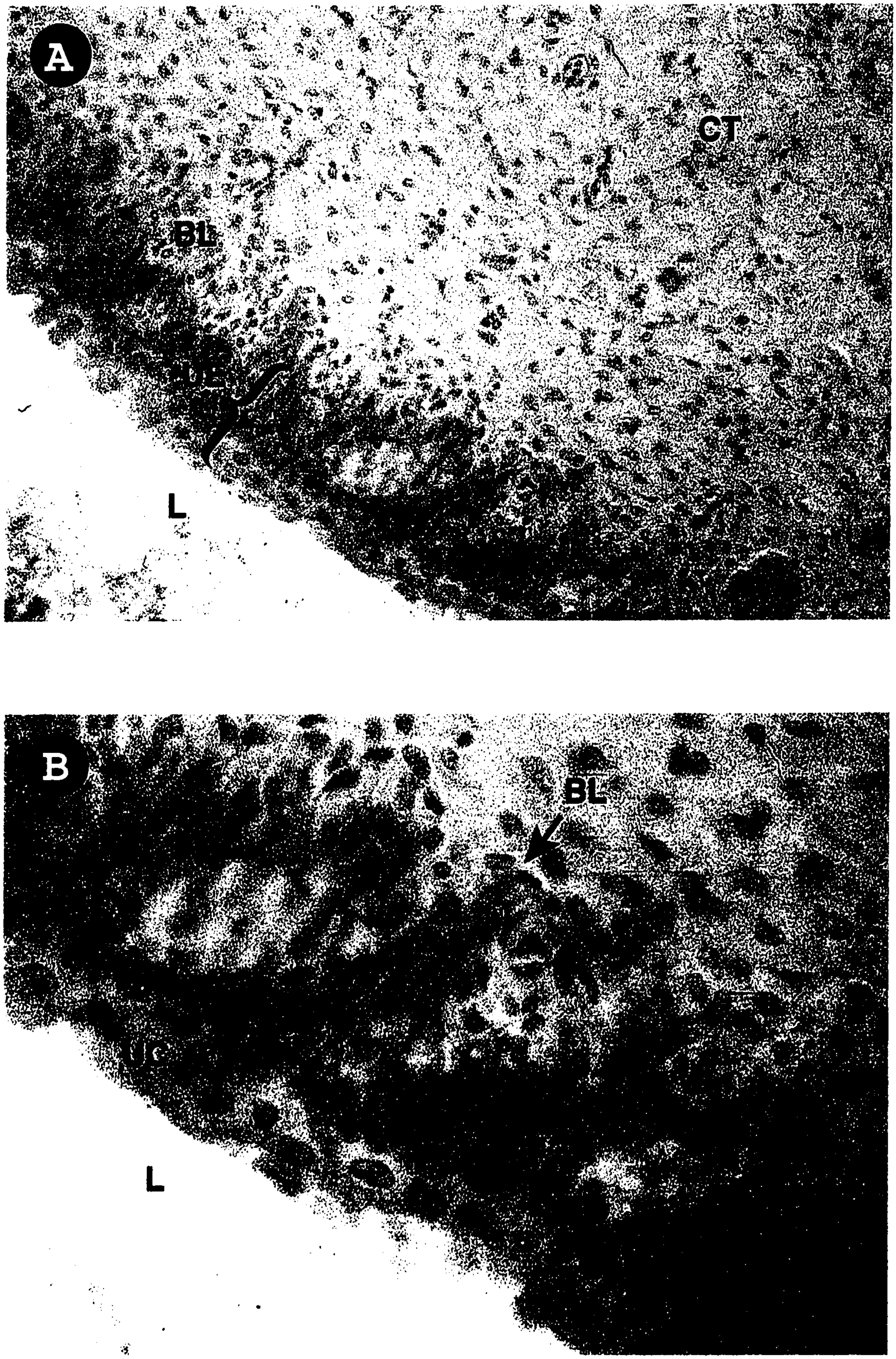
Figure 2: Micrographs of negative control slides illustrating human and rat urinary bladder histology. The umbrella cells (UC) face the lumen (L) at the surface of the uroepithelium (UE). The arrow indicates the area where one would expect to find the basal lamina (BL) which is connected to the underlying loose connective tissue (CT). Gill's hematoxylin stain.

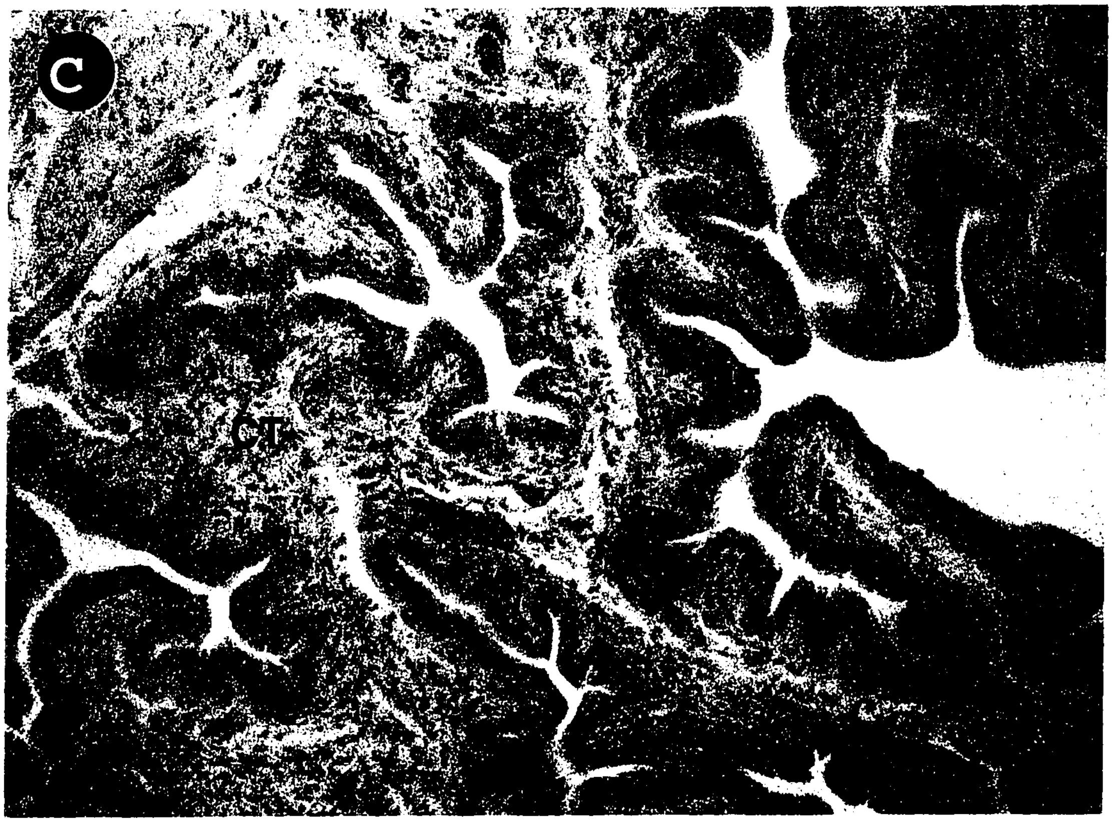
A. An example of a negative control slide of normal human urinary bladder tissue. Magnification approximately x260.

