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# **EXPERIMENTAL BLADDER RECONSTRUCTION**

**Hans Frederiksen**



**LUND UNIVERSITY**

2007



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## LIST OF PAPERS

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I. Frederiksen H, Davidsson T, Månsson W and Uvelius B. 1999. Sprouting of bladder nerves into cystoplastic cecal segment in the rat. *Urol. Res.* 27:476-482.
- II. Frederiksen H, Sjuve R, Arner A, Uvelius B. Regeneration of detrusor muscle after subtotal cystectomy in the rat; effects on contractile proteins and bladder mechanics. *Neurourol & Urodyn.* 20:685-697, 2001.
- III. Frederiksen H, Arner A, Malmqvist U, Sjuve R, Uvelius B. 2004. Nerve induced responses and force-velocity relations of regenerated detrusor muscle after subtotal cystectomy in the rat. *Neurourology and Urodynamics* 23:159-165.
- IV. Semerdzhiev Y, Frederiksen H, Hedlund P, Davidsson T, Månsson W, Uvelius B. Cystometric and in-vitro muscle studies of cystoplastic appendiceal segments in the rat. *Neurourol Urodyn* 2006; 25:259-267.
- V. Frederiksen H, Davidsson T, Gabella G, Uvelius B. Nerve distribution in rat urinary bladder after incorporation of acellular matrix graft or subtotal cystectomy. Submitted 2007.

## ABBREVIATIONS

ATP	Adenosine triphosphate
ATP- $\gamma$ -S	Adenosine 5'-O-(3-thio)triphosphate
BAMG	Bladder acellular matrix graft
ECP	Enterocystoplasty
EFS	Electrical field-stimulation
EGTA	Ethylene glycol tetraacetic acid
DPX	Di-n-butylphthalate in xylene
IF	Intermediate filaments
ISO-OMPA	Tetra(monoisopropyl)pyrophosphoramidate
$K_{\max}$	Force at maximal contraction elicited by high $K^+$ -concentration in the presence of $Ca^{2+}$
mN	Millinewton
NANC	Non-adrenergic, non-cholinergic
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIS <sup>®</sup>	Small intestinal submucosa
TTX	Tetrodotoxin
$V_{\max}$	Maximal shortening velocity



## INTRODUCTION

A reduced urinary bladder capacity may result from various causes, e.g. surgical resection, infectious or inflammatory disease. In neurogenic bladder disturbances, the overactivity will cause an elevated bladder pressure, posing a threat to kidney function in the long run. Often these problems are combined in a small bladder with significant overactivity. The symptoms caused by these conditions are often quite troublesome for the patient, not least socially. When pharmacological therapy fails to resolve the problem, surgical treatment remains.

Surgically enhancing bladder capacity will improve the storage function of the bladder, at the same time reducing pressure levels. The method of incorporating an intestinal segment into the bladder to achieve this was first used towards the end of the nineteenth century. Today, the routine procedure in these patients is enterocystoplasty (ECP), incorporating an intestinal segment into the opened bladder, most often using an ileal segment. This procedure can, in addition to peri-operative problems, cause a number of complications in the long run, such as metabolic acidosis with possible interference with bone mineralization, tumour formation, mucus production interfering with bladder emptying, urinary tract infections, gastrointestinal malfunction, and bladder rupture (McInerney and Mundy, 1995; Rink and Adams, 1998). A clinically important drawback is the risk of impaired bladder emptying, necessitating clean intermittent self-catheterisation (CISC) for a substantial number of patients. This in turn reduces the number of suitable patients for the procedure, since CISC requires a good hand function, which many of the patients with neurogenic disorders lack. Not much is known about the functional changes of the intestinal muscle following ECP.

It has been shown in the rabbit that the response to  $\alpha$ -agonists and ATP, as well as the response to electrical field stimulation, can differentiate intestinal from detrusor smooth muscle. Interestingly, intestinal smooth muscle showed a shift in these responses from that of intestine towards that of detrusor weeks to months after enterocystoplasty (Batra et al, 1987; Gill et al, 1989) provided that the intestinal segment was detubularized (Batra et al, 1992). However, little is known about the causes of these changes. A number of possible explanations have been put forward, such as the exposure to urine, the changed pattern of contraction and the possible effects of bladder nerves.

In the experiments described in paper I, we wanted to study what happens to the intrinsic innervation in the intestinal segment in ECP and to see whether nerves from the bladder could be demonstrated growing into it.

When subjected to increased functional demands, smooth muscle has a pronounced ability for compensatory growth (Gabella, 1987). Hypertrophy and



hyperplasia of the detrusor smooth muscle cells is seen with infravesical outlet obstruction (Gilpin et al, 1985; Gabella and Uvelius, 1990; Saito et al, 1996; Levin et al, 1995), which causes stretching of the detrusor muscle cells as residual urine develops, and increased workload during micturition. Growth of the detrusor is also seen secondary to increased workload and distension in rats with diabetes insipidus and experimental diabetes insipidus (Malmgren et al, 1992; Uvelius, 1996; Malmgren et al, 1989). Denervation of the bladder results in paralysis and distension; these bladders also increase their weight rapidly and detrusor muscle hypertrophy is seen (Uvelius and Mattiasson, 1986). It has been shown that stretch of isolated detrusor muscle rapidly stimulates de novo synthesis of DNA in the smooth muscle cells (Karim et al, 1992).

Following subtotal cystectomy in the rat, a rapid recovery of bladder capacity and bladder weight to near normal values is seen (Saito et al, 1996; Piechota et al, 1998). A reasonable assumption is that growth of the detrusor smooth muscle that remains after the operation is important for the recovery of the bladder. Since these bladders are not distended and the workload during each micturition is not increased (although the total number of micturitions per day is increased due to a reduced capacity), subtotal cystectomy represents an interesting model to study smooth muscle growth. Paper II is based on studies of newly formed muscle after subtotal cystectomy, aiming at determining to what extent adult animals can produce new, contracting bladder muscle, and if the mechanical properties of this muscle are different from control detrusor. Experiments on active and passive tension were performed as well as measurements of actin, myosin and cytoskeletal proteins.

Different parts of the normal urinary bladder have different patterns of innervation and receptor properties, reflecting their different roles in the micturition cycle. The detrusor of the bladder body, consisting of the parts above the ureteral orifices, is predominantly parasympathetically innervated, and has a high density of muscarinic receptors. It contracts strongly following muscarinic and purinergic stimulation, and relaxes following adrenergic stimulation (reflecting its role of storing urine between and contracting during micturitions). The bladder base, on the other hand, shows a dense adrenergic innervation and has a high density of alpha-adrenergic receptors. It contracts strongly following adrenergic stimulation but less following cholinergic and purinergic stimulation (reflecting its role of participating in maintaining continence between micturitions and relaxing during micturition)(Levin et al, 1980; Alm and Elmér, 1975; Alm et al, 1995).

Other investigators (Lin et al, 1995) have found that the growth of the bladder after subtotal cystectomy is mainly through distension of the bladder base, while we found that growth was mainly through growth of the bladder body, with a net

synthesis of contractile proteins and normalization of passive and active length-tension properties. This poses interesting questions regarding the properties of the regenerated bladder after subtotal cystectomy. Will it have properties resembling those of the bladder base or mimic the properties of the bladder body that it replaces? In paper III, we have investigated whether the motor nerve innervation of the new bladder body after subtotal cystectomy was dense enough to allow normal activation of the smooth muscle cells, by comparing force production through nerve stimulation and direct muscle cell stimulation and by determining possible supersensitivity to muscarinic activation. We have also examined if there were any regional differences in pharmacological properties and force-velocity relations in the normal bladder body, and if such differences were reflected in the properties of the newly formed bladder body.

In paper IV, we continued our investigation of the possible functional integration of intestinal segments that have been incorporated in the bladder by enterocystoplasty. Only limited information is available on the function of the enterocystoplastic muscle during filling and emptying. Also, it is not known whether the intestinal muscle cells will eventually be replaced by detrusor muscle cells, or their precursors, moving into the intestinal segments in rather the same way as cells migrate into acellular matrix grafts (e.g., Piechota et al, 1998). The changed pattern of response to electrical field stimulation and agonist stimulation seen in intestinal muscle some time after enterocystoplasty (Gill et al, 1989; Batra et al, 1988; Batra et al, 1992) might be influenced by several factors, such as a changed pattern of distension and contraction, urine exposure and the influence of bladder nerves. The fact that detubularization is necessary to bring about the changes (Batra et al, 1992) indicates that the increased contact area between detrusor and intestinal segment and the exposure to the pattern of stretch and relaxation of the bladder that result from detubularization might be important, and that the importance of exposure to urine is less, if any. It has been shown that in the detrusor itself, rhythmic stretch and relaxation influences muscarinic receptor density (Chun et al, 1989). Our results in (I) indicate that the changes might be brought about by atrophy of the intrinsic intestinal innervation and by influence from bladder nerves entering the intestinal segment. In paper IV, we test this hypothesis by *in vivo* cystometry in conscious rats that have undergone ECP, and by *in vitro* experiments on detrusor, intestinal and enterocystoplastic smooth muscle.

Different methods to avoid the clinical problems associated with enterocystoplasty, such as mucus secretion and urine resorption, have been tried and are under development. One example is autoaugmentation with removal of detrusor muscle, but leaving the mucosa intact (e.g., Swami, 1998; Stöhrer, 1997). Matrix grafts used as a scaffold, into which muscle, blood vessels, connective tissue and nerves from the bladder can grow, have been used in a

number of studies, several of them using bladder acellular matrix grafts (BAMG) from the same species or small intestinal submucosa (SIS<sup>®</sup>) grafts from pig. Excellent results have recently been reported (Atala et al, 2006) in a patient series with collagen/polyglycolic acid grafts into which autologous detrusor muscle cells and urothelial cells were seeded prior to surgery.

In experiments with BAMGs in rats, urothelial lining on the luminal surface was completed already at 2 weeks (Probst et al, 1997). Fibroblasts and capillaries could be seen growing into the grafts as early as after 1 week (Probst et al, 1997; Wefer et al, 2001) and muscle cells after 3 weeks. 12 weeks after the operation the muscle layer was so well developed that it was difficult to delineate the graft from the original bladder tissue (Probst et al, 1997).

SIS grafts seem to be incorporated in the rat bladder as fast as the BAMGs (Kropp et al, 1995), despite consisting of non-bladder tissue and deriving from a different species. SIS cystoplasty in larger animals as dog (Kropp et al, 1996) and pig (Merguerian et al, 2000) demonstrate vessels and smooth muscle in the matrix almost as early as in the rat.

Nerves can be demonstrated in histological sections from rat BAMGs as early as 4 weeks after surgery (Probst et al, 1997; Sutherland et al, 1997). Tissue strips from BAMG 4 months (Piechota et al, 1998) and from SIS 11 months (Vaught et al, 1996) after surgery have been shown to contract when field stimulated in vitro, indicating the presence of functioning motor nerve endings.

We have previously shown on rat bladder wholemounts (Uvelius and Gabella, 1998) that on the ventral wall of the bladder the detrusor muscle is arranged in well-defined longitudinal bundles with discrete longitudinal nerve trunks, while in the lateral and dorsal walls muscle and nerves are less regularly arranged. In paper V, we have compared the nerve arrangement pattern in bladders with SIS implants with the pattern in bladders regenerated after subtotal cystectomy and control bladders. Will the muscle and nerve arrangement in the SIS implant show a different pattern than the native bladder; will the arrangement in the remaining part of the partially resected bladder, into which the SIS graft has been incorporated, be affected? We compared wholemounts, stained for demonstration of acetylcholinesterase, of bladders with SIS implants, bladders that had developed after subtotal cystectomy, and control bladders.

## **AIMS OF THE STUDY**

- To determine if bladder nerves grow into the intestinal segment after enterocystoplasty in the rat.
- To study what happens with the intrinsic innervation in the intestinal segment after enterocystoplasty.
- To determine whether a functional integration of the intestinal smooth muscle takes place after enterocystoplasty.
- To determine to what extent adult rats can produce new contracting bladder muscle.
- To investigate if the mechanical and pharmacological properties of such newly formed muscle differs from that of native bladder muscle.
- To study the nerve distribution pattern after matrix grafting and after subtotal cystectomy.

## **MATERIALS AND METHODS**

The animal experiments were approved by the local animal research ethics committee.

Female rats of the Sprague-Dawley strain, weighing 200-250 g, were used in all the experiments.

### **Surgery**

Anaesthesia was accomplished throughout using ketamine (100 mg/kg body weight) and xylazine (15 mg/kg body weight) injected intramuscularly.

In all cases, access to the bladder was made through a lower abdominal midline incision, which after completed surgery was closed in two layers, using interrupted 3-0 Dexon sutures.

Appendiceal enterocystoplasty (in I called cecocystoplasty) was performed using a detubularized segment measuring approximately 1 x 1 cm (I) or 7 x 7 mm (IV) from the mid-portion of the appendix isolated on its vascular supply. The anastomosis was completed with running 5-0 Dexon sutures.

Subtotal cystectomy (II, V) was accomplished by dissecting the bladder body free, ligating the bladder immediately above the ureteral orifices with 4-0 Dexon, then removing the bladder cranial to the ligature.

Small intestinal submucosa (SIS<sup>®</sup>) grafts sized about 5 x 7 mm were sewn in with running 5-0 Dexon<sup>®</sup> after resecting part of the top of the bladders (V).

When sham operations were used for the controls (IV), the bladders were exposed but not opened.

The animals were killed either by cervical dislocation or CO<sub>2</sub> asphyxia, followed by exsanguination.

### **Nerve staining, wholemounts**

Bladders to be examined as wholemounts (I, V) were removed from the animal immediately after sacrifice, the ureters ligated, the urethra cannulated and the bladder distended by 0,7 – 1 ml of saline. After fixation and longitudinal opening of the dorsal side of the bladders, they were transferred to Krebs' solution containing hyaluronidase and ISO-OMPA for 4 – 12 hours. They were then stained for acetylcholinesterase according to a variation of the method of

Karnovsky and Roots (1964) proposed by Baker et al (1986). This method not only gives a general nerve stain but also a weak staining of the detrusor muscle bundles. After the incubation, usually overnight, the bladders were opened further by scalpel, enabling us to flatten the whole bladder out with minimal folds. After dehydration, the bladders were mounted on slides, and examined under a light microscope.

Control appendiceal segments, taken from the animals at sacrifice, were fixed, stained and mounted in the same manner (I).

Planimetry on prints was used to determine the area of implants and – in the subtotal cystectomies – the bladder surface above the ureteral orifices in relation to total bladder area (V) and to determine the relative area within implants with preserved innervation (I).

### **Histology**

Segments containing the anastomotic regions to be examined in cross-section by light microscopy were embedded in paraffin and cut sections stained for connective tissue and muscle using the Van Gieson method or with hematoxylin-eosin. Specimens from bladder wall regenerated after subtotal cystectomy and the corresponding controls were examined in a phase-contrast microscope after fixation in glutaraldehyde in cacodylate buffer and embedding in araldite.

### **Protein analysis**

For the analysis of protein contents in the bladder wall, detrusor specimens were taken at sacrifice and then stored at  $-86^{\circ}\text{C}$  until analysis. They were then thawed and homogenized in SDS buffer (for composition, see II), boiled and stored at  $-20^{\circ}\text{C}$  prior to SDS polyacrylamide gel electrophoresis (SDS-PAGE). Four different volumes of sample and of skeletal muscle actin with a known concentration were run in parallel on each gel. The gels were then stained with Coomassie Blue and scanned in a densitometer. The areas under the curve for the myosin heavy chain, the actin, and the intermediate filament bands were measured. The tissue concentration of actin and the ratios myosin/actin and intermediate filament/actin were then calculated.

## **Organ bath experiments**

Tissue strips for organ bath experiments were obtained under a dissecting microscope with the bladders and appendiceal parts submerged in Krebs solution. The strips were trimmed free of mucosa/submucosa before being transferred to the organ baths, where they were mounted with silk ligatures between a fixed rod and a force transducer connected to a polygraph. The organ baths contained oxygenated 37°C Krebs solution gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> giving pH 7.4.

### *Length-Tension Relations After Subtotal Cystectomy*

The muscle strips were allowed to accommodate for one hour at a preload of 5 mN.

Maximal contractions were produced by increasing K<sup>+</sup> and Ca<sup>2+</sup> concentrations after an initial 15 minutes in Ca<sup>2+</sup>-free Krebs solution. After each contraction the strips were shortened to a length at which no passive tension could be recorded, and relaxed in Ca<sup>2+</sup>-free Krebs for 5 minutes before being stretched and contraction at a new length was initiated.

The strips were stretched in steps until there was an obvious drop in active tension on further stretching. At each length step passive and active tension were recorded.

After completion of the active force experiments, a second passive tension curve was obtained by relaxing the strips with papaverine and EGTA in Ca<sup>2+</sup>-free Krebs solution, the strips were then stretched again from their shortest length in similar length steps at 10 minute intervals, long enough for a stable passive tension to develop.

After the experiment the strips were fixed at their optimum length for active force development and strip length was measured (enabling us to transform strip length to bladder circumference in the following analysis). They were then embedded in araldite and examined in a phase-contrast microscope where cross-sectional muscle area, relative to which all force values are given, was determined.

### *In Vitro Pharmacological Experiments After Subtotal Cystectomy*

Strips were taken from two circular rings of bladder tissue, one taken immediately above the ureteric orifices (supratrigonal preparation), the other from the equatorial region (equatorial preparation).

The strips were stretched to a preload of 5 mN and allowed to accommodate for 1 h.

In one series of experiments they were subject to electrical field stimulation (EFS) at 20 Hz at 3 min intervals (0.5 ms pulse duration, 7 s train duration, supramaximal voltage) until contractions were stable. Stimulation frequency was then changed in steps from 0.5 to 60 Hz.

Then the 0.5 to 60 Hz stimulation protocol was repeated in the presence of scopolamine, where-upon the strips were left to recover for 30 min.

Next, dose-response curves for first carbachol and then phenylephrine were registered by cumulative addition of the respective drug, with a 30 min washout in-between.

Contractions in high-K<sup>+</sup> Krebs solution were induced before and after the EFS experiments and after the dose-response experiments.

In a second series of experiments, the above-described protocol was followed until after the high-K<sup>+</sup> contraction after the first frequency-response determination. Frequency-response determinations were then made in the presence of first prazosin, then both prazosin and scopolamine. After recovery, non-cumulative dose-response relations to  $\alpha$ - $\beta$ -methylene-ATP were determined, where-upon the strips were desensitised to  $\alpha$ - $\beta$ -methylene-ATP by repeated exposures. In the continuing presence of  $\alpha$ - $\beta$ -methylene-ATP, prazosin and scopolamine were again added, and a fourth frequency-response curve was registered. All frequency-response determinations were followed by high-K<sup>+</sup> contractions.

Contraction responses to field stimulation and agonists were expressed as percentages of the first high-K<sup>+</sup> contraction; there was virtually no difference in amplitude between the first and last high-K<sup>+</sup> contraction in each experiment.

### *In Vitro Force-Velocity Experiments After Subtotal Cystectomy*

In one series of experiments on non-operated rats, we compared shortening velocity between equatorial and supratrigonal segments (n=6 for each group).

In a second series of experiments, equatorial strips were compared to the corresponding segment of age-matched controls at 6 weeks, 15 weeks and 9 months after surgery (n=6 for each group).

Chemically skinned preparations were used to ensure maximal activation of the contractile system, achieved by thiophosphorylation of the regulatory light



chains. The muscle strips were dissected to a length of about 3 mm and a diameter of 0.2 mm and mounted in a device for isotonic quick release, where one end was connected to a force transducer and the other to a lever for adjustment of afterload, that could be released or clamped. Then the strips were stretched to a length where passive tension was just noticeable and strip length measured. The strips were incubated for 15 min in an ATP-free rigor solution containing  $\text{Ca}^{2+}$ , calmodulin and ATP- $\gamma$ -S, and contracted by introducing MgATP. Shortening velocity, expressed in muscle lengths/second, was recorded 100 ms after release at different afterloads.

#### *In Vivo Cystometry After Enterocystoplasty*

11 rats underwent cystometry 1 month after enterocystoplasty (ECP), 6 sham-operated rats served as controls. Another 11 ECP operated rats underwent the same procedure 3 months postoperatively; here 8 sham-operated controls were used.

The bladder catheters were put in place through a lower midline incision under general anaesthesia 3 days before scheduled cystometry, in order for the bladders to accommodate.

A thin polyethene catheter was inserted and sutured into the bladder, then tunnelled subcutaneously to the back of the animal.

The animals were placed in metabolic cages, the catheter connected to a pressure transducer and an infusion pump, with which saline was infused at a rate of 10 ml/h. Micturition volumes were collected in a cup, their weight measured. Variables used in the study were recorded during a 30 min period when the micturition pattern had stabilized after onset of the infusion, which usually took about an hour. The cystometries were carried out during daytime. Micturition volume, residual volume (measured after a micturition at the end of the experiment, by lowering the catheter tip below the animal), threshold pressure (i.e., bladder pressure immediately before a micturition), basal pressure (i.e., bladder pressure immediately after a micturition) and spontaneous activity were recorded. Bladder capacity was calculated for each animal by adding its mean micturition volume and residual volume. Bladder compliance was calculated by dividing mean micturition volume ( $\Delta$ volume) with the difference between mean threshold pressure and mean basal pressure ( $\Delta$ pressure).

#### *In Vitro Pharmacological Experiments After Enterocystoplasty*

Tissues from enterocystoplasty segments, control bladders and appendix were harvested at 1 month (8 ECP operated animals, 6 sham-operated controls) and 3 months (13 ECP animals, 10 sham-operated controls) after surgery. Strips sized approximately 5 x 2 x 0.5 mm were dissected, trimmed free of mucosa and

suspended with silk ligatures between metal hooks, one of which was connected to a force transducer, measuring isometric tension, in organ baths with Krebs solution thermostatically kept at 37°C, bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, giving pH 7.4.

Passive tension after an initial 45 min calibration was measured to 2-3 mN.

Contractile ability was then tested by high-K<sup>+</sup> stimulation.

