Urol Res (1993) 21:383–388

Urological Research

Post-irradiation bladder dysfunction: development of a rat model

J. A. Vale[1], W. G. Bowsher[2], K. Liu[3], A. Tomlinson[4], H. N. Whitfield[1], K. