Nerve fibres in urothelium and submucosa of neuropathic urinary bladder: an immunohistochemical study with S-100 and neurofilament

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Intravesical administration of drugs has been used commonly in spinal cord injury patients to suppress detrusor hyperreflexia (eg oxybutynin, verapamil, terodiline) or, to initiate a micturition reflex (eg 15S 15-methyl prostaglandin F₂ alpha, prostaglandin E₂); however, the response has been variable and sometimes, unpredictable. This prompted us to study the presence of nerve fibres in the vesical urothelium and submucosa in mucosal biopsies taken from the dome and trigone (obtained prior to performing a therapeutic procedure eg, vesical lithotripsy, or a diagnostic cystoscopy) in 47 consecutive, unselected paraplegic/tetraplegic patients with a neuropathic urinary bladder. Nerve fibres were demonstrated by routine immunohistochemical technique using commercially available monoclonal and polyvalent antibodies against S-100 (DAKO A/S, Glostrup, Denmark) and Neurofilament (MILAB, Malmo, Sweden). Biopsy specimens were graded for the presence of nerve fibres on a 0–3 scale for urothelium, and superficial/deep submucosa separately in a blind and randomised manner. Virtually no fibre presence was found in one paraplegic patient and no superficial or single fibres were noted in a tetraplegic patient. Absence of C-fibre hyperplasia (Grade 0) was found in nine cases (paraplegic: 4; tetraplegic: 5); Grade 1 hyperplasia was observed in 17 cases (paraplegic: 4; tetraplegic: 13); Grade 2 hyperplasia was seen in 11 cases (paraplegic: 7; tetraplegic 4); and Grade 3 hyperplasia was noticed in eight cases (paraplegic 3; tetraplegic: 5). The magnitude of C-fibre hyperplasia was not significantly different between paraplegic and tetraplegic patients ($\chi^2=4.64$; $P=0.3262$). The relationship, if any, between the degree of C-fibre hyperplasia and duration of paralysis was studied by categorising patients as <5 years, and >5 years of paralysis. No evidence of single fibre or fibre bundle hyperplasia (Grade 0) was seen in five and six cases, grade 1 hyperplasia in six and 11 cases, grade 2 hyperplasia in two and nine cases, and grade 3 hyperplasia in three and five cases respectively in these two categories of patients. ($\chi^2=1.92$; $P=0.58$). The possible relationship between C-fibre hyperplasia in the vesical mucosa/submucosa and (i) the vesical response to intravesical drug administration; (ii) the vesical urothelial proliferation arrest; (iii) the electrical stimulation of urinary bladder by implanted electrodes (sacral anterior root stimulator); and (iv) long-term indwelling urethral Foley catheter drainage, are discussed with illustrative case reports. In conclusion, mucosal biopsy and study of nerve fibres in urothelium and submucosa of neuropathic bladder has helped to generate hypotheses on the association between C-fibre hyperplasia and response to intravesical pharmacotherapy and the predictive value of such a study in identifying those patients who are likely to respond to intravesical pharmacotherapy.

Keywords: spinal cord injury; neuropathic bladder; bladder biopsy; immunohistochemistry; C-fibres; hyperplasia

Introduction

The management of the neuropathic bladder in spinal cord injury patients has changed from an emphasis on a surgical procedure such as transurethral resection of the bladder neck and division of the external urethral sphincter to a non-surgical method viz. intermittent

catheterisation (whenever feasible) as this has been shown to achieve complete, low pressure emptying of the urinary bladder with few complications. However, intermittent catheterisation regimes often require adjunctive intravesical pharmacotherapy (i) to increase the bladder capacity in patients with a small capacity bladder so that they do not have to catheterise themselves very frequently, and (ii) to abolish detrusor hyperreflexia when present, in order to minimise

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urinary leakage between catheterisations. The success of intravesical pharmacotherapy depends on (1) the pharmacological properties of the drug administered; (2) penetration of the drug across the vesical mucosa and its absorption from the bladder; and (3) qualitative and quantitative presence of nerve fibres in the urothelium, submucosa, and detrusor muscle. Ishizuka *et al*¹ demonstrated that intravesically instilled capsaicin (in normal, unanesthetized rats) induced reflex-mediated hyperactivity, probably via stimulation of neurokinin₂ receptors. Intravesically administered capsaicin released neuropeptides from nerves in or immediately beneath the urothelium in the bladder and/or outflow region, and these peptides initiated a micturition reflex by afferent stimulation. Like capsaicin, intravesically infused prostaglandin E₂ facilitated micturition which was attenuated by selective blockade of Neurokinin₁ or Neurokinin₂ receptors and, most effectively, by a combined blockade (Ishizuka *et al*).² This suggested that tachykinins, both neurokinin A (NK₂ receptors) and substance P (NK₁ receptors), may be released from nerves and mediate part of the effects of PGE₂. The location of such nerves may be in and/or immediately below the urothelium.

We observed that the vesical response to intravesically administered drugs in spinal cord injury patients is sometimes unpredictable and the magnitude of response varies from patient to patient. This prompted us to study the presence of nerve fibres in bladder mucosal biopsies obtained from spinal cord injury patients while they were undergoing some other diagnostic or therapeutic procedure on the urinary bladder. Although the presence of nerve fibres in the detrusor muscle of denervated bladders has been documented, to our knowledge, the extent and presence of (abnormal) nerve fibres in the vesical urothelium and in the vesical submucosa of neuro-pathic bladders from mucosal biopsy specimens has not been characterised by immunohistochemical studies. We believe that such a study of the presence of nerve fibres in the urothelium and submucosa of spinal cord injury patients with a neuropathic bladder may contribute towards a better understanding of the vesical response to intravesically administered drugs in a given individual and also provide guidance towards selection of suitable patients who are likely to benefit by such a therapeutic regime. As of now, the choice of patients for intravesical administration of a drug is often on an empirical basis in that a given drug, say, oxybutynin is administered intravesically, and if the individual spinal cord injury patient shows a satisfactory therapeutic response, drug administration is continued; and intravesical pharmacotherapy is discontinued if the patient fails to show an optimal response. Quantitative and qualitative study of nerve fibres and receptors in the urothelium and vesical submucosa in spinal cord injury patients may help towards selection of cases who may have higher probability of responding to a given drug regime.