Electrical field stimulation: square-wave pulses with a duration of 0.5 ms were delivered through two platinum electrodes placed in parallel to the strips in the organ baths, frequency from 1 to 32 Hz, train duration 5 s and train interval 120 s.

In addition to plain frequency-response registrations, the impact of different concentrations of scopolamine on contractions induced by electrical field stimulation was investigated.

To ensure neuronal origin of the contractions in the EFS experiments, some of the strips were exposed to tetrodotoxin, after which virtually no contractile force remained.

Concentration-response curves for carbachol were determined by cumulative addition of this agonist. The response to a single concentration of  $\alpha$ - $\beta$ -methylene-ATP was tested in some preparations.

## RESULTS

### Enterocystoplasties

#### Microscopy, sections

When examined by microscopy in sections, bladders subjected to appendiceal enterocystoplasty showed an abrupt transition from urothelial to intestinal mucosa. The luminal surface of the intestinal segment was covered by a thin mucus layer. The anastomotic region was rich in collagen, containing little muscle and with no inflammation. In both the intestinal segment and the bladder, the musculature was well preserved; the bladder muscle was arranged in bundles, whereas in the intestinal segment the muscle layer was more homogenous and often thicker.

#### Microscopy, wholemounts

The acetylcholinesterase staining used for the wholemounts gave a faint staining of the muscle in both the bladder and the ECP segment, revealing a well-preserved smooth muscle with an even thickness in the whole segment. The myenteric plexus of the intestinal segment was heavily stained. While the control intestine throughout showed a very regular pattern of innervation, almost geometrical in appearance, the ECP segments showed a varied pattern; in all such segments, there were areas lacking the original nerve plexus pattern, showing various degrees of degeneration with loss of ganglion cells, loss of interconnecting axons and in some areas even a complete lack of ganglia and nerve trunks but still with well-developed muscle.

We found no correlation between the relative area with a preserved innervation and the time after the operation. The best-preserved areas of the myenteric plexus were generally found in the mid-portion of the intestinal patch, i.e. away from the line of anastomosis.

In contrast to the degeneration found in the myenteric plexus, we could observe nerves sprouting from the cut bladder nerves in the vicinity of the anastomosis. Several nerve bundles originating from cut bladder nerves could be seen entering the enterocystoplastic segment across the anastomotic line and then branching into smaller nerves. Some of these nerves reached lumps of surviving ganglion cells.

The nerves could also all be followed into areas of the intestinal patch devoid of ganglion cells and their course in the patch did not resemble that of myenteric nerves normally found in the intestine.

The rat bladder does not normally contain ganglia. In the ECP bladders, we found small ganglia in the bladder wall only in a few cases and they were located close to the anastomosis. These ganglia seemed to have contact with nerves from both the bladder and the intestinal segment.

### Cystometry

The in vivo cystometry experiments showed a significantly lower basal pressure in ECP bladders than in controls, one month as well as three months after surgery, with no signs of detrusor overactivity. The threshold pressure was also lower in the ECP bladders than in controls, although significant only at one month. Micturition pressure was significantly lower in the operated animals. Bladder capacity was significantly higher in the operated animals, as were micturition volumes, in spite of a residual volume of about 0,5 ml at both one and three months. There was virtually no residual urine in the controls. Bladder compliance was higher in the operated animals than in controls at one month, but showed no difference at three months.

The calculated threshold tension showed no difference between control and ECP bladders. The calculated maximum micturition tension (based on maximal micturition pressure and bladder capacity, since time resolution was too slow to determine at which volume the maximal pressure was developed; hence the true value of the maximum micturition tension will be lower than calculated in both controls and operated animals) was significantly lower in the ECP bladders than in controls.

An estimate of the force produced by the whole bladder circumference is given by multiplying the calculated maximum micturition tension by bladder circumference at bladder capacity. In one-month control and ECP bladders these estimates are  $824 \pm 129$  and  $514 \pm 110$  mN, respectively. In the three-month groups the corresponding estimates are  $1228 \pm 279$  and  $680 \pm 95$  mN. None of the differences reach significance.

### Functional Experiments on Isolated Tissues

*Agonist-induced responses.* Concentration-dependent contractions following cumulative addition of carbachol were registered in all preparations from implants, control detrusor and control appendix.

1 month after the sham operation, control detrusor strips showed maximal contraction at 10  $\mu$ M of carbachol,  $125 \pm 6\%$  of that elicited by maximal  $K^+$ -contraction ( $K_{max}$ ). Strips from implants and control appendix produced weaker contractions than the detrusor strips; for control appendix, significant differences versus control detrusor were obtained above 3  $\mu$ M. For implants, significantly weaker contractions were recorded at 3 and 10  $\mu$ M of carbachol.

Although implants seemed to respond with stronger contractions than control appendix (mean maximal contractions to carbachol of  $75 \pm 6\%$  for implants at  $30 \mu\text{M}$  and  $46 \pm 9\%$  at  $10 \mu\text{M}$  for control appendix expressed relative to  $K_{\text{max}}$ ) the difference was not significant.

At three months, similar response patterns were seen. Mean maximal detrusor strip contractions were  $121 \pm 6\%$  of  $K_{\text{max}}$  at  $10 \mu\text{M}$  of carbachol. At the same carbachol concentration, preparations from implants responded with maximal contractions of  $66 \pm 11\%$  ( $p < 0.05$  vs. detrusor and control appendix).

Corresponding figures for control appendix were  $24 \pm 4\%$  ( $p < 0.05$  vs. detrusor and implants).

When exposed to  $10 \mu\text{M}$  of  $\alpha$ ,  $\beta$ -methylene-ATP, control detrusor responded with contractions amounting to  $29 \pm 8\%$  and  $35 \pm 2\%$  of  $K_{\text{max}}$  at 1 and 3 months, respectively.

Exposure of implants and control appendix to the agonist resulted in a reduction in tension, with a similar response in both. There was no difference in response observed at 1 and 3 months after surgery.

*Electrically induced responses.* 1 month after surgery, tissue strips all showed frequency-dependent contractions, but the responses did not differ significantly at any investigated frequency. Maximal responses of  $92 \pm 3\%$  (32 Hz),  $92 \pm 14\%$  (24 Hz) and  $73 \pm 12\%$  (24 Hz) of  $K_{\text{max}}$  were reached in preparations from detrusor, implant and control appendix, respectively.

In the corresponding experiments 3 months after surgery, implants gave responses significantly higher than control appendix, except at 4 and 8 Hz. The implants also gave higher responses than detrusor, but significant only at 1 and 2 Hz.

When exposed to scopolamine ( $0.01 \text{ nM} - 1 \mu\text{M}$ ), the strips showed a concentration-dependent inhibition of EFS responses. At 1 month, inhibitory effects of  $69 \pm 3\%$ ,  $71 \pm 4\%$ , and  $34 \pm 10\%$  were obtained for detrusor, implant and control appendix, respectively, at  $1 \mu\text{M}$  of scopolamine. Control appendix differed significantly from the others ( $p < 0.05$ ).

At 3 months, implants were significantly more inhibited by scopolamine than the control appendix at  $1 \text{ nM} - 1 \mu\text{M}$ ;  $69 \pm 3\%$  vs.  $43 \pm 6\%$  reduction at  $0.1 \mu\text{M}$ , respectively. At the same concentration, detrusor strip contractions were reduced by  $59 \pm 4\%$ .

## **Subtotal Cystectomies**

### ***Bladder Weight And Shape***

As would be expected, the bladder weight decreased dramatically following subtotal cystectomy. Four days after the operation, bladder weight, including the base, was about 50% of that in controls. After 10 days, a slight increase could be seen, and after 10 weeks, bladder weight had normalized. The shape of the operated bladders was, as in controls, oval to almost spherical. Most of the bladders consisted of supratrigonal tissue, although a shift upwards of the ureteral entrances on the dorsal side was noted, the distance between the ureteral orifices was also wider than in controls, indicating some distension of the trigonal area as well.

### ***Microscopy, sections***

10 weeks after the operation, light microscopy of sections of specimens from the ventral part of the bladders showed practically no difference compared to controls; there was a continuous urothelial lining, a submucosa and a well-developed muscle layer organized in bundles, running at different angles, with a size of the individual cross-sectioned muscle fibres that seemed to be the same as in controls.

Examination with phase-contrast microscopy of sections from the strips used in the length-tension experiments showed densely packed smooth muscle bundles separated by small amounts of interstitial connective tissue; the picture was similar in operated animals and controls. The diameter of nucleated smooth muscle cell profiles seemed to be equal in both groups.

### ***Microscopy, wholemounts***

The detrusor muscle of the control bladders stained lightly for acetylcholinesterase and was arranged in bundles in a characteristic pattern with a longitudinal orientation from the level of the ureterovesical junction to the dome of the bladder on the ventral side. On the lateral and dorsal sides, the layer was more faintly stained and arranged in a more criss-cross fashion. No intramural ganglion neurons were found. The nerves reaching the bladder from the pelvic ganglia spread from the area around the ureterovesical junction, with one left and one right ventral nerve running obliquely to the ventral muscle bundles in the most caudal part; cranial to this, the nerves branched and the branches ran parallel to the muscle bundles. Small branches could frequently be

seen crossing the midline along the ventral bladder wall, running almost transversely, and anastomosing with ventrolateral nerves.

The nerves dorsolateral to the ureteral orifices were thinner than the ventral nerves, varied in number, and branched frequently and irregularly. Most of them followed muscle bundles; occasionally nerves crossing the midline could be seen.

Using planimetry, we found that the total surface of the operated bladders was in the same range as in controls; however, the portion of the surface located cranial to the ureteral orifices was significantly lower ( $p < 0.01$ ) in the operated bladders ( $71 \pm 4\%$ ) than in controls ( $88 \pm 1\%$ ).

The detrusor muscle in the operated bladders showed the same arrangement as in controls, with a ventral longitudinal orientation and in the dorsolateral region a mesh of bundles with a criss-cross orientation. We found a weaker staining of the muscle layer than in the controls.

As in the controls, the ventral nerves passed ventromedially to the ureterovesical junctions, and then branched. The branches ran obliquely to the ventral longitudinal muscle bundles throughout the whole ventral surface, the pattern with smaller nerves running parallel to the muscle bundles seen in the cranial parts of the control bladders could not be found here. Nerves crossing the midline were frequently found, always in an oblique direction.

The course of the dorsolateral nerves did not differ from that observed in the controls.

### ***Circumference-Stress Relations***

#### *-Passive stress*

All strips were stretched up to circumferences (lengths) giving 50-70 mN/mm<sup>2</sup>. Only small differences were noted between the first and second passive stress curves, except for the 10 week-group, where the second curve had length-stress values much lower than in the first, suggesting that the strips had been overstretched.

The passive stress curves for the operated animals showed a leftward shift, i.e. their circumferences were smaller for corresponding stresses, in all groups compared to controls. This was also true for the 10 week-group, where there was a leftward shift up to a circumference of about 30 mm; when stretched beyond this, passive stress remained almost constant (first stretch) or even fell (second stretch), probably due to the suggested over-stretch mentioned above.

### *-Active stress*

Circumference-active stress curves showed a rising phase, a plateau phase and a falling phase.

Control strips developed maximal active stress ( $81 \pm 9 \text{ mN/mm}^2$ ) at a circumference of  $23.6 \pm 0.3 \text{ mm}$ , corresponding to a bladder volume of 0.23 ml, assuming a spherical shape.

At 4 days, optimum circumference had fallen ( $18.1 \pm 0.2 \text{ mm}$ ) and the maximum active stress produced was very low,  $14 \pm 3 \text{ mN/mm}^2$ .

At 10 days some recovery had occurred, but active stress ( $31 \pm 9 \text{ mN/mm}^2$ ) and optimum circumference ( $15.6 \pm 0.2 \text{ mm}$ ) of the plateau phase were still significantly lower than in controls.

Further recovery was observed in the 10-week bladders, where optimum circumference ( $23.8 \pm 0.2 \text{ mm}$ ) was identical to that of the control group. Active stress values ( $49 \pm 7 \text{ mN/mm}^2$ ) also had risen, but were still significantly lower than in controls.

### *Contractile and cytoskeletal proteins*

Mean detrusor actin concentration did not differ significantly between any of the groups. In the four-day group, there was a considerable difference in concentration from one animal to the other.

The detrusor myosin/actin ratio was similar in the controls ( $0.77 \pm 0.09$ ) and the 10-week group ( $0.75 \pm 0.11$ ). There was a tendency towards a decreased ratio a 4 and 10 days, although it did not reach significance.

The detrusor intermediate filament (IF)/actin ratio had increased at 4 days, and was significantly higher than in controls ( $0.145 \pm 0.007$ ) in the 10-day group ( $0.275 \pm 0.045$ ). By 10 weeks, the IF/actin ratio was similar ( $0.173 \pm 0.022$ ) to that in controls.

### *Response to nerve stimulation*

Maximal response to nerve stimulation of strips from the supratrigonal area amounted to 60-70% of  $K_{\text{max}}$ . The response in equatorial strips reached about 50% of  $K_{\text{max}}$ . No significant difference was seen between strips from operated animals and their respective controls.

Adding prazosin ( $10^{-6} \text{ M}$ ) had no effect on the frequency-response relations in any of the groups. Adding scopolamine ( $10^{-5} \text{ M}$ ) as well as prazosin decreased the maximal response in all strips. In the supratrigonal strips, the decrease in response was somewhat more pronounced for the operated strips, but this difference was not significant. However, in the equatorial strips, there was a



significantly ( $p < 0.05$ ) more pronounced decrease in maximal response in the operated strips, showing that the nerve responses in these were more inhibited by the muscarinic blocker (i.e., the atropine resistance was lower).

In a separate experiment, the influence of scopolamine ( $10^{-5}$  M) on nerve-induced responses was tested in the absence of prazosin. In strips from the supratrigonal area, the maximal response was similar in operated and controls, but in equatorial strips it was significantly lower in the operated than in the controls.

Frequency-response relations in the presence of prazosin, scopolamine (concentrations as above) and  $\alpha$ ,  $\beta$ -methylene-ATP ( $10^{-5}$  M) after desensitization to  $\alpha$ ,  $\beta$ -methylene-ATP revealed a pronounced additional inhibition in the supratrigonal strips, with a partial recovery at 60 Hz. There was no difference between operated and controls. For equatorial strips from control animals, an almost complete inhibition was seen at all frequencies, whereas in the operated animals, there was an inhibition up to 20 Hz, but not at 40 and 60 Hz, the remaining response at 60 Hz was significantly ( $p < 0.01$ ) higher in the operated group than in the controls.

#### ***Carbachol dose-response relations***

Cumulative dose-response relations to carbachol showed maximum response about 50% higher than  $K_{max}$  in all four groups. The  $-\log EC_{50}$  was within the range 5.5 to 5.7  $-\log$  M, with no significant difference between the groups.

#### ***$\alpha$ , $\beta$ -methylene-ATP dose-response relations***

Non-cumulative dose-response relations to  $\alpha$ ,  $\beta$ -methylene-ATP showed a maximal response of about 30-40% of  $K_{max}$  with no significant difference between the four groups.

#### ***Phenylephrine dose-response relations***

Cumulative dose-response relations to phenylephrine showed maximal responses of only about 10-20% of  $K_{max}$  with no significant difference between the four groups. A considerable variation in response between strips was noted.

### *Force-velocity relations*

We first measured  $V_{\max}$  in detrusor strips from unoperated animals, and found no significant difference between strips from the supratrigonal area and equatorial strips.

We then determined force-velocity data in equatorial strips from animals operated 6 weeks, 3 months and 9 months earlier and in their matched controls. At all points in time,  $V_{\max}$  was significantly lower in the operated animals than in their matched controls.

### **Matrix implants**

The animals with bladder matrix implants were sacrificed 12 to 45 weeks after operation. When prepared for wholemounds, the bladders could readily be filled to the same volume (0.7 ml) as the control bladders without reaching maximum distension. The shape of the bladders was the same as in the controls, and no trigonal distension was seen. The arrangement of the detrusor muscle and the ventral and dorsolateral nerves was similar to that in controls, and the intensity of the nerve staining appeared to be the same.

In all specimens, well stained muscle bundles from the detrusor reached into the matrix from the edges, occasionally well into the core of it. The matrix itself stained only very weakly, and showed no obvious structures.

The nerve distribution pattern in the native part of the bladders was the same as in controls. Some nerve trunks, direct continuations of ventral and dorsolateral nerves, could be seen entering the matrix, usually following its muscle bundles. Generally, branching of the nerves was not seen when they passed the suture line, they continued as single nerves into the matrix and showed some limited branching within the muscle bundles. Almost all the bladder nerves reaching the suture line issued branches into the matrix.

No ganglion cells were seen in the operated bladders, neither in the native part nor in the matrix.

## DISCUSSION

The method of augmenting the urinary bladder by incorporating a bowel segment, enterocystoplasty (ECP), to achieve an increased capacity and decreased pressure levels has been in clinical use for a long time. More recently, acellular matrix grafts and matrix grafts seeded with autologous bladder cells have been used for the same purposes (Atala et al, 2006). For a good functional outcome, it would be desirable with a true functional integration into the bladder of the inserted segment. This seems not to be the case in a majority of ECP patients. A large number of those patients need intermittent catheterisation to empty properly (Blaivas et al, 2005; Hasan et al, 1995).

From a physiological point of view, this kind of surgery creates an interesting situation: two smooth muscle organs with different characteristics of their smooth muscle and of their innervation, and with different pharmacological properties (Batra et al, 1987), are brought into close contact.

Not much is known about the functional changes of the intestinal segment after enterocystoplasty. It has been shown in the rabbit (Batra et al, 1987, 1988, 1992) that the pharmacological properties of the intestinal muscle shift with time towards that of detrusor muscle. Several tentative explanations have been put forward, such as exposure to urine, a changed pattern of contraction and relaxation or a direct influence from detrusor muscle cells on intestinal muscle cells, e.g. via myogenic propagation, as suggested by Gill et al (1989). None of them are, however, without objections. The fact that detubularization is necessary for the changes to come about (Batra et al, 1992) speaks against exposure to urine as an important factor. The anastomotic area contains little muscle, which makes it improbable that myogenic propagation is responsible. It has previously not been reported if bladder nerves reach smooth muscle in the intestinal segment.

Since the innervation of the urinary bladder shows a considerable plasticity, with a balance between the functional demands of the bladder and its innervation (Gabella et al, 1992; Gabella and Uvelius, 1993), it is not surprising to find nerves sprouting from the cut nerve trunks in the bladder wall.

In our model with nerve-stained wholemounts, we have shown that such sprouting nerves frequently cross the anastomosis into the intestinal segment in ECP. The pattern of growth of these nerves makes it unlikely that they originate from intestinal ganglia, a possibility also in conflict with our finding of a pronounced degeneration of the intestinal ganglia (I).

The ganglionic degeneration found in the intestinal segment cannot be explained by lack of vascular supply or smooth muscle atrophy, since we found a well

preserved vasculature and a thick and well preserved muscle layer. Over-stretch of the nerves due to a stretch of the intestinal segment during bladder filling beyond that in native intestine could contribute, a theory though somewhat contradicted by the finding that the ganglionic atrophy was most pronounced in the peripheral areas of the ECP segments.

This finding would lend more support to the idea that the atrophy was caused by the nerves growing in from the bladder. However, since we do not know if nerve ingrowth or ganglion degeneration came first, there is also the possibility that the nerve sprouting is secondary to the degeneration; if this is the case, the degeneration must then have been caused by other factors.

It has been shown for skeletal muscle that a changed motor nerve innervation can change the contractile properties of the muscle, so that for example a fast-twitch muscle cross-innervated with a nerve from a slow-twitch muscle will change its ultrastructure to that of a slow-twitch muscle, even if impulse activity is excluded (Feng et al, 1982). This suggests the existence of an activity-independent trophic factor. It has, however, also been suggested that the nerve activity pattern is of importance for the differentiation of muscle type (Kuno, 1984). Even if it remains to be demonstrated, similar mechanisms could be involved as a factor explaining the shift in properties in the ECP segment.

We think it is reasonable to speculate that the bladder nerves demonstrated growing into the intestinal segment are involved in and may be responsible for the changes in receptor pharmacological properties of the intestinal smooth muscle towards that of bladder muscle, and that possibly also trophic factors or a changed contraction pattern are involved.

Our *in vitro* experiments with detrusor strips after ECP (IV) show results partly similar to the findings of Batra et al in ECP segments in the rabbit (Batra et al, 1987, 1988, 1992), with an increased response to muscarinic stimulation in the ECP strips to levels intermediate between that of control intestine and detrusor. Maximal response to nerve stimulation in ECP strips was the same as in detrusor, and (at three months) significantly higher than in control intestine. However, the vigorous contraction seen in detrusor strips on purinergic stimulation was not found in the ECP segments or in control intestine, and in that respect the ECP segments still behaved like intestine (cf. Batra et al, 1987). In rabbit, it was found that ECP muscle showed increased contractile response to both nerve stimulation (Gill et al, 1989) and purinergic activation (Batra et al, 1988, 1992). The results in our study thus indicate that rat ECP segments retain more of their intestinal pharmacological properties than is the case in rabbit. One interesting observation in this study is that the ECP segment after muscarinergic blockade retained a similar nerve stimulation response as the detrusor muscle, but did not contract on purinergic stimulation. This seems to

indicate a NANC response that is not purinergic. We did not in this study perform  $\alpha$ -blockade, but Batra et al (1988) found no effect of  $\alpha$ -stimulation on muscle tone in rabbit ECP muscle.

It is not known whether the changing properties of the intestinal segment also means that it, with time, could participate in the normal filling and emptying of the bladder, i.e. if it is functionally integrated.

Our *in vivo* cystometry experiments showed residual urine constituting only 25-30% of bladder capacity in the operated animals despite a capacity exceeding that of controls by about 100%. Calculation of force produced by the whole bladder circumference showed lower values for the ECP bladders, though not reaching significance. Calculated threshold tension was similar in operated and control bladders, suggesting that the bladder wall stretch needed to elicit micturition was unchanged. Should the intestinal segment be completely passive during micturition, we think that a higher residual volume would have been expected in the operated animals, as would a more pronounced effect on bladder wall force. Hence, we find that the cystometry results suggest that the incorporated intestinal segment, at least to some extent, participates in the bladder wall contraction during micturition.