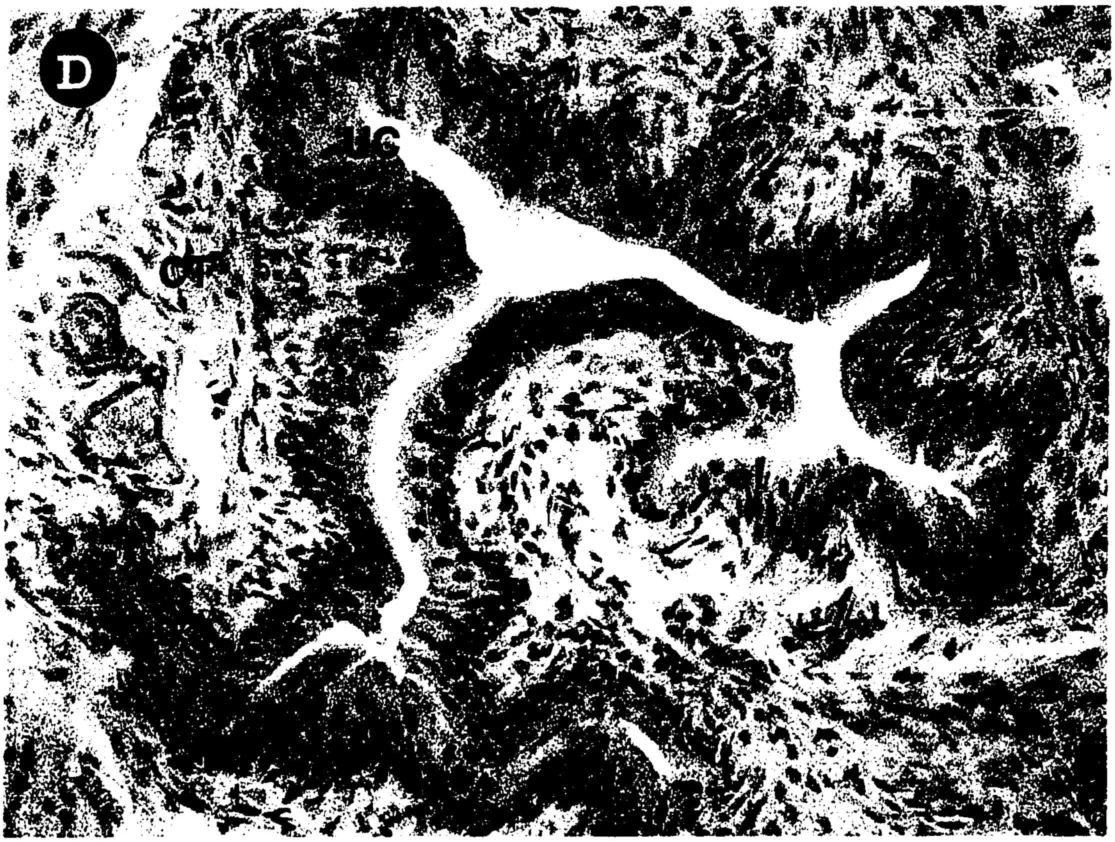
B. An example of a negative control slide of normal human urinary bladder tissue. Magnification approximately x720.

C. An example of a negative control slide of normal rat urinary bladder tissue. Magnification approximately x155.

D. An example of a negative control slide of normal rat urinary bladder tissue. Magnification approximately x345.


A
CT
BL
L
B
BL
L


C
CT


D
UG
CT

E. DENUDATION OF THE UROEPITHELIUM

In normal uninflamed regions of the bladder, all of the layers of the uroepithelium were present, including the umbrella cells. As the severity of submucosal inflammation increased, there was a significant increase in the level of denudation. This denudation ranged from the loss of the superficial umbrella cells at lower levels of inflammation, to complete loss of the uroepithelium, exposing the underlying connective tissue to the lumen. The slide in Figure 3 illustrates a highly inflamed and denuded bladder section. An undenuded human section was seen in Figure 2. The bar graph in figure 4 summarizes the analyzed data.

Figure 3: Micrographs of an inflamed region of human bladder tissue. Diseased human bladder tissue showing the highest degree of submucosal inflammation (SI) and partial uroepithelial denudation (arrow) adjacent to complete denudation. Gill's hematoxylin stain.

A. Magnification approximately x112.

B. Magnification approximately x300.


A

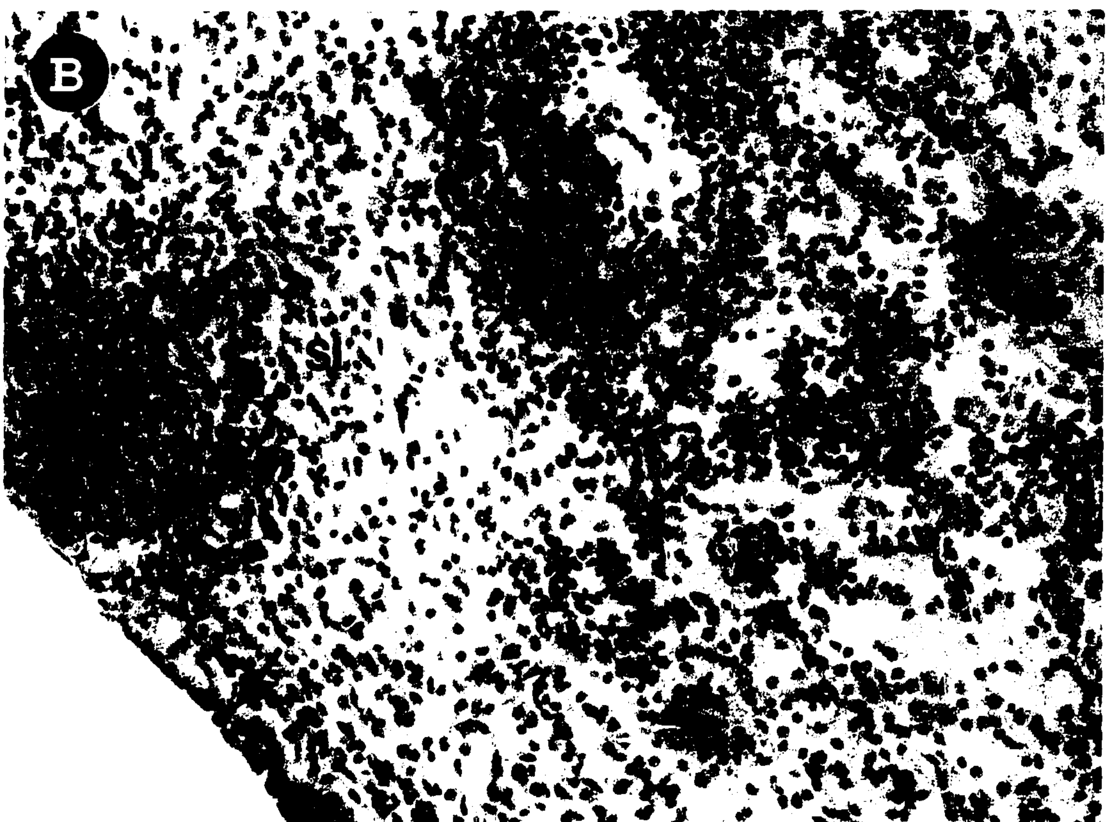
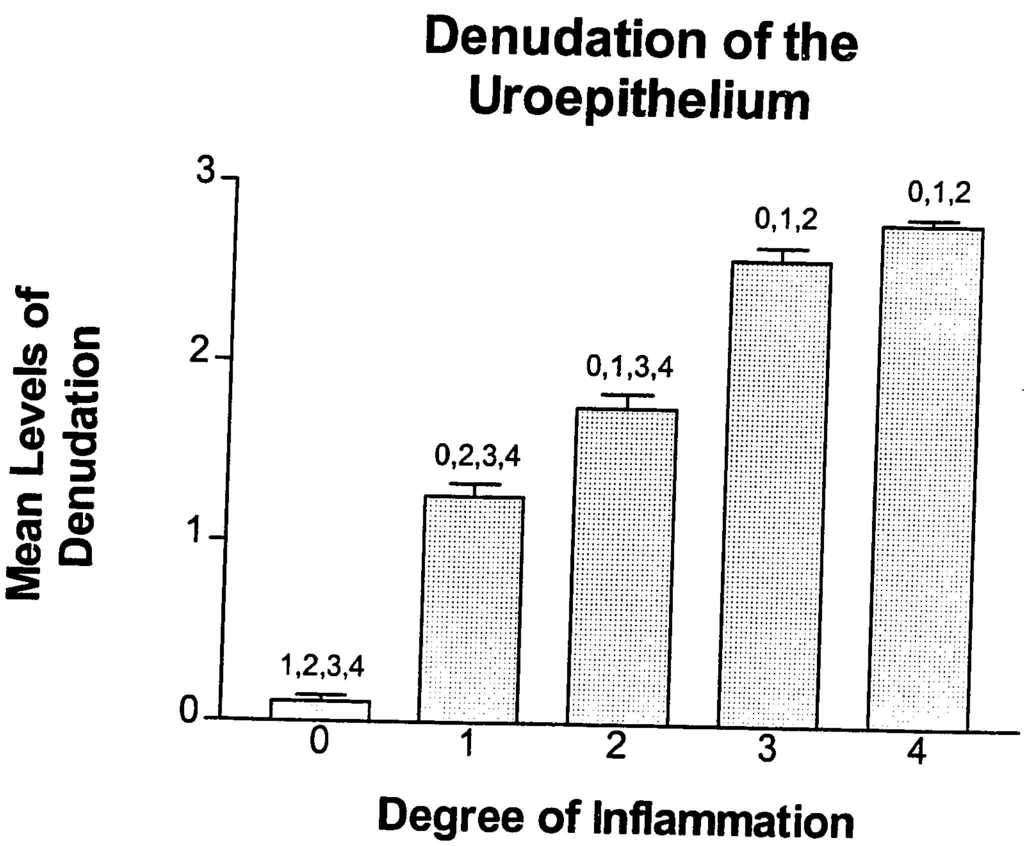

B

Figure 4: A bar graph which illustrates the mean levels of uroepithelial denudation at each degree of submucosal inflammation. Variability is measured as standard error. Significant differences ($P < 0.05$) of medians are reported at the top of each bar as degrees of inflammation, where applicable.



Denudation of the Uroepithelium
Mean Levels of Denudation
3
2
1
0
1,2,3,4
0,2,3,4
0,1,3,4
0,1,2
0,1,2
0
1
2
3
4
Degree of Inflammation

F. IMMUNOSTAINING

1. Human Studies

In normal uninflamed regions of the human bladder, HS, lectin plus mucin and low levels of C-4-S co-localized in the umbrella cells at the surface of the uroepithelium. Examples of these are shown in Figure 5. In these uninflamed regions, none of the markers were detected in the basal lamina. However, as the degree of submucosal inflammation increased, the following changes occurred:

1) There was a coordinate decrease in surface cell staining for all of the markers.
2) The levels of HS in the basal lamina increased.
3) There was a slight increase in the levels of PLL in the basal lamina.

Figure 6 illustrates the deficit of surface HS at high levels of submucosal inflammation. The increase of HS in the basal lamina can also be seen in this figure. The analyzed data for the decreases in surface staining of HS, lectin plus mucin and C-4-S are summarized in bar graphs in Figures 7, 8 and 9, respectively. The data demonstrating the increases in HS and PLL levels in the basal lamina are summarized in Tables 4 and 5, respectively.

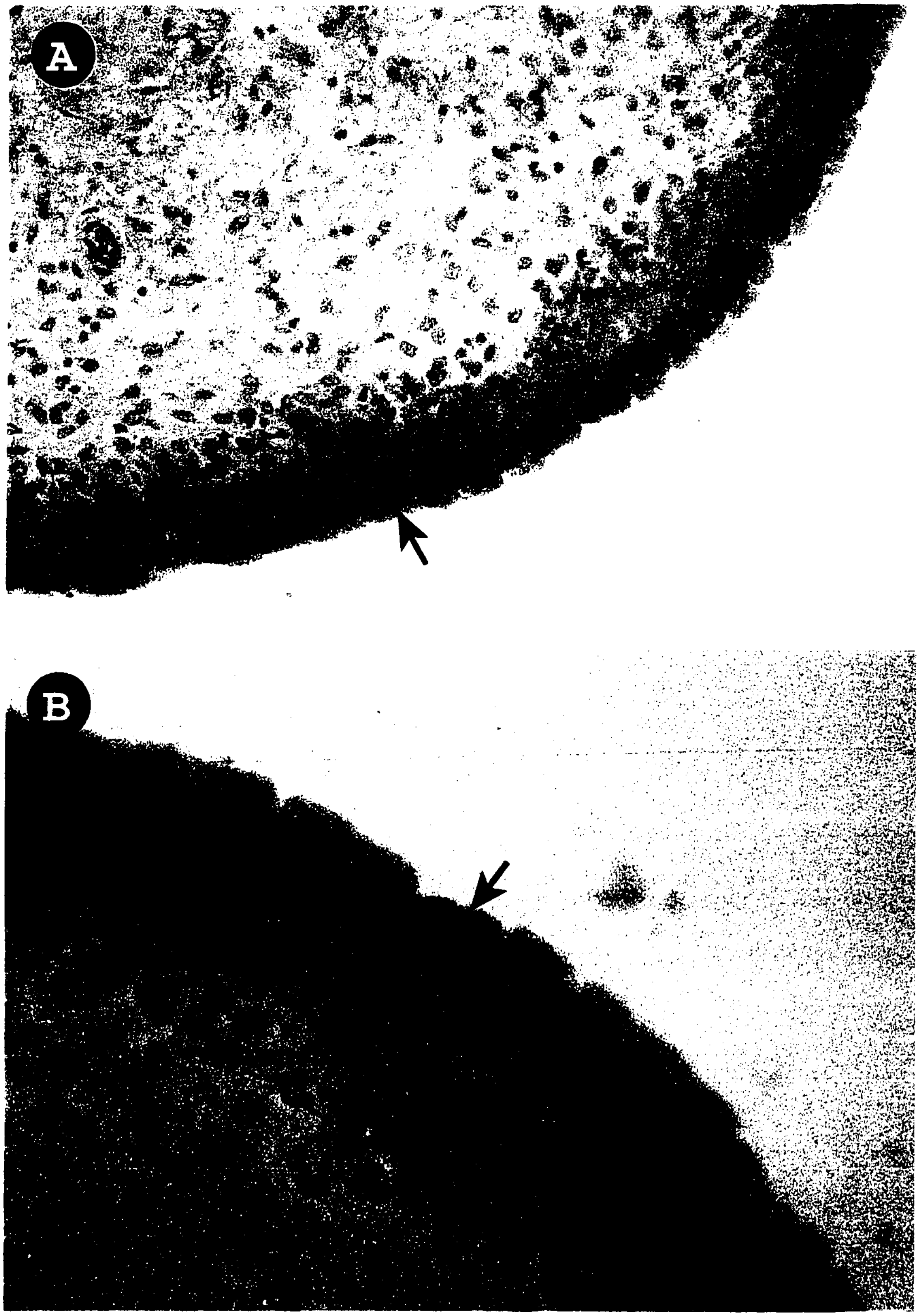
Figure 5: Micrographs of uninflamed regions of human bladder tissue immunostained for HS, C-4-S and PLL. Gill's hematoxylin counterstain.

A. *This tissue was stained with monoclonal anti-HS antibodies. High levels of* HS were localized in the umbrella cells (arrow) and HS was undetectable in the basal lamina. Magnification approximately x320.

B. This tissue was stained with monoclonal anti-HS antibodies. High levels of HS were localized in the umbrella cells (arrow) and HS was undetectable in the basal lamina. Magnification approximately x560.

C. This tissue was stained with monoclonal anti-C-4-S antibodies. Low levels of C-4-S were localized in the umbrella cells (arrow). C-4-S was undetectable in the basal lamina. Magnification approximately x240.

D. This tissue was stained with polyclonal anti-PLL antibodies. High levels of PLL and were localized in the umbrella cells (arrow). PLL was undetectable in the basal lamina. Magnification approximately x480.


A
B


C

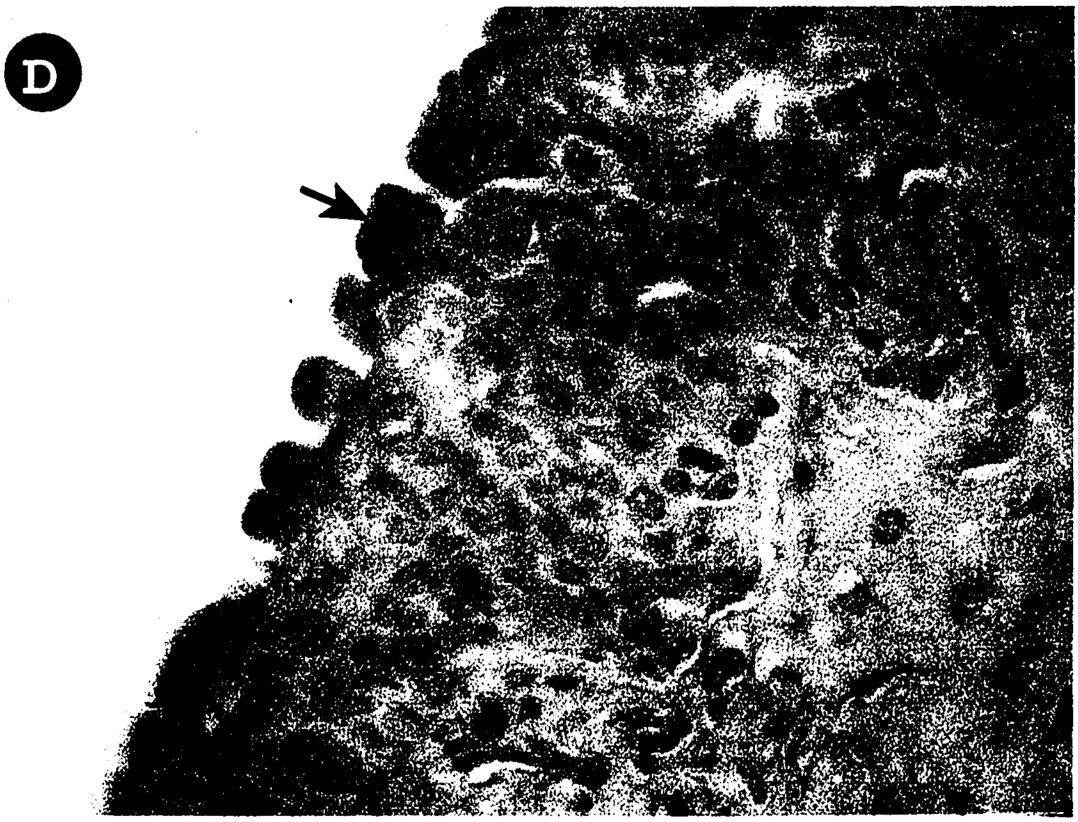

D

Figure 6: Micrographs of a highly inflamed region of human bladder tissue immunostained for HS. Note the lack of staining at the uroepithelial surface and the increased levels of HS in the basal lamina (arrow). Gill's hematoxylin counterstain.

A. This tissue was stained with monoclonal anti-HS antibodies. Magnification approximately x149.

B. This tissue was stained with monoclonal anti-HS antibodies. Magnification approximately x282.

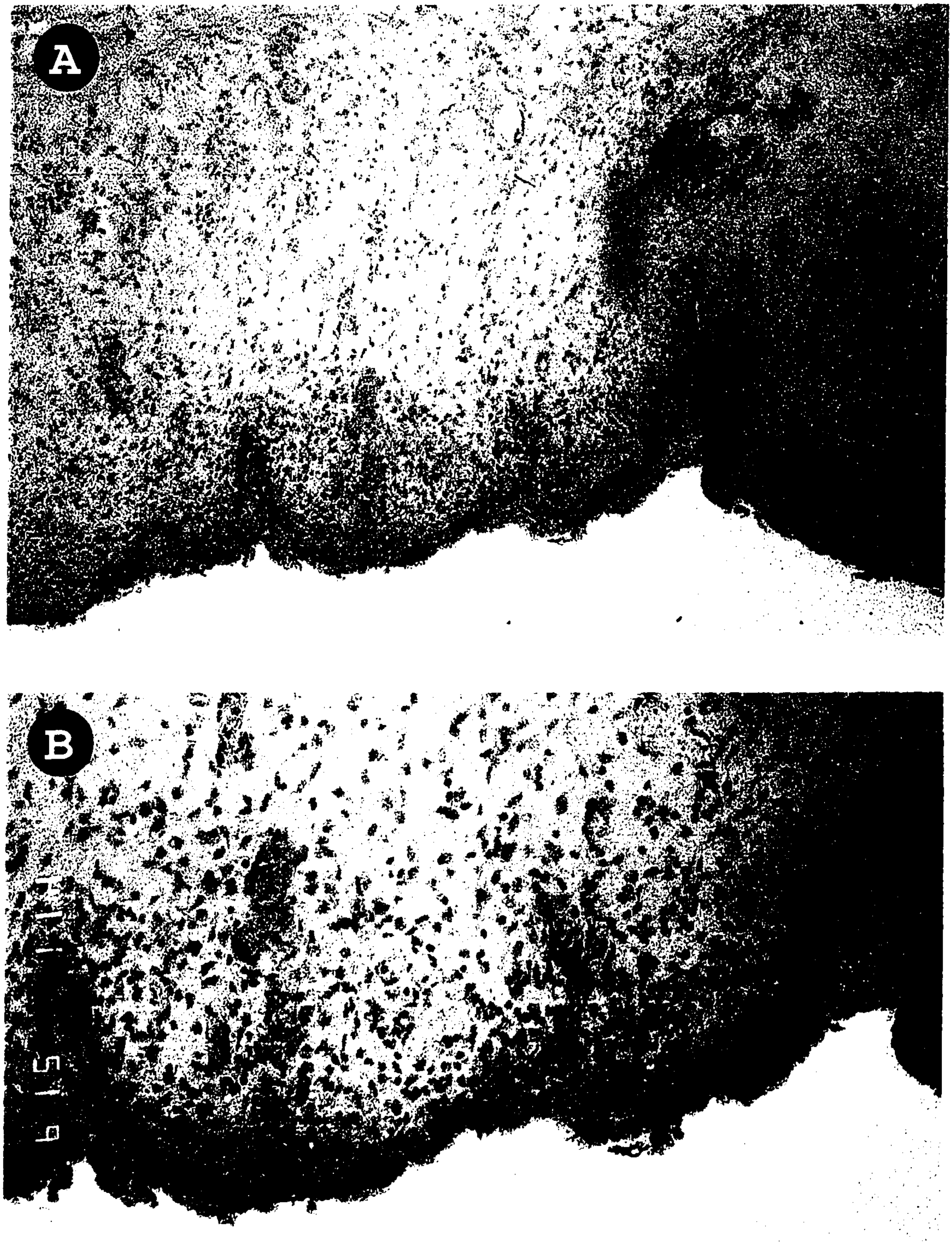

A
B

Figure 7: A bar graph which illustrates the mean surface staining levels of HS at each degree of submucosal inflammation. Variability is measured as standard error. Significant differences (P <0.05) of medians are reported at the top of each bar as degrees of inflammation, where applicable.

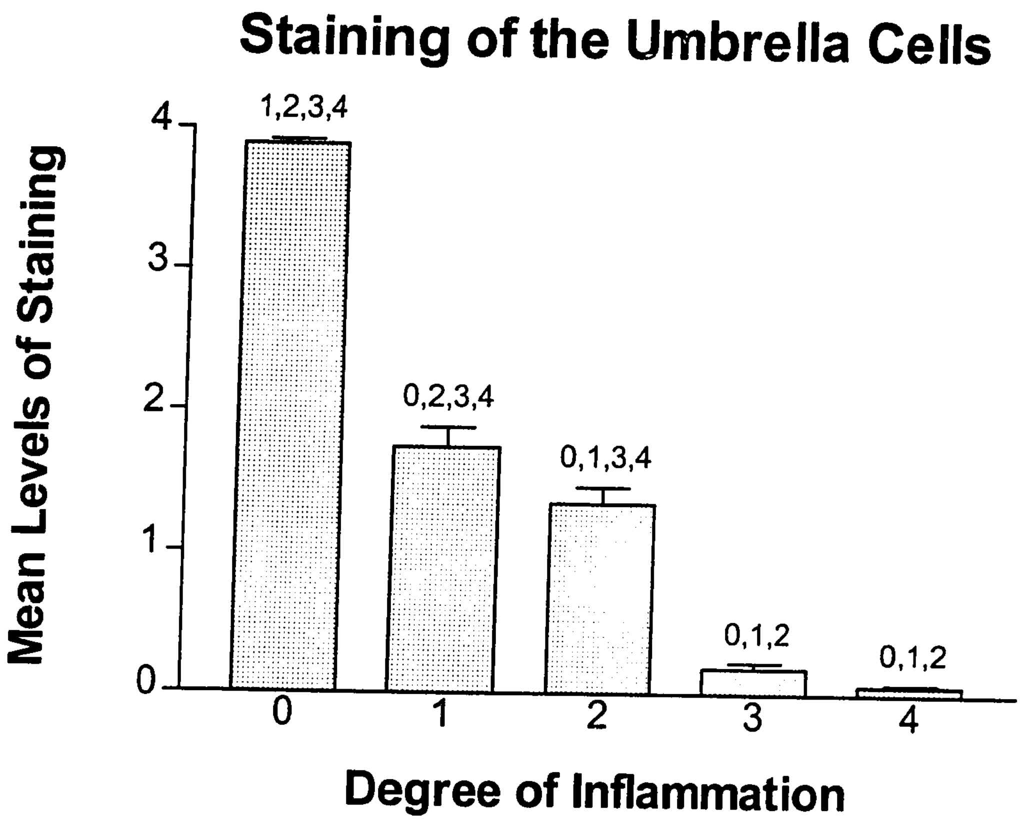
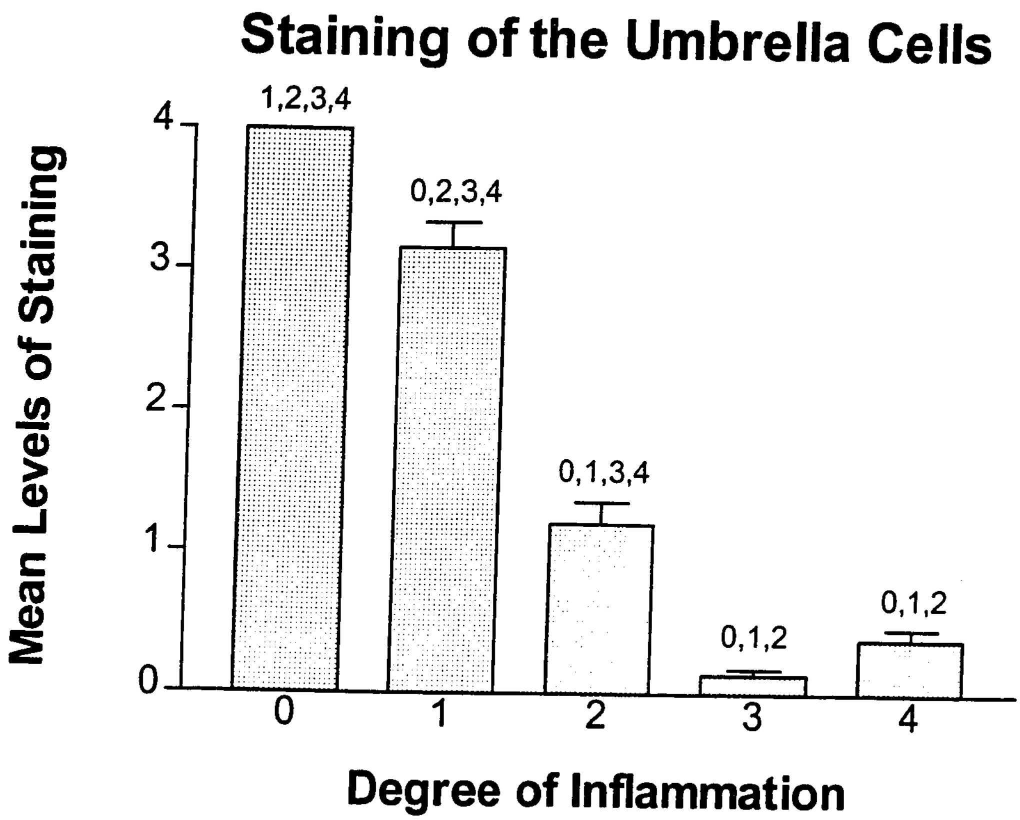

Staining of the Umbrella Cells
Mean Levels of Staining
4
3
2
1
0
1,2,3,4
0,2,3,4
0,1,3,4
0,1,2
0,1,2
0
1
2
3
4
Degree of Inflammation

Figure 8: A bar graph which illustrates the mean surface staining levels of PLL and mucin at each degree of submucosal inflammation. Variability is measured as standard error. Significant differences ($P < 0.05$) of medians are reported at the top of each bar as degrees of inflammation, where applicable.



Staining of the Umbrella Cells
1,2,3,4
0,2,3,4
0,1,3,4
0,1,2
0,1,2
4
3
2
1
0
Mean Levels of Staining
0
1
2
3
4
Degree of Inflammation

Figure 9: A bar graph which illustrates the mean surface staining levels of C-4-S at each degree of submucosal inflammation. Variability is measured as standard error. Significant differences ($P < 0.05$) of medians are reported at the top of each bar as degrees of inflammation, where applicable.

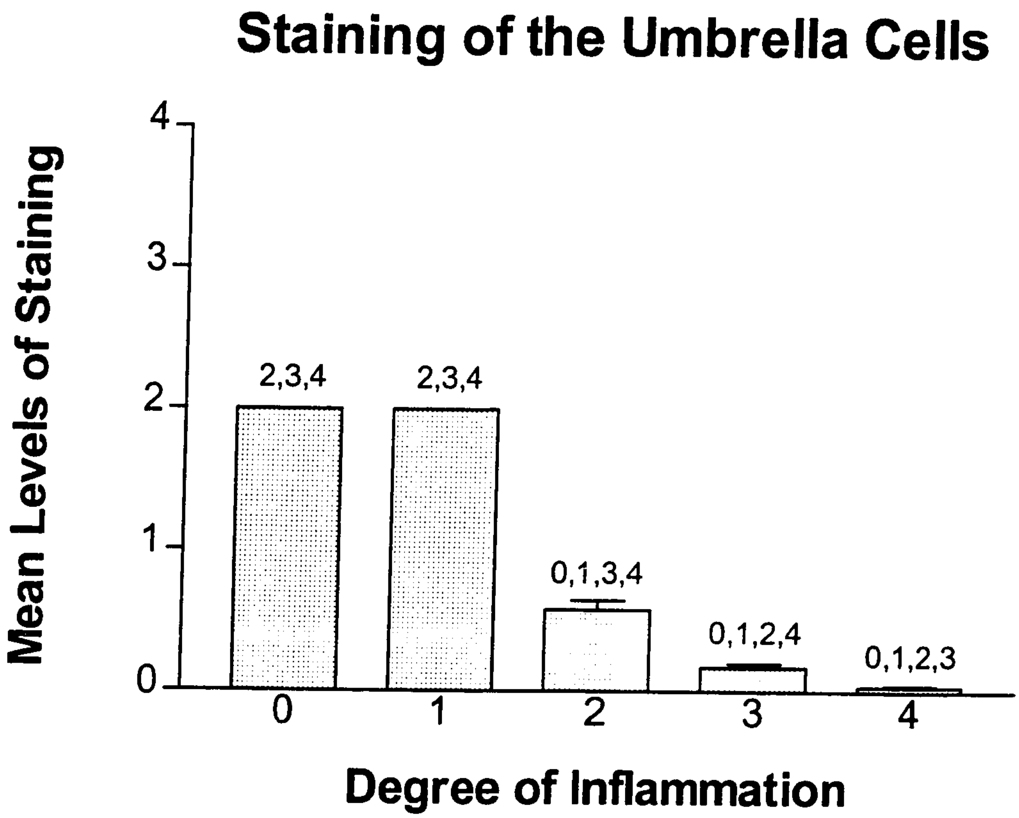

Staining of the Umbrella Cells
Mean Levels of Staining
4
3
2
1
0
2,3,4
2,3,4
0,1,3,4
0,1,2,4
0,1,2,3
0
1
2
3
4
Degree of Inflammation

Table 4: A summary of the mean levels of HS in the basal lamina at each degree of submucosal inflammation. Variability is measured as standard error. Significant differences ($P < 0.05$) of medians are reported as degrees of inflammation, where applicable.

Degree of Inflammation	Mean Levels of Staining ± SEM	Significant Differences ($P < 0.05$)
0	0.00 ± 0.00	1,2,3,4
1	0.98 ± 0.19	0,3,4
2	1.49 ± 0.14	0,3,4
3	3.24 ± 0.12	0,1,2
4	3.61 ± 0.07	0,1,2

Table 5: A summary of the mean levels of PLL in the basal lamina at each degree of submucosal inflammation. Variability is measured as standard error. Significant differences ($P < 0.05$) of medians are reported as degrees of inflammation, where applicable.

Degree of Inflammation	Mean Levels of Staining ± SEM	Significant Differences ($P < 0.05$)
0	0.00 ± 0.00	3
1	0.00 ± 0.00	3
2	0.00 ± 0.00	3
3	0.95 ± 0.12	0,1,2,4
4	0.21 ± 0.07	3

2. Rat Studies

Heparan sulfate and C-4-S GAGs were not detected at the mucosal surfaces of any of the rat bladders using immunohistochemistry. Although, HS and C-4-S were widely distributed throughout the connective tissue which was used as a positive control. Unlike the GAGs investigated in this study, PLL and human mucin were localized in the umbrella cells of the normal, untreated rat bladders and the DNBS-treated control bladders. An example of this is shown in Figure 10. Treatment with ethanol or PS resulted in a loss of staining at this location and some mild submucosal inflammation on occasion. These results can be seen in Figure 11. The rat bladders which were pre-treated with the barrier breaker (PS or ethanol) followed by DNBS resulted in extreme inflammation and an almost complete loss of tissue integrity as seen in Figure 12. These experiments were terminated after 12 hours due to a rapid decrease in survival rate of the test animals past this point. Post-mordem analyses on the test animals suggested that the tissue damage and inflammation resulted in an obstruction of the urethra which prevented voiding.

Figure 10: Micrographs of a normal, untreated rat bladder stained for PLL. Placental lactose lectin is found in the umbrella cells of normal rat bladders (arrow). Gill's hematoxylin counterstain.

A. This tissue was stained with polyclonal anti-PLL antibodies. Magnification approximately x500.

B. This tissue was stained with polyclonal anti-PLL antibodies. Magnification approximately x700.

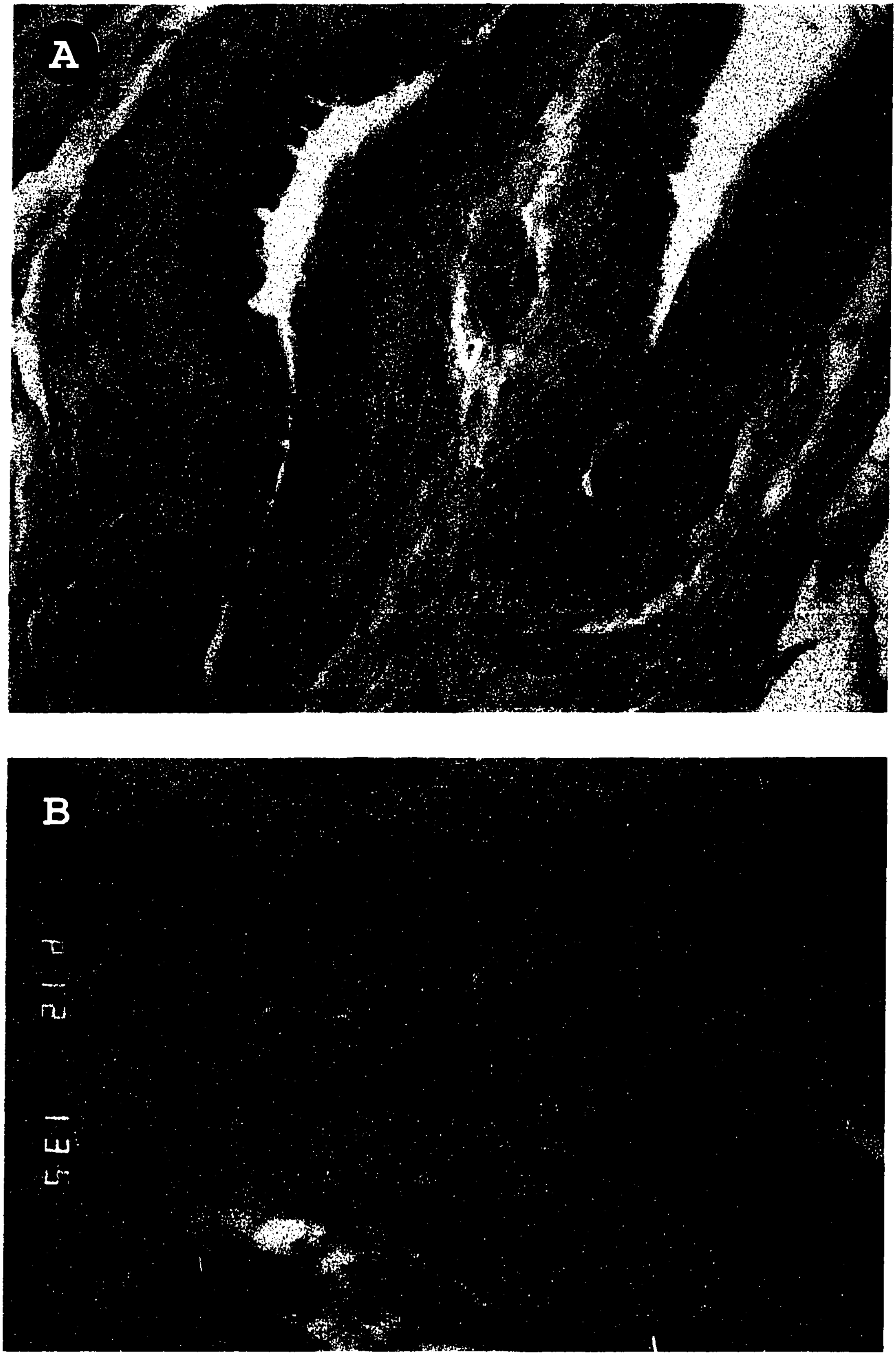

A
B

Figure 11: Micrographs of ethanol- and PS-treated rat bladder tissue immunostained for PLL. This micrograph shows the loss of PLL in the umbrella cells of ethanol- and PS-treated rat bladders (arrows). Gill's hematoxylin counterstain.

A. This tissue was treated with ethanol and stained with polyclonal anti-PLL antibodies. Magnification approximately x400.