Neural input has a significant role in regulating the growth of the bladder and innervation influences tissue levels of nerve growth factor in the urinary bladder.³ The presence of nerve fibres in the urothelium and submucosa may provide trophic factors for vesical urothelial proliferation. Vesical urothelial proliferation arrest has been demonstrated in spinal cord injury patients by immunohistochemical study with proliferating cell nuclear antigen (PCNA) and Ki 67 antigen (using the monoclonal antibodies suitable for detection of the recombinant DNA based MIB-1 fragment of the Ki 67 antigen).⁴ The observed urothelial proliferation arrest in the spinal cord injury patients may be a contributory factor for their becoming prone to the development of cystitis. Thus the presence of adequate number of normally (physiologically) functioning nerve fibres or conversely, paucity of nerve fibres or presence of abnormal nerve fibres in the vesical urothelium and submucosa may have a bearing on the proliferation status of vesical urothelium in spinal cord injury patients and, in turn, to their propensity for development of recurrent cystitis. Further, in cystitis, there may be an exaggerated prostanoid production leading to intense activation of sensory nerves, increasing the afferent input from the bladder mucosa.⁵ This may possibly explain why some spinal cord injury patients develop a severe degree of autonomic dysreflexia during an episode of acute cystitis whereas many other tetraplegic and paraplegic patients with a lesion above T-6 level do not exhibit features of autonomic dysreflexia during an episode of acute urinary tract infection and cystitis.

Continuous irritation of the bladder mucosa, for example by an indwelling catheter, over a period of time (many months to years as we have seen female patients with a neuropathic bladder who have been on indwelling urethral catheter regime for over three decades), may result in neural stimulation with secondary changes in the bladder mucosa. It is thus possible that squamous metaplasia observed in those spinal cord injured patients on long-term indwelling urethral catheter drainage may indeed be a nerve-mediated phenomenon. In order to understand these complex issues on altered anatomy and physiology of the neuropathic bladder, we carried out a preliminary investigation by studying prospectively the pattern of C-fibres in the vesical urothelium and vesical submucosa in bladder mucosal biopsy specimens of spinal cord injury patients by immunohistochemical studies.

Patients and methods

Study population

Forty-seven consecutive adult spinal cord injury patients who were otherwise scheduled for a cystoscopy prior to treatment of bladder stone(s) ($n=22$), urethral diverticulum ($n=2$), urethral calculus ($n=1$), urethral stricture ($n=1$), and prostatic urethral calculus

($n=1$), division of external urethral sphincter and transurethral resection of bladder neck ($n=6$), insertion of JJ ureteral stent (as the initial step before shock wave lithotripsy of renal calculus) ($n=3$); removal of JJ stent ($n=2$), for evaluation of haematuria ($n=8$), or prior to performing ascending ureteropyelography in a case of pelviureteric junction obstruction were the subjects of this study. The nature of the investigation was explained to the patient and to his/her carer(s) and written informed consent was obtained. Cystoscopy was performed with a Storz 22 Fr. rigid cystoscope in the lithotomy position. Cold-cup biopsy was taken randomly under vision from the dome and from the trigone of the urinary bladder. Any bleeding point was then fulgurated with diathermy electrode. All participants underwent the procedure without any intra-operative or post-operative complication. Performance of bladder biopsy did not result in any additional morbidity to any of the patients and did not increase the duration of hospitalisation; the cervical spinal cord injury patient with C-5 tetraplegia who underwent JJ stenting of the ureter (as the initial procedure prior to shock wave lithotripsy of renal calculus), and bladder biopsy was discharged home the same evening after the procedures were carried out uneventfully.

Tissue processing, histology

Bladder biopsies were fixed by immediate immersion in 0.1 M, phosphate buffered, 4% formaldehyde, pH 7.4 for a minimum of 6 and a maximum of 12 h. After dehydration, specimens were routinely processed to paraplast using vacuum processing and a maximum temperature of 53°C. Five micron sections were cut and mounted routinely for Haematoxylin and Eosin staining. All the biopsy specimens were evaluated together by a Pathologist who was unaware of the site of biopsy and of relevant clinical details.

Immunohistochemistry

Nerve tissue of sympathetic/parasympathetic and sensory type was identified using a combination of two antibodies. A polyvalent antibody against S-100 (neural crest antigen found in high density on all neural crest derived tissue, ie on both axons and Schwann cells) was chosen as the screening antibody in view of the high contrast provided in routine immunohistochemistry. The disadvantage of using this antibody is that the circulating macrophages are also stained positive (being similarly neural crest tissue-derived). But this was overcome by additionally staining for Neurofilament, a well defined antigen epitope found on the 200 kD neurofilament protein specifically found in the axons of peripheral nerve tissue as part of the axon located neurofilaments. With lower antigenic density, this antibody is less suitable for screening purposes.

Five micron sections were routinely stained for Neurofilament and S-100 using commercially available

monoclonal and polyvalent antibodies (Neurofilament: Catalogue no. MC 001, MILAB, S-200 74 Malmö, Sweden; and S-100: Code no. Z311, DAKO A/S, DK-2600 Glostrup, Denmark), routinely contrasted using avidin-biotin and alkaline phosphatase methods.