\* \* \*

Smooth muscle shows an ability to react with compensatory growth when subjected to increased functional demands. One example is the increased detrusor workload following infravesical outlet obstruction, resulting in detrusor hypertrophy and hyperplasia (Gabella 1987, Gilpin et al 1985, Gabella and Uvelius 1990, Saito et al 1994, Levin et al 1995). Another example is the detrusor growth resulting from the increased workload and bladder distension in rats with experimentally induced diabetes mellitus or diabetes insipidus (Uvelius 1996, Malmgren et al 1989 and 1992). It has been shown that stretch of isolated detrusor muscle rapidly stimulates *de novo* DNA synthesis in the smooth muscle cells (Karim et al 1992). Also, denervated bladders will become paralysed and distend, then develop detrusor hypertrophy and rapidly gain weight (Uvelius and Mattiasson 1986).

Induction of detrusor growth constitutes an interesting perspective on bladder reconstruction.

It has been shown that rat bladders rapidly regain capacity and weight following subtotal cystectomy, a procedure in which nearly all supratrigonal tissue is removed. In the experiments of Saito et al (1996) the bladder capacity reached about 60% and bladder weight about 75% of controls 4 weeks after surgery. Piechota et al (1998) found that after 4 months capacity had recovered even further.

Subtotal cystectomy as a model to study smooth muscle growth is interesting in the sense that the bladder is not distended and that the workload during each micturition cycle is not increased, although micturition frequency increases due to the decreased bladder volume.

Lin et al (1989) found that the regeneration of the rabbit bladder after subtotal cystectomy was mainly a result of distension of the bladder base, supported by the finding of a ratio of  $\alpha/\beta$  receptor response to epinephrine in *in vitro* whole-bladder much higher in operated animals than in controls, which would then reflect the higher concentration of  $\alpha$ -adrenoceptors in the bladder base than in the bladder body. Goldstein et al (1970) found, also in the rabbit, that the increasing capacity of the bladder following subtotal cystectomy was the result of expansion of the remaining parts of the bladder rather than tissue regeneration.

Saito et al (1996) found, on the other hand, that in the rat bladder there was not only an increase in weight and capacity after subtotal cystectomy, but also a partial restoration of *in vitro* whole-bladder response to electric field stimulation and to bethanechol, after an initial decrease in these responses. At the same time, there was an increased response to phenylephrine immediately after surgery, which then gradually returned to control level. They did not comment on the general appearance of the regenerated bladder, but the mentioned findings support the view that the restoration of the bladder is not only the result of mere distension of the remaining bladder base, but that a true growth of the remaining bladder body takes place. The bladder base is rich in  $\alpha$ -adrenergic receptors, whereas these are scarce in the bladder body, which instead is rich in muscarinic receptors (Lin et al, 1989; Andersson and Arner, 2004; Alm and Elmér, 1975; Levin et al, 1980). A relatively more pronounced growth of the remaining bladder body would thus explain the changing pattern of reactions to stimulation that they found, provided that the newly formed bladder tissue develops pharmacological properties that resemble those of the normal bladder body from which it stems.

Our experiments (II) showed some distension of the bladder base compared to controls, but the main part of the regenerated bladders consisted of supratrigonal tissue. The results of the analysis of contractile proteins showed a rapid increase in the intermediate filament (IF)/actin ratio, reaching significance at 10 days. At 10 weeks, the ratio was normalized. In growing smooth muscle, an increase in the desmin/actin ratio is seen (Malmqvist et al, 1991; Vinter-Jensen et al, 1995). Our analysis did not distinguish between the IF fractions vimentin and desmin, but the interstitial vimentin-containing cells (Levin et al, 1995; Andersson and Arner, 2004) are few, and the vimentin concentration in both normal and hypertrophic detrusor constitutes less than 5% of the total desmin-vimentin concentration (Malmqvist et al, 1991). Light microscopy of cross-sectioned

muscle strips at 4 days, 10 days and 10 weeks showed that they were all practically indistinguishable from controls, speaking against an increase in interstitial cells as the source of the IF increase. We are therefore convinced that the significant increase in IF/actin ratio seen after 10 days is due to an increase in desmin. The myosin/actin ratio was similar in controls and the 10-week group, with a tendency towards decreased ratios at 4 and 10 days, which however did not reach significance. There was no significant difference in actin concentration between any of the groups.

Bladder weight recovered completely in 10 weeks.

Light microscopy of the bladder body wall at 10 weeks showed that there was practically no difference compared to controls.

All this taken together suggests that a net synthesis of contractile proteins has taken place and that new detrusor muscle has formed from the remaining part of the bladder body after the subtotal cystectomy.

4 days after subtotal cystectomy, bladder circumference-tension relations showed a pronounced leftward shift, i.e. corresponding stress was developed at a smaller circumference compared to controls. Circumference for maximum active stress then gradually normalized, and at 10 weeks was back at control level.

The successive rightward shift of the circumference-stress curves seems to parallel the weight recovery of the bladders. The mechanism for the rightward shift could be sought in the cell-to-cell connections, with muscle cells sliding past each other, which has been shown to occur in distended denervated rat bladders (Ekström and Uvelius, 1983), or in changes in the sarcomere equivalents within the muscle cells, or a combination of them.

Even if the circumference for optimum force production returned to normal with time, force production per muscle cross-sectional area did not. Lowered maximum active force has been shown to occur in hypertrophic smooth muscle. The reason for this reduction is not clear, but has been suggested to be caused by a decreased cellular myosin concentration and a lowered myosin/actin ratio (Malmqvist et al, 1991). In our experiments, we saw a tendency towards a lowered myosin/actin ratio, but it was back to normal at 10 weeks, whereas the maximum force producing ability was still low compared to controls. Another possible explanation could be a decreased relative amount of smooth muscle in the strips, but we found no signs of that. Myofibrocytes induced in the rabbit detrusor by outflow obstruction have been shown to have a poor contractile ability (Roelofs et al., 1995). An increased relative amount of such cells could also contribute to decreased force production ability. We think that the amount of these cells was limited in our preparations, but this remains to be demonstrated.

Thus, we have not found a convincing explanation for the lasting decrease in active force in the newly formed bladders.



The relatively high active force, 50-70% compared to  $K_{max}$ , generated when strips from regenerated bladders were field stimulated (III) showed that they had a functioning motor nerve innervation. The fact that we found no supersensitivity to carbachol or phenylephrine is in accordance with this. In rat bladders, as opposed to rabbit bladders, all ganglion cells are located outside the bladder (Gabella and Uvelius, 1990). This could facilitate reinnervation in the newly formed bladder after subtotal cystectomy in the rat. This could also partly explain the differences found in responses between rabbit and rat bladders after subtotal cystectomy.

The strips from both controls and operated animals responded readily to carbachol and weakly to adrenergic stimulation, equatorial and supratrigonal preparations alike. This pattern is expected in the controls and shows that the regenerated muscle consists of detrusor and not bladder base muscle.

Alpha adrenoceptor blockade with prazosin did not affect field stimulation response in any of the preparations, a characteristic typical of the bladder body (Dean and Downie, 1978; Sanctioli, 1983).

Comparison of supratrigonal and equatorial strip responses in the controls showed very similar frequency-response relations and atropine resistances. Only one significant difference between them was seen; when simultaneous muscarinic, alpha adrenergic and purinergic (P2X1) blockage was applied, the equatorial strips lost all contractility, whereas the supratrigonal strips still showed about 20% of the response without blockers at 60 Hz. There was no mucosa in the strips, ruling out the possibility of mucosal mechanisms. Another possibility would be ganglionic mechanisms, but since the rat bladders lack intramural ganglia this can also be ruled out.