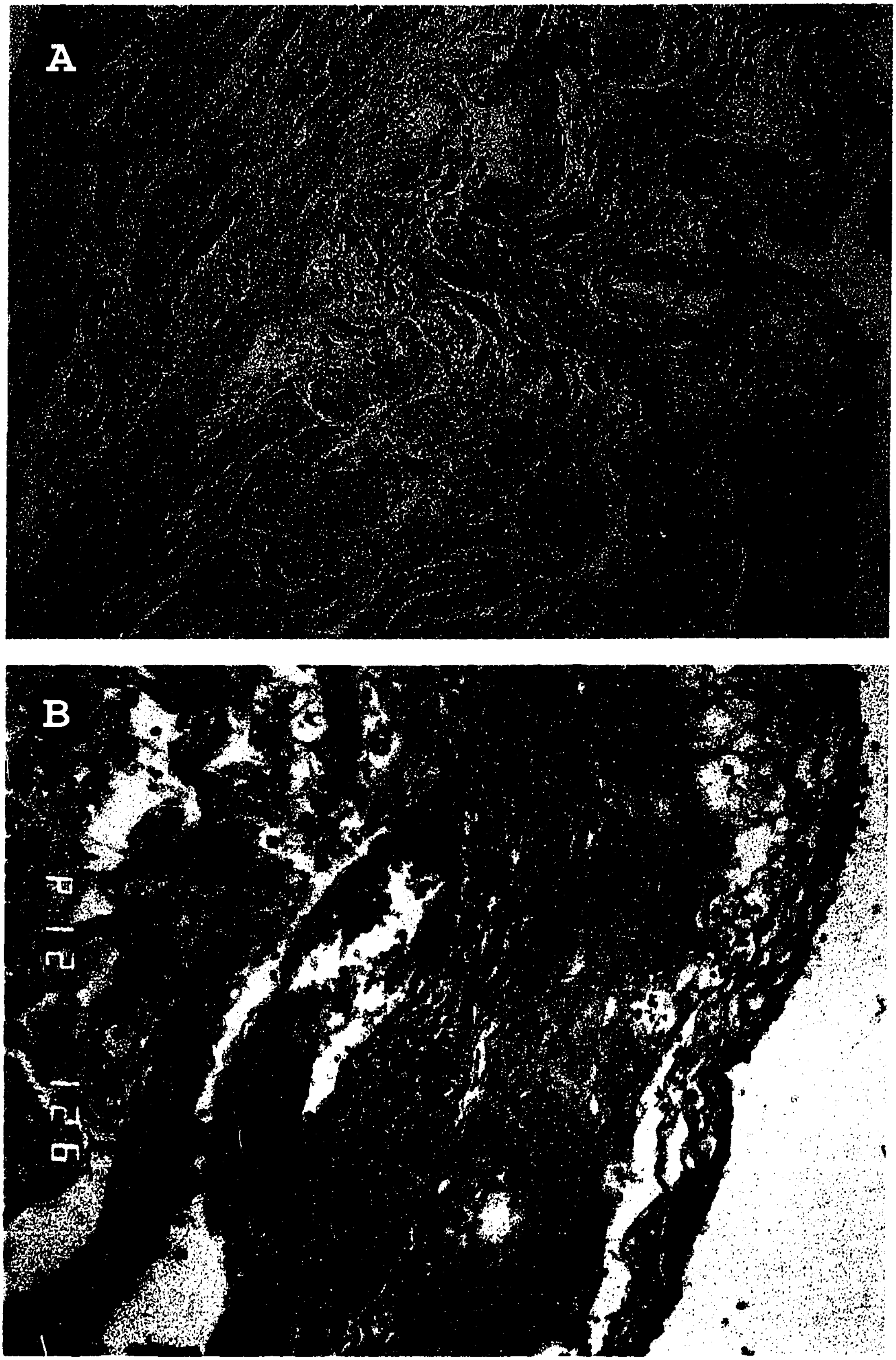
B. This tissue was treated with PS and stained with polyclonal anti-PLL antibodies. Magnification approximately x400.


A
B

Figure 12: Micrographs of a rat bladder pre-treated with ethanol followed by DNBS and immunostained for PLL. These micrographs show the extreme inflammation and loss of tissue integrity in rat bladders treated with the combination of ethanol and DNBS. Gill's hematoxylin counterstain.

A. This tissue was stained with polyclonal anti-PLL antibodies. Magnification approximately x173.

B. This tissue was stained with polyclonal anti-PLL antibodies. Magnification approximately x325.


A
B

DISCUSSION

The human bladder tissues chosen for this study were from patients diagnosed with IC. Like ulcerative colitis, IC is a "patchy" disease with localized areas of submucosal inflammation and denudation. Thus, in the human studies it was quickly discovered that most of the bladders investigated had healthy regions in addition to the more abundant diseased regions along the uroepithelium. This reflects the skewed frequency distribution in Figure 1. As a consequence, the bladders were scored in small increments which allowed the investigators to analyze the relationship between the distribution of mucosal surface molecules, denudation and submucosal inflammation.

There are certain symptoms which suggest that a patient is suffering from IC, such as pain in the area of the bladder. However, before a concrete diagnosis can be made, a histological examination is done. There are only two consistent histological markers of IC which are submucosal lymphocytes and denudation of the uroepithelium (Lynes *et al.*, 1990). This is consistent with the findings of this project. The areas that had the greatest numbers of submucosal lymphocytes correlated with statistically higher levels of uroepithelial denudation (see Figure 4). Although there were other submucosal inflammatory cells present in the inflamed regions of the IC bladder, the principal cell type was the lymphocyte. Neutrophils were also prominent but it was not necessary to identify each cell for the purposes of this study.

This project demonstrated that HS, C-4-S, PLL and human mucin colocalize in the umbrella cells at the mucosal surface of healthy human urinary bladders. This supports the first hypothesis which stated that, "HS, C-4-S, PLL and human mucin would be found at the mucosal surface of the uninflamed human urinary bladder". It also suggests that these surface cells are responsible for the secretion of these molecules. Chondrocytes and fibroblasts are well-known as major producers of PGs but it has also been reported that tracheal, uterine, glomerular and mammary epithelial cells secrete PGs as well (Clark *et al.*, 1994; Lohmander *et al.*, 1979; Mapstone & Culp, 1976; Boat *et al.*, 1992; Carson *et al.*, 1988; Bustos *et al.*, 1995; Gowda *et al.*, 1986). Furthermore, galectins have been found in intestinal epithelial cells and directly shown to be secreted in to the intestinal lumen (Beyer & Barondes, 1982). Likewise, mucin is secreted by intestinal epithelial cells (Ceri *et al.*, 1998). Because of their properties and their location, it is thought that the function of these mucosal surface molecules is to help protect the underlying tissue from the contents of the lumen. Evidence for this lies in the fact that these molecules became absent from this location whenever the histological signs of IC were witnessed (see Figures 7, 8 and 9). This supports the second hypothesis which stated that, "as the degree of submucosal inflammation and denudation increased, there would be significant decreases in the levels of...[HS, C-4-S, PLL and human mucin]...at the mucosal surface". Furthermore, experiments have been done which demonstrate the following:

1) When the GAG barrier is neutralized, the protective properties (both chemical and bacterial) of the barrier are lost (Parsons, 1994a; Parsons, 1994b; Parsons *et al.*, 1988; Stein *et al.*, 1996).

2) The exogenous administration of GAGs restore the protective properties of the barrier (Parsons, 1994b).

3) The exogenous administration of GAGs has a therapeutic effect in IC patients (Parsons *et al.*, 1994).

The idea that GAGs, lectins and mucins protect mucosal surfaces isn't unique to the bladder. Similar functions have also been suggested in the stomach, intestine and prostate, for example (Yamamoto *et al.*, 1995; Ceri *et al.*, 1998; Oke, 1996).

An animal model was used to support these claims. First, the distribution of these molecules in the normal rat bladder was characterized. The results suggested that although none of the GAGs were found at the mucosal surface, PLL and mucin did play a role in protecting the underlying tissue. Both lectin and mucin were found in the umbrella cells of the healthy rat bladder (see Figure 10). It was demonstrated in the rat model that when these molecules are removed from the mucosal surface, the underlying tissue becomes susceptible to inflammation. Since HS and C-4-S were not found at the mucosal surface of the rat bladder, the third hypothesis is only supported in part. The third and final hypothesis stated that, "[HS, C-4-S, PLL and human mucin]...would contribute to the protection of the underlying tissue which would otherwise become susceptible to irritation and inflammation". In this animal model, the chemical irritant used was DNBS. Healthy, otherwise untreated rat bladders were resistant to the inflammatory effects of the DNBS. However, when the rat bladders were pre-treated with PS or ethanol, both of which disrupted the mucosal integrity of the bladder (Figure 11), the bladders became susceptible to the effects of the DNBS (see Figure 12). Similar processes may also be happening in the human bladder.

If IC is preceded by a breakdown of mucosal integrity, what causes the breakdown in the first place? Certainly not PS or ethanol. Changes in the distribution of GAGs, lectins and mucins have been known to occur during development and certain disease states such as colon cancer (Schamhart & Kurth, 1994; Oke, 1996). Therefore, it is possible that a change in the distribution of these molecules occurs at the bladder mucosa prior to IC. If this change involved a loss of the protective mucosal surface molecules, it could render an individual susceptible to irritation and inflammation. Unlike the rat, the human bladder would not normally be exposed to DNBS. However, it could be exposed to potentially harmful microbes and it would certainly be exposed to the toxic contents of the urine.

An alternative possibility is that the submucosal inflammation associated with IC precedes changes at the uroepithelium and contributes to the following:

1) tissue destruction and a breakdown of mucosal integrity.

2) changes in the distribution of mucosal surface molecules.

3) an influx of urine solutes which could sustain or augment the inflammation.