Grading of C-fibres in vesical urothelium and submucosa

The presence of thin single axons, intermediate calibre bundles of 3–10 axons, and thick, peripheral nerve-like bundles of nerve fibres was separately scored for vesical urothelium, superficial and deep submucosa and deep musculature if present in the biopsy, on a 0 to 3 scale as defined below:

- Grade 0: No evidence of single fibre or fibre bundle hyperplasia.
- Grade 1: Increase in the presence of superficial naked axons, the distribution of which is patchy (Figure 1).
- Grade 2: Considerable excess of nerve fibres both at deeper level and superficially, most excessive fibres are of single axon distribution (Figure 2).
- Grade 3: Excessive presence of all types of fibres in superficial and deeper parts of the mucosa and submucosa (Figure 3).

In case no C-fibre was (virtually) present, this observation was recorded separately.

Results

The clinical details and results of immunohistochemical studies with S-100 and neurofilament on bladder biopsies are given in Table 1. Virtually no fibre presence was found in one paraplegic patient and no superficial or single fibres were noted in a tetraplegic patient. Absence of C-fibre hyperplasia (Grade 0) was found in nine cases (paraplegic: 4; tetraplegic: 5); Grade 1 hyperplasia was observed in 17 cases (paraplegic: 4; tetraplegic: 13); Grade 2 hyperplasia was seen in 11 cases (paraplegic: 7; tetraplegic: 4); and Grade 3 hyperplasia was noticed in eight cases (paraplegic: 3; tetraplegic: 5). The magnitude of C-fibre hyperplasia did not vary significantly between paraplegic and tetraplegic patients ($\chi^2=4.64$; $P=0.3262$). The magnitude of C-fibre hyperplasia in patients with bladder stone(s) ($n=22$) did not differ significantly from those with other clinical conditions ($n=25$) ($\chi^2=0.603$; $P=0.9626$).

Intra-urothelial presence of nerve fibres was detected in a patient with evident squamous metaplasia (Figure 4). The development of thick calibre, peripheral nerve like fibre bundles was limited to cases with Grade 3 hyperplasia, but in such cases very thick bundles could be found in an abnormal, very superficial location (Figure 5). Granulation tissue development, such as in the presence of calculus was generally associated with the emergence of large amounts of small and intermediate calibre axon bundles, closely associated with the proliferating

Figure 1 Microphotograph of trigone urinary bladder biopsy featuring Grade 1 degree of nerve fibre increase. Note the relative absence of stained axons in the very superficial mucosa and the relatively small calibre of the stained axon bundles in deeper parts of the mucosa. Five micron paraffin section, stained with monoclonal antibody against Neurofilament, Haematoxylin counterstain. Microscopical magnification 100×

Figure 2 Microphotograph of trigone urinary biopsy featuring Grade 2 nerve fibre increase in a SCI patient. Note patchy distribution of excess fibres, increased in comparison to grade 1, with more axons in superficial position present as both single and thin axon bundles. Five micron section, stained with monoclonal antibody against Neurofilament antigen, Haematoxylin counterstain. Microscopical magnification 100×

blood vessels (Figure 6) and could easily be differentiated from the pattern of fibre hyperplasia under study.

Four patients underwent bladder biopsies twice (IP; BC; JJ; PF); the time interval between the two biopsies was 4, 11, 21, and 8 months respectively; the indications for cystoscopy were insertion/removal of JJ stent in IP; electrohydraulic lithotripsy of bladder calculus in PF and BC; and removal of prostatic urethral calculi/transurethral resection of prostate in JJ. Overall grading of C-fibre hyperplasia was 0 and 3 in IP; 1 and 3 in BC; 1 and 1 in JJ and 3 and 1 in PF in the two sets of biopsies taken at different time points. This interesting observation suggests possible temporal variation in the magnitude of bladder mucosal and submucosal C-fibre hyperplasia in a given spinal cord injury patient. Thus the degree of C-fibre hyperplasia may vary in the same patient over

a period of time and the factors which may contribute to such changes in the degree of C-fibre hyperplasia include (1) the duration of neuropathic bladder dysfunction, (2) therapeutic interventions such as the presence of an indwelling urethral Foley catheter (in which case the balloon of the Foley catheter may be irritating the trigone of the bladder and the tip of the catheter may be abutting against the dome of the bladder), (3) presence of an indwelling urethral JJ stent with the distal end of the stent coiled inside the urinary bladder, and (4) presence of vesical or prostatic urethral stone(s).

Discussion

Multiple factors may contribute to the development of C-fibre hyperplasia in the vesical mucosa/submucosa and these include (i) denervation/decentralization of

Figure 3 Microphotograph of dome biopsy of urinary bladder featuring Grade 3 nerve fibre increase in a SCI patient. Note excessive fibre presence as both single and thin axon bundles superficially in submucosa and thick, peripheral nerve like bundles deeper in submucosa. Five micron section, stained with monoclonal antibody against Neurofilament antigen, Haematoxylin counterstain. Microscopical magnification 200 ×

urinary bladder; (ii) duration of paralysis; (iii) presence of an indwelling urethral catheter; and (iv) electrical stimulation of neural innervation of the urinary bladder. The relationship between the degree of C-fibre hyperplasia and duration of paralysis was studied by categorising patients into (i) <5 years, and (iii) >5 years after developing neuropathic bladder. No evidence of single fibre or fibre bundle hyperplasia (Grade 0) was seen in five and six cases; grade 1 hyperplasia in six and 11 cases; grade 2 in two and nine cases; and grade 3 in three and five cases respectively in these two categories of patients. ($\chi^2=1.92$; $P=0.58$). Presence of an indwelling urethral Foley catheter, by constant mechanical irritation, may induce C-fibre hyperplasia. Of the eight patients with grade 3 C-fibre hyperplasia, four were on long-term indwelling urethral Foley catheter drainage. Possible contributory factors to the development of grade 3 hyperplasia in the other four cases with grade 3 C-fibre hyperplasia are: electrical stimulation of the bladder in one (PR); presence of JJ ureteral stent in another (IP), this tetraplegic patient had grade 0 hyperplasia before insertion of JJ stent and 4 months later was found to have developed grade 3 hyperplasia; long-term indwelling catheter drainage in PR although at present she is practising intermittent catheterisation with adjunctive pharmacotherapy; repeated electro-ejaculation in MS.

Ishizuka *et al*² demonstrated that prostaglandin E₂

when given intravesically, may stimulate micturition by releasing tachykinins from nerves in and/or immediately below the urothelium. These tachykinins, in turn, initiate a micturition reflex by stimulating neurokinin₁ and neurokinin₂ receptors. This illustrates the important role played by nerve fibres in the vesical urothelium and submucosa in the context of intravesical drug administration, a therapeutic modality which is becoming increasingly popular for the management of the neuropathic bladder in spinal cord injury patients. The drugs which are being administered intravesically in spinal cord injury patients include oxybutynin, prostaglandin E₂, 15(S) 15-methyl prostaglandin F₂ alpha, verapamil, atropine, phentolamine, terodiline, bupivacaine, and capsaicin.

The following three case reports illustrate the possible relevance of C fibre hyperplasia in vesical mucosa and submucosa to clinical pharmacotherapy of intravesical drug administration in spinal cord injury patients with a neuropathic bladder.

Case 1

AR, a 50 year old male with T-7 paraplegia due to transverse myelitis and detrusor hyperreflexia was advised to have intermittent catheterisation and adjunctive pharmacotherapy with oxybutynin

Table 1 Clinical details and results of immunohistochemical studies with S-100 and neurofilament on bladder biopsies in paraplegic/tetraplegic patients

Patient	Sex	Date of birth	Date of onset of paralysis	Diagnosis	Grading ¹	S-100/Neurofilament immunohistochemical staining of urothelium and submucosa
1. H95/0267-68	F	12-8-1924	1969	Multiple Sclerosis. Incomplete tetraplegia	1	Increase in the presence of superficial naked axons the distribution of which is patchy
2. H95/0486-87	M	11-4-1926	1975	Transverse myelitis. Paraplegia T-10	2	No evidence of excess axonal profiles other than in patchy areas, but in such areas, a 2-3 + increase in naked axons is achieved which are very closely approximated to the superficial lining mucosa
3. H95/0488-89	M	21-9-1971	18-12-1991	Traumatic tetraplegia. C-5	3	2-3 + excess of small fibres in superficial position of mucosa diffusely throughout the biopsy. (Date of biopsy: 17-02-1995)
4. H95/0490-91	F	02-05-1965	20-06-94	Traumatic incomplete perineal paraplegia	2	Considerable excess of nerve fibres both at deeper level and superficially, most excessive fibres are of single axon distribution
5. H95/0110-11	M	04-03-1953	24-11-1994	Traumatic tetraplegia C-5	0	No evidence of axonal fibre increase; no evidence of axonal abnormalities
6. H94/3259-60	M	13-06-1938	06-02-1961	Traumatic paraplegia T-9	0	No evident increase of axonal profiles
7. H94/2869-70	M	23-12-1939	03-11-1993	Traumatic tetraplegic C-5	1	Trigone biopsy revealed thick nerve bundles in deeper mucosa, though perhaps slightly increased calibre and a patchy distribution in the deeper mucosa of bundles of intermediate calibre. Slightly patchy increase of single axons in superficial position, to some extent related to the presence of lymphoid follicles. Dome biopsy revealed granulation tissue with fibre increase
8. H94/2302-03	M	06-02-1921	15-12-1962	Traumatic paraplegia L-1	1	Trigone biopsy revealed a clear increase in very superficially located medium-sized bundles of axons. Dome biopsy showed patchy distribution of (0-1+) naked axons in superficial locations and no evident increase in deeper layers
9. H94/0472-73	M	28-11-1937	11-04-1978	Traumatic paraplegia L-3	2	Trigone biopsy showed 2-3 + presence of single naked axons in superficial location. Thick nerve bundles in deep location regularly seen especially in association with large blood vessels. Dome biopsy revealed limited presence of 1+ to (focally) 2+, of single naked axons, although over some areas of the biopsy they are almost absent
10. H94/1677-78	F	25-06-1964	24-09-1983	Guillain Barre Syndrome Tetraplegia C-4	1	Trigone biopsy showed a clear increase in very superficially located medium-sized bundles of axons. Dome biopsy showed large lymphoid follicles; although many fibres are associated directly with the large lymphoid follicle, there is an excess of medium sized bundles of axons in relatively deep but also superficial location. (Date of biopsy: 03-06-1994)
11. H94/1675-76	M	12-03-1928	06-09-1985	Traumatic tetraplegia C-5	0	Trigone biopsy showed normal presence of medium sized bundles in deeper and intermediate locations; no evidence of fibre increase. Dome biopsy revealed normal amounts nerve fibre; no evidence of superficial fibre increase