Supratrigonal strips from controls and operated animals showed virtually no differences at all and we conclude that the surgery and subsequent growth has no impact on the pharmacological properties of this segment.

Strips from the equatorial region of the operated animals differed in two ways from the controls. Their atropine resistance was lower, and they showed, as the supratrigonal strips, a partially preserved ability to react to field stimulation at high frequencies that was not blocked by scopolamine, prazosin and  $\alpha$ - $\beta$ -methylene-ATP desensitisation. In this respect they behave like the supratrigonal strips in both operated animals and controls, suggesting that the newly formed equatorial segment of the bladder retains pharmacological properties of the supratrigonal segment from which it has developed. The lowered atropine resistance could reflect a decreased relative contribution of non-cholinergic nerves to the contraction, and thus an increased relative amount of cholinergic activator nerves.

The non-cholinergic, non-adrenergic, non-purinergic response seen in the bladder base and in the equatorial strips of the operated bladders at high stimulation frequencies, in contrast to the controls, might be explained by an



increased excitability of the muscle cells or by nerves using other transmitters. The TTX effect supports the last suggestion.

We found that maximal shortening velocity ( $V_{\max}$ ) was equal in equatorial and supratrigonal segments from controls. It has been shown that smooth muscle from different parts of the same organ might have different  $V_{\max}$  (Arner et al, 1998), but since that was not the case here, we chose to study shortening velocity only in equatorial strips at different points in time after subtotal cystectomy. In all three groups (6 weeks, 3 months and 9 months after surgery),  $V_{\max}$  was found to be significantly lower in the operated animals compared to controls.

$V_{\max}$  in hypertrophic detrusor muscle secondary to experimentally induced infravesical outflow obstruction has been found to be lower than in controls (Sjuve et al, 1996). They found an increased relative amount of the basic isoform of the essential 17-kDa myosin light chain ( $LC_{17b}$ ), which has previously been found to correlate with a lowered  $V_{\max}$  (Malmqvist and Arner, 1991). They also found a decreased relative amount of myosin heavy chains with a 7 amino acid insert; a high relative amount of myosin heavy chains with the insert has been associated with a higher  $V_{\max}$  (Kelley et al, 1993).

Whether or not the strips in our experiments had any alterations in the 17-kDa myosin light chain isoform composition or in the relative amount of myosin heavy chains with the 7 amino acid insert was not analysed. Another explanation to the decreased  $V_{\max}$  could be changes in cell-to-cell coupling, e.g. cells being coupled in parallel instead of in series, maybe to compensate for a decreased active force of the individual smooth muscle cells. However, this remains to be investigated.

\*                    \*                    \*

It has been shown that in parallel to the hypertrophy of the bladder following outlet obstruction there is also a pronounced hypertrophy of the ganglion neurons projecting to the bladder (Steers et al 1990; Gabella et al, 1992). Unilateral denervation by removal of the pelvic ganglion leads to sprouting of axons from the contralateral to the denervated side (Uvelius and Gabella, 1998). Hypertrophy of the nerve cell bodies in the remaining ganglion occurs, reflecting the increased target organ mass (Gabella and Uvelius, 1993). Normally there are no ganglion cells in the rat bladder; after denervation, however, small ganglia appear along the axon-free nerves (Uvelius and Gabella, 1995).

These are examples of the high degree of plasticity of the bladder innervation seen in the adult bladder. The normal distribution of nerves in the rat bladder has been described in conjunction with studies of partial denervation (Uvelius and Gabella, 1998). We have shown that bladder nerves sprout into intestinal

segments used for augmentation in the rat, and that the intrinsic ganglion neurons of the intestinal segment degenerate (I). Less is known about the distribution of nerves in bladders regenerated after subtotal cystectomy or after incorporation of acellular matrix grafts. The latter has been used in bladder augmentation to avoid the drawbacks encountered when using intestine for the same purpose. Since it has been shown that such segments in rat bladders contract when subjected to electrical field stimulation (Vaught et al, 1996; Piechota et al, 1998), indicating functioning motor-nerve innervation, studies of nerve origin and distribution in these are of great interest.

Previous descriptions of the presence of nerves in ECP segments and acellular matrix grafts have mainly been based on cross-sections of the bladder wall (Probst et al, 1997; Sutherland et al, 1996), studies of which do not allow analysis of the nerve and muscle bundle architecture; the wholemounds used in (V) made it possible to do this.

Distribution of both nerves and muscle bundles in control bladders corresponded well with the picture described earlier by Uvelius and Gabella (1998). The new bladder developed after subtotal cystectomy showed a muscle architecture similar to controls, but the regular pattern of ventral nerve branches running in parallel with the muscle bundles, as found in the control bladders, was absent. Instead, the nerves were running obliquely to the muscle bundles ventrally, resembling the picture found in the basal parts of the control bladders and thus reflecting the nerve/muscle anatomy in the part of the bladder from which it had developed.

Almost all of the cut bladder nerves close to the SIS graft anastomosis were observed to have a continuation into the graft but without branching or changing direction at entering. Limited branching was seen further into the matrix, where the nerves typically followed along the muscle bundles. Since only the uppermost part of the bladders were replaced by SIS grafts, and the muscle arrangement pattern in the grafts was found to be criss-cross rather than the longitudinal pattern usually found in the ventral part of the bladders, it would have been interesting to study the arrangement of muscle and nerves in wholemounds from bladders with the whole bladder dome, or the entire ventral surface, replaced by SIS scaffolds.

Confirming the findings of others (Kropp et al, 1995; 1996) we did not find any shrinkage of the SIS grafts, as opposed to what has been found for bladder acellular matrix grafts (Reddy et al, 2000; Probst et al, 2000; Brown et al, 2002). The graft material in itself is quite rapidly resorbed, Badylak et al (1998) found only scattered remnants histochemically four weeks after SIS grafting in the dog. A small series of patients operated with grafts seeded with autologous cells has shown promising results (Atala et al, 2006).

It seems that the use of scaffolds for regeneration of the bladder can produce a bladder segment with well-organised functioning muscle, mucosa and nerve components and thus might be a realistic alternative to the use of bowel segments in bladder reconstruction in man.

## IN CONCLUSION

The experiments on bladder reconstruction performed in this study – regeneration after subtotal cystectomy, replacement with bowel segments and matrix grafts – show that the bladder has a high capacity for regenerative growth. After enterocystoplasty, nerves from the bladder grow into the intestinal segment, in which ganglionic atrophy appears. This is a possible explanation to the shift in pharmacological properties shown to occur in such segments with time, a phenomenon also found in our experiments. The cystometry results in animals operated with enterocystoplasty suggest that the bowel segment, at least to some degree, participates in the emptying of the bladder, i.e. is functionally integrated and not merely a passive part during emptying. The findings after subtotal cystectomy show that the rat bladder can produce new, contracting muscle, albeit with lowered maximal shortening velocity and lowered maximal force production ability compared to controls. The nerves found in the new bladder that develops after subtotal cystectomy are the result of growth and branching of pre-existing nerves. The nerves found in the SIS matrix grafts are a continuation of nerve trunks from the bladder reaching the grafts at the suture line. Neither in the subtotally cystectomized bladders nor in the grafts do the growth pattern of nerve trunks nor muscle bundles fully attain the pattern found in normal bladders.

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