Evidence has been found which suggests that IC has an autoimmune etiology. Many IC patients have other autoimmune diseases such as rheumatoid arthritis and Hashimoto's thyroiditis and 50% of IC patients have autoantibodies (Shipton, 1965; Silk, 1970; Ochs, 1997). Furthermore, several autoimmune models of IC which show features similar to those of human IC have been developed (Luber-Narod *et al.*, 1996; Bullock *et al.*, 1992). It is well-known from cartilage studies that inflammatory mediators such as transforming growth factor-β (TGF-β) can affect the distribution and structure of proteoglycans. Serine proteinases of the tertiary granules of neutrophils are also thought to degrade PGs in the cartilage of rheumatoid arthritis patients (Weiss, 1989; Bryson *et al.*, 1998). Transforming growth factor-β is a cytokine produced by T lymphocytes, platelets and monocytes (Feghali *et al.*, 1997). It plays several important roles in acute and chronic inflammation including neovascularization, wound healing and chemoattractant effects on neutrophils, T lymphocytes and unactivated monocytes (Stvrtinova *et al.*, 1995). Bujia *et al.* (1996) demonstrated that TGF-β upregulates the synthesis of PGs and GAGs in nasal septum chondrocytes *in vitro*. Likewise, Zanni *et al.* (1995) showed that TGF-β alters the structure and sulfation pattern of newly synthesized GAGs. Furthermore, TGF-β is known to have antiproliferative effects on epithelial cells (Stvrtinova *et al.*, 1995). Thus, inflammatory mediators including TGF-β and serine proteinases could contribute to some of the pathological changes seen at the uroepithelium of IC patients. In this scenario, the inflammation could cause the underlying bladder tissue to become exposed to potentially harmful urine contents which would in turn perpetuate the disease. It is also important to note that changes in the structure of the GAGs could theoretically render them undetectable by the antibodies used in this study.

This project also revealed that in patients with IC, there is an upregulation of HS and, to a lesser extent, PLL in the basal lamina in the areas of greatest surface destruction and submucosal inflammation (Tables 4 and 5). However, this was often seen only when there were remnants of the uroepithelium left. This phenomenon relates back to developmental changes in the distribution of GAGs and lectins and was particularly interesting for the following reasons:

1) The structure of the basal lamina affects the proliferation, movement and differentiation of the overlying epithelial cells (Fuchs *et al.*, 1997).

2) Both HS and PLL were thought to contribute to the physical protection of the underlying tissue when found in the umbrella cells.

This raises the following questions:

1) Is this upregulation a result of an almost complete denudation of the overlying uroepithelium?

2) Could this be a trigger for the remaining cells to divide and restore the uroepithelium?

3) Could this upregulation result in some physical protection for the underlying tissue in the meantime?

The HS and PLL of the basal lamina may be interacting with other components in the basal lamina to form a network. For example, collagen is a major component of the basal lamina in the bladder and it has been demonstrated that galectins have the capacity to bind collagen strands through a galactose-hydroxylysine linkage (Wilson *et al.*, 1995; Chang *et al.*, 1987; Soderstrom *et al.*, 1987). Furthermore, galectin-3 has been shown to colocalize with laminin in the basal lamina (Bao *et al.*, 1995; Le Marer *et al.*, 1996). Heparan sulfates of the syndecan families of PGs have also been known to interact with collagen fibers and it is well-known that HS PGs are abundant in the glomerular basement membrane of the kidney (Iozzo, 1997; Scott, 1988; Oldberg & Svensson, 1997; Elenius & Jalkanen, 1994; Groffen *et al.*, 1997).

CONCLUSIONS

The molecules that were investigated in this study, namely HS, C-4-S, human mucin and PLL are found at the mucosal surface of the human urinary bladder. They co-localize in a certain subpopulation of cells at the uroepithelial surface, known as umbrella cells. This suggests that they could interact with each other at this location. In fact, it has been demonstrated that PLL binds, colocalizes and affects the secretion of mucin (Fang *et al.*, 1993; Oke, 1996; Ceri *et al.*, 1991; Ceri *et al.*, 1998). Furthermore, certain PGs have lectin-like domains along their protein cores which could also interact with mucins (Halberg *et al.*, 1986). However, further studies are required to determine whether GAGs (or PGs), mucins and/or lectins;

1) interact with each other at the mucosal surface of the urinary bladder.
2) affect their own secretion at the mucosal surface of the urinary bladder.

From this project it may be concluded that lectins and mucins play a protective role at the mucosal surface of the rat bladder. It may also be concluded that there is a loss of complex carbohydrates and lectins at the mucosal surface of IC bladders. These results demonstrate that a breakdown in the integrity of a mucosal barrier consisting of complex carbohydrates and lectins in the human urinary bladder results in or from IC. Furthermore, the findings of this project may be used in conjunction with clinical findings to further characterize and diagnose IC.

FUTURE STUDIES

This project posed more questions than it answered. Further experiments are required before the etiology and the progression of IC can be characterized. These would ultimately involve;

1) characterizing the structural associations between the mucosal surface molecules.

2) defining the specific interactions between these molecules and components of the mucosal membrane.

3) identifying any intracellular events which result from these interactions and changes in their distributions.

4) defining the specific interactions and effects of inflammatory mediators on mucosal surface molecules.

5) the development of PG, GAG and galectin knockout mice as animal models of IC.

6) the study of animal models of autoimmune IC.

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APPENDICES

Appendix 1. The data used in the statistical analyses. The mean, sample size, standard deviation (SD), standard error (SEM), median, minimum value and maximum value of denudation or staining is summarized for each degree of submucosal inflammation.

Uroepithelial Denudation					
Inflammation	0	1	2	3	4
Mean	0.1098901	1.25581395	1.75510204	2.58895706	2.7987013
Sample Size	91	86	196	163	308
SD	0.3144855	0.67189016	1.1507966	0.92115106	0.6239505
SEM	0.032967	0.07245178	0.08219976	0.07215012	0.355529
Median	0	1	2	2	3
Minimum	0	0	0	1	2
Maximum	1	2	4	4	4

Surface HS Staining					
Inflammation	0	1	2	3	4
Mean	3.89	1.7441861	1.3469388	0.1840491	0.0649351
Sample Size	91	86	196	163	308
SD	0.314	1.2942047	1.6523614	0.5799028	0.2468122
SEM	0.033	0.1395577	0.1180258	0.0454215	0.0140634
Median	4	1.5	1	0	0
Minimum	3	0	0	0	0
Maximum	4	4	4	3	1

Surface PLL Staining					
Inflammation	0	1	2	3	4
Mean	4	3.15873016	1.2	0.1375661	0.38829787
Sample Size	71	63	100	189	188
SD	0	1.43915689	1.5891043	0.5574604	1.05123878
SEM	0	0.18131673	0.1589104	0.0405493	0.07666947
Median	4	4	0	0	0
Minimum	4	0	0	0	0
Maximum	4	4	4	3	4

Surface C-4-S Staining					
Inflammation	0	1	2	3	4
Mean	2	2	0.5789474	0.1743119	0.03773585
Sample Size	18	27	152	218	318
SD	0	0	0.8017061	0.3802506	0.19085702
SEM	0	0	0.065027	0.0257538	0.01070273
Median	2	2	0	0	0
Minimum	2	2	0	0	0
Maximum	2	2	2	1	1

Basal HS Staining					
Inflammation	0	1	2	3	4
Mean	0	0.97674419	1.4897959	3.2392638	3.61038961
Sample Size	91	86	196	163	308
SD	0	1.72849301	1.9387805	1.5746227	1.18795084
SEM	0	0.1863882	0.1384843	0.123334	0.06768978
Median	0	0	0	4	4
Minimum	0	0	0	0	0
Maximum	0	4	4	4	4

Basal PLL Staining					
Inflammation	0	1	2	3	4
Mean	0	0	0	0.952381	0.21276596
Sample Size	71	63	100	189	188
SD	0	0	0	1.7081959	0.90005752
SEM	0	0	0	0.124253	0.06564344
Median	0	0	0	0	0
Minimum	0	0	0	0	0
Maximum	0	0	0	4	4