Table 1 cont'd

<i>Patient</i>	<i>Sex</i>	<i>Date of birth</i>	<i>Date of onset of paralysis</i>	<i>Diagnosis</i>	<i>Grading¹</i>	<i>S-100/Neurofilament immunohistochemical staining of urothelium and submucosa</i>
12. H94/0806-07	M	14-06-1950	09-10-1992	Traumatic paraplegia incomplete L-4	0	Trigone biopsy showed no increase of fibres either superficially or in deeper layers. Dome biopsy revealed no evident increase in naked axons fibres superficially or medium-sized bundles in deeper layers
13. H94/0644-45	M	07-07-1958	10-12-1991	Traumatic paraplegia L-1	0	Trigone biopsy revealed medium-sized axon bundles in deeper layers but no hyperplasia and no excess single fibres in superficial layers. Dome biopsy showed no evident increase of naked single axons superficially or in deeper layers. No medium or thick calibre bundles were seen
14. H94/0095-96	M	06-06-1973	29-07-1993	Traumatic tetraplegia C-4/5	3	Trigone biopsy revealed 2-3 + presence of thin naked axons in superficial mucosa directly adjacent to basement membrane with medium-sized and medium calibre nerve bundles. Dome biopsy showed thin axon hyperplasia only in deeper layers of submucosa and no evident thick nerve bundles other than within deep muscle layers. (Date of biopsy: 14-01-1994)
15. H94/1461-62	M	12-03-1950	23-05-1992	Traumatic paraplegia T-12	3	Trigone biopsy 3 + positivity of single naked axons in superficial position. Dome biopsy showed variable distribution of excessive numbers of single naked axons (3-4+) and also superficially positioned medium calibre bundles
16. H94/2905-06	M	11-09-1934	29-04-1967	Traumatic paraplegia T-10	2	Trigone biopsy shows patchy presence of filaments close to the mucosa. Dome biopsy revealed diffuse presence 2-3+ of naked axons in submucosal position directly adjacent to urothelium
17. H94/1552-53	M	20-07-1945	07-09-1992	Traumatic tetraplegia C-4/5	1	Trigone biopsy showed no evidence of any fibre increase. Dome biopsy showed relatively high numbers of single naked axons in relation to deep muscle, but in superficial mucosa, there was no evident increase of nerve fibres
18. H95/1626-27	M	03-12-1971	09-1988	Traumatic paraplegia T-6	2	Superficially located single axons were seen in increased numbers in trigone biopsy. Dome biopsy showed the presence of evenly distributed small and medium fibre bundle hyperplasia both superficially in the mucosa and slightly deeper both beneath the squamous metaplasia and between the pre-existent urothelial lining
19. H94/2585-86	M	23-03-1958	29-11-1980	Traumatic tetraplegia C-5	1	Trigone biopsy revealed thick nerve bundles in deeper layers associated with muscle bundles of normal calibre. An occasional group of nerve fibres close to the urothelium but no evident increase of single axons. Dome biopsy revealed mild increase of single fibres in deeper parts of the mucosa and 1-2+ increase of single naked fibres in superficial mucosa. (Date of biopsy: 09-09-1994)
20. H94/3157-58	M	30-09-1931	24-01-1994	Traumatic tetraplegia C-5/6	1	Trigone biopsy showed patchy increase of axonal fibres mainly associated with proliferating blood vessels. Dome biopsy showed some increase of thick bundles in deeper layers of mucosa

Table 1 cont'd

Patient	Sex	Date of birth	Date of onset of paralysis	Diagnosis	Grading ¹	S-100/Neurofilament immunohistochemical staining of urothelium and submucosa
21. H94/2525-26	M	10-04-44	17-8-1990	Transverse myelitis Paraplegia T-7	0	Trigone biopsy showed no evident increase of naked single axon fibres in superficial submucosa, a 1+ increase in bundles in the deeper level of the submucosa. Dome biopsy showed slightly patchy increase of axonal bundles but single axons were not evidently increased
22. H94/2903-04	M	24-06-1960	22-08-1984	Traumatic tetraplegia C5/C6	3	Trigone biopsy showed occasional patch presence of bundles of filaments close to the mucosa. Dome biopsy showed an excessive thin fibre increase in submucosa. Thick nerve bundle increase was associated with blood vessels
23. H93/3250	M	23-02-1958	29-11-1980	Tetraplegia C-5	1	Well defined thick nerve bundle in deeper layer and relatively close to mucosa both in neurofilament and S-100 staining. (Date of biopsy: 13-12-1993)
24. H93/3337-38	F	30-07-1946	01-01-1969	Tuberculous meningitis Paraplegia L-1	3	Trigone biopsy showed extensive hyperplasia of thin naked axons in superficial submucosa. Dome biopsy revealed naked fibres and in deeper layers, medium-sized and medium calibre axons and nerve bundles
25. H94/0097-98	M	23-04-1963	06-09-1991	Traumatic tetraplegia C-6	2	Trigone biopsy revealed presence of relatively thick nerve bundles of naked axons, but single fibres were present only 1-2+. Dome biopsy showed relatively rich presence of single naked axons in most superficial mucosa
26. H94/0155-56	M	06-03-1947	04-06-1990	Traumatic tetraplegia	2	Trigone biopsy revealed 2+ presence of single naked axons in submucosa. Dome biopsy showed thick nerve bundles in relation to deep muscle. Slightly patchy presence of single naked axons of density between 1 & 2+. In addition to thick nerve bundles, very rich presence of nerve fibres in smooth muscle bundles
27. H94/0184-85	F	01-10-1934	01-01-1986	Traumatic tetraplegia C-4	3	Trigone biopsy revealed 3+ presence of thin naked axon fibres in single distribution and axon fibre bundles of medium calibre in very superficial location in continuity with medium to thick bundles in deeper position associated with smooth muscle. Biopsy of the dome revealed 2-3+ and focally 3-4+ presence of naked axons in very superficial position in addition to slightly deeper positions of medium calibre nerve fibres
28. H94/0293-94	F	19-06-1958	01-08-1981	Traumatic paraplegia T-5	2	Trigone biopsy revealed 1-2+ presence of single naked axons in superficial location, but regular presence of medium-sized bundles in very superficial location. Dome biopsy revealed 2-3+ proliferation of thin single naked axons in superficial and very superficial location and medium-sized deeper bundles and relatively rare, large very deep bundles in association with muscle regularly present
29. H94/0470-71	M	24-01-1935	March 1990	Tetraplegia C-6 (post-disectomy)	2	Trigone biopsy revealed only focal increased presence superficially of naked single axons, and in most places only occasional bundles of medium calibre nerve bundles in superficial location. Dome biopsy revealed 2-3+ (slightly patchy) to 1-2+ presence of single naked nerve fibres in superficial location; in slightly deeper location 2-3+ presence of single naked nerve fibres often associated with relatively thin bundles of fibres

Table 1 cont'd

<i>Patient</i>	<i>Sex</i>	<i>Date of birth</i>	<i>Date of onset of paralysis</i>	<i>Diagnosis</i>	<i>Grading¹</i>	<i>S-100/Neurofilament immunohistochemical staining of urothelium and submucosa</i>
30. H94/0642-43	M	25-06-1964	23-10-1983	Traumatic paraplegia T-6	2	Trigone biopsy: Although axons are seen in bundles in association with blood vessels in papilla-like structures between crypts with intestinal metaplasia, thin naked single axons are rarely seen. Dome biopsy showed only 1-2+ presence of single naked axons in very superficial location where there is inflammatory infiltrate. Patchy distribution of (2-3+) thin naked axons in deeper layers
31. H94/2505-25-13	M	06-06-1973	29-07-1993	Traumatic tetraplegia C-4/5	1	Trigone biopsy revealed no evident increase of neurofilaments in superficial location either singly as naked axons or in bundles. Dome biopsy revealed slight increase in naked axons in deeper layers and 1-2+ increase superficially. (Date of biopsy: 02-09-1994)
32. H94/0808-09	M	17-12-1965	December 1983	Multiple sclerosis Tetraplegia	1	There is no evidence of nerve fibres <i>superficially</i> . Well developed thick nerve bundles are seen in association with big blood vessels in deeper <i>location</i>
33. H95/1275-76	F	25-06-1964	24-09-1983	Guillian Barre syndrome Tetraplegia C-4	3	Trigone biopsy shows clear hyperplasia of intermediate axon bundles between three to five single fibres up to very superficial layers of the biopsy. In slightly deeper parts, occasional bundles of up to ten axons are seen. Dome biopsy shows the presence of large numbers of thin and single axons, and bundles of between three to five axons in the superficial mucosa and also in deeper parts. (Date of biopsy: 09-05-1995)
34. H94/1554-55	M	01-01-1975	10-05-1992	Traumatic paraplegia T-5	1	Trigone biopsy showed no evident increase in nerve fibres. Occasionally, 1-2+ presence of naked fibres in superficial location
35. H94/3413-14	M	27-11-1944	29-07-1989	Traumatic tetraplegia C-5	2	Trigone biopsy showed excess of thick bundles of axonal profiles in superficial location, in addition to 2-3+ excess of thin axons. Dome biopsy showed considerable excess of single naked axons in superficial location
36. H94/3415-16	M	19-09-1950	21-09-1976	Traumatic tetraplegia C-5	1	Trigone biopsy showed no evidence of axon profile increase. Dome biopsy showed 1+ increase in axon profiles in a patchy distribution related to the superficial mucosa
37. H95/0256-66	F	13-05-1926	1974	Multiple sclerosis Flaccid paraplegia	1	Trigone biopsy showed light increase of axon fibres in deeper layers but no abnormality of axons related to superficial mucosa. Dome biopsy showed some increase in medium and thick bundles in relatively superficial position
38. H95/0770-71	M	22-02-1944	1970	Multiple sclerosis Tetraplegia C-4 incomplete	1	Trigone biopsy revealed limited nerve fibre presence. Dome biopsy revealed thin nerve fibres proliferating in the area under squamous metaplasia. In the area of granulation tissue, nerve fibres were not found superficially
39. H94/3004	M	21-09-1971	18-12-1991	Traumatic tetraplegia C-5	0	Only very small deep bundles of 3-10 axons are seen. No superficial or single fibres are noted. There are no deep peripheral nerve type bundles and only in association with large blood vessel are some bundles of higher size found. (Date of biopsy: 25-10-1994)

Table 1 cont'd

<i>Patient</i>	<i>Sex</i>	<i>Date of birth</i>	<i>Date of onset of paralysis</i>	<i>Diagnosis</i>	<i>Grading¹</i>	<i>S-100/Neurofilament immunohistochemical staining of urothelium and submucosa</i>
40. 95/1006-07	M	12-06-1943	25-08-1994	Traumatic tetraplegia C-4	1	Trigone biopsy revealed limited presence of nerve filaments 0-1+ in submucosa adjacent to basement membrane. Dome biopsy revealed 1-2+ presence of naked fibres directly adjacent to urothelium
41. H95/1008/09	M	19-10-1948	June 1967	Paraplegia T-6	1	Trigone biopsy revealed very limited thin axon proliferation in submucosa directly adjacent to basement membrane. Thicker nerves present in deeper layers. Dome biopsy shows normal amount of nerve fibres. Thick nerve bundles are seen especially associated with blood vessels in submucosa, with limited presence of naked axons in submucosa
42. H95/1131-32	M	31-12-1929	11-12-1982	Traumatic tetraplegia C-5	1	Trigone biopsy: With antibodies to neurofilament, very little positivity of medium and thick nerve bundles in deep layers. The very limited presence of nerve fibres is confirmed by S-100 staining which highlights the relatively thick calibre nerve bundles in deeper layers and the severe paucity of fibres in submucosa. Dome biopsy: With antibodies to neurofilament, very little axons are seen in superficial location. This is confirmed by staining with S-100. Naked nerve fibres in association with deeper blood vessels and lymphoid follicles are seen
43. H95/1232-33	M	15-02-1930	17-12-1951	Traumatic paraplegia T-5	0	Trigone biopsy revealed a very focal, patchy presence of occasional thin single axons in superficial location, usually associated with the presence of lymphocytes. Throughout the remainder of the biopsy (superficial mucosa and muscle), there is no suggestion of nerve fibre hyperplasia. Dome biopsy showed one focus of thin single axons present in the submucosa, associated with a lymphoid follicle. Throughout the remainder of the biopsy, there is no evidence of axon proliferation either in superficial mucosa or deep muscle
44. H95/1273-74	M	23-01-1935	20-12-1991	Traumatic tetraplegia C-5, incomplete	0	Trigone biopsy showed an occasional thin axon in the submucosa with no definite hyperplasia. Dome biopsy showed no evidence of single fibre or fibre bundle hyperplasia in the mucosa
45. H95/1575-1576	M	03-07-1933	30-08-1976	Traumatic paraplegia T-10	0	Trigone biopsy revealed no proliferation of single fibre axons or fibre bundles in the superficial or deep mucosa and submucosa or in the muscle tissue. Dome biopsy showed virtually no fibre presence with S-100 and neurofilament stain which is confirmed by the absence of single axons

Table 1 cont'd

Patient	Sex	Date of birth	Date of onset of paralysis	Diagnosis	Grading ¹	S-100/Neurofilament immunohistochemical staining of urothelium and submucosa
46. H95/1407-08	M	29-09-1962	05-02-1982	Traumatic paraplegia T-6	3	Trigone biopsy revealed extensive increase in the superficial submucosa of single thin axons. Slightly deeper but still superficially, there is an increase of bundles of axons of intermediate calibre. Deeper in the wall, associated with smooth muscle, there are thick nerve bundles of peripheral nerve morphology. Dome biopsy showed an extensive increase in the superficial submucosa of single thin axons. Slightly deeper but still superficially, there is an increase of bundles of axons of intermediate calibre, very much associated with the presence of intermediate calibre arterial blood vessels. Deeper in the wall, associated with smooth muscle, are thick nerve bundles of peripheral nerve morphology
47. H95/1405-06	M	19-08-1938	12-06-1957	Traumatic tetraplegia C-5/6	0	Trigone biopsy revealed limited presence of thin single axons in the submucosal areas (sparse) in between lymphoid follicles. Dome biopsy showed no evident increase of thin single axons or thicker bundles in deeper layers

1. Overall grading of C-fibres in vesical urothelium and submucosa, on a 0 to 3 scale as defined under 'Patients and methods'.

Figure 4 Microphotograph of intra-urothelial nerve fibre hyperplasia in squamous metaplastic trigone biopsy of SCI patient. Note focal presence of single axon fibres, contrasted by Neurofilament stain with urothelium. Five micron section, stained with monoclonal antibody against Neurofilament antigen, Haematoxylin counterstain Microscopical magnification 400 ×

Figure 5 Microphotograph of detail of patient of Figure 4. Note presence of thick, peripheral nerve calibre nerve bundles in very superficial location in bladder. Five micron section, stained with monoclonal antibody against Neurofilament antigen, Haematoxylin counterstain. Microscopical magnification 200 ×

administered intravesically three times a day in the form of a commercially available solution containing 5 mg of oxybutynin in 30 ml. He diligently carried out this regime, but there was no clinical response to intravesical oxybutynin therapy; he continued to leak urine between catheterisations even though he was performing intermittent catheterisation six times a day. He ultimately preferred to have an indwelling urethral catheter drainage despite its potential disadvantages. Immunohistochemical study of bladder mucosal biopsy with S-100 and neurofilament showed no evidence of (grade 0) nerve hypertrophy.

Case 2

MC, a 36 year male with spinal cord injury at L-1 level did not demonstrate a satisfactory clinical response to intravesical oxybutynin therapy (5 mg of oxybutynin in 30 ml administered intravesically three times a day), and he discontinued its use. Immunohistochemical study of bladder mucosal biopsy in this paraplegic also showed absence of (grade 0) nerve hypertrophy.

Case 3

In contrast to the above two cases, PR, a 48 year old female with paraplegia due to tuberculous meningitis,

showed a satisfying clinical response to intravesical oxybutynin therapy (5 mg of oxybutynin in 30 ml, administered intravesically three times a day) which was prescribed as an adjunctive measure to intermittent catheterisation to prevent urine leaks between catheterisation. Bladder mucosal biopsy showed extensive hypertrophy of thin naked axons in superficial submucosa seen both in S-100 and neurofilament immunohistochemical studies.

Although the above three patients represent only anecdotal case reports, these observations suggest a possible functional relationship between the presence/virtual absence of, and hypertrophy of nerve fibres in mucosa and vesical submucosa in spinal cord injury patients and the response to intravesical drug (eg oxybutynin) therapy justifying further clinical research aimed at recognising the patient who may have a lower probability of responding to a given drug regime and in whom this treatment modality may not be effective.

Paucity of, or virtual absence of C-fibres, a source of trophic factors such as VIP, in the vesical mucosa and submucosa may be considered a possible cause of urothelial proliferation defect or arrest. Immunopathological analysis of the bladder trigone biopsy of AN (H95/1576), (a 61 year old male traumatic paraplegic with T-10 level since 30-08-76), using

Figure 6 Microphotograph of submucosal granulation tissue in trigone urinary bladder biopsy of SCI patient with vesical calculus. Note extensive proliferation of small blood vessels with increased numbers of small and medium sized bundles closely associated with and following individual small vessels. Five micron section, stained with monoclonal antibody against Neurofilament antigen, Haematoxylin counterstain. Microscopical magnification 200 ×

monoclonal antibodies against neurofilament and S-100 showed no proliferation of single fibre axons or fibre bundles in the superficial, or deep mucosa, submucosa, and muscle tissue. With antibodies to proliferating cell nuclear antigen, approximately 20%–30% labelling was found in areas linked by urothelium; with antibodies to MIB-1, in the urothelial layers, only 1% of the nuclei stained positive indicating complete PCNA, MIB-1 dissociation and proliferation arrest. Similarly, immunopathological analysis of the bladder dome biopsy of BL (H95/1273, a 60 year old male traumatic tetraplegic patient), using monoclonal antibodies against S-100 and neurofilament showed no evidence of single fibres or fibre bundle hyperplasia in the mucosa. Monoclonal antibodies against proliferating cell nuclear antigen showed approximately 30–40% positive labelling over two-thirds of the height of the mucosa. With antibodies to MIB-1, virtually no effective labelling was seen. Thus in this patient with no evidence of single fibre or fibre bundle hyperplasia, complete PCNA, MIB-1 dissociation with proliferation arrest was observed. Although at present insight into the cause/effect nature of this association does not allow for conclusive interpretation, the relation-

ship between fibre hyperplasia and proliferation-dependent cell surface changes facilitating bacterial colonisation require further exploration.

Electrical stimulation of urinary bladder by implanted electrodes (sacral anterior root stimulator) has been shown to decrease the frequency of urinary tract infection. The reduction in the episodes of cystitis in these spinal cord injury patients following implantation of sacral anterior root stimulator was, so far, believed to be due to more efficient emptying of the urinary bladder and consequent decrease in residual urine. It is also possible that electrical stimulation of urinary bladder leads to release of trophic factors from the vesical mucosal and submucosal nerves and improved proliferation status of vesical urothelium. Two patients in this study had undergone implantation of an anterior sacral root stimulator. PR, a 33 year old traumatic paraplegic patient since 05-02-1982, had had an anterior sacral root stimulator with extradural placement of electrodes inserted on 29-05-1992. The bladder biopsy taken on 22-05-1995 (H95/1407-08) showed grade 3, C-fibre hyperplasia. Trigone biopsy revealed there was extensive increase of single axons in the superficial submucosa. Slightly deeper but still superficially, there

was an increase of bundles of axons of intermediate calibre. Even deeper in the wall, associated with smooth muscle, were thick nerve bundles of peripheral nerve morphology. Dome biopsy, featuring almost identical changes, showed extensive increase in the superficial submucosa of single thin axons. The bladder biopsy of the other patient JJ, a 37 year old male who sustained traumatic tetraplegia at C-5 level on 29-11-1980, had undergone sacral anterior root stimulator implantation in March 1982 and bilateral posterior rhizotomy S₂ to S₅ on 15-09-1987 showed well-defined thick nerve bundles in deeper layers as well as relatively close to vesical mucosa both with neurofilament and S-100 staining (H93/3250/2). At present, it is not known what role electrical stimulation of anterior sacral roots S₂, S₃, and S₄ may have on the development of such C-fibre hyperplasia in the vesical mucosa and submucosa. In contrast however, the availability of such a stimulus dispersing fibre system as in the above patients with C-fibre hyperplasia, will modulate the effect of sacral anterior root stimulation at the pre-ganglionic level. Subsequent changes in the kinetics of epithelial turnover may well be explained either by the changes in bladder function or through alterations in the release of trophic factors. This opportunity for further elucidating the beneficial effects of neural stimulation warrants further research in experimental models. If alterations of cell proliferation occur in the absence of, or preceding, fibre hyperplasia, a direct effect of (neuro-mechanical) stimulation may be assumed. If however, such changes are observed only after, and only in cases where nerve fibre hypertrophy has occurred, a direct relationship through the release of trophic factors may be assumed.

Thus in summary, although clarifications on cause and affect relationship of C-fibre hyperplasia in vesical mucosa/submucosa observed in patients with a neuropathic bladder requires further study, a previously unappreciated fact remains that a significant proportion of spinal cord injury patients with a neuropathic bladder exhibit this phenomenon of C-fibre hyperplasia whereas some paraplegic and tetraplegic patients do not exhibit it for reasons presently not well understood. Due to the limited number of cases available for assessment, it was not possible to draw any firm conclusion on the possible clinical relationship between C-fibre hyperplasia and response to intravesical pharmacotherapy. However, this concept, if proved correct, may open new avenues for future investigations in terms of qualitative and quantitative assessment of nerve fibres and receptors in vesical mucosa/submucosa to predict those patients who may have low probability for exhibiting a therapeutic response to intravesical pharmacotherapy with a particular drug so that alternative strategies may then be considered earlier to the benefit of the patient. The possibility that sacral nerve stimulation has a trophic effect on urothelium, even if this requires mediation through (not always achieved/found) nerve

fibre hyperplasia may further add to justifications of the bladder biopsy (in itself an invasive test, although with the advent of flexible cystoscopy, the morbidity of bladder biopsy may be less) for study of nerve fibres hyperplasia, cholinergic, adrenergic, peptidergic, and growth factor receptors and urothelial proliferation status. Why some spinal cord injury patients develop C-fibre hyperplasia relatively early and why some do not exhibit this at all, requires further study, especially as the overall effect associated with this change is perhaps, positive as a whole and not detrimental to the bladder function. The possible confounding role of the chronic mechanical stimulation of urothelium by indwelling catheters (at least at some time in the past for many patients) and functional relationship between C-fibre hyperplasia and squamous metaplasia (three of the eight patients with grade 3 C-fibre hyperplasia (trigone biopsy of BC who has been on indwelling catheter drainage, dome and trigone biopsy of PR who was on indwelling catheter drainage, and dome biopsy of IP who had JJ stent of left ureter) showed features of squamous metaplasia) requires further assessment. It should be emphasised that unlike a laboratory model, in a clinical study on spinal cord injury patients, many variables are inherently present viz. duration of paralysis, level of paralysis, whether the lesion is complete or incomplete, presence of a syrinx, number of episodes of acute urinary tract infection, therapeutic interventions such as type and duration of a particular form of bladder drainage, presence of bladder stone(s) and if so, the duration of its presence in the bladder before it was removed, whether a sacral anterior root stimulator was implanted and if so, how long after the spinal cord injury was it implanted, the duration of its use, whether a baclofen pump for intrathecal delivery of baclofen was implanted and if so, how long after the injury it was implanted and how long has it been in use, etc. All these variables may affect bladder physiology and functional anatomy and what we observe at a particular time point in a given spinal cord injury patient is only a cross-sectional view and indeed, no two spinal cord injury patients are identical. It is important that we keep this issue of diversity in mind while drawing any inferences on pathophysiology of neuropathic bladder in patients with spinal cord injury.

In conclusion, mucosal biopsy and study of nerve fibres in the urothelium and submucosa of the neuropathic bladder has helped to generate hypotheses on the association between C-fibre hyperplasia and response to intravesical pharmacotherapy and predictive value of such a study in identifying those patients who are likely to respond to intravesical pharmacotherapy.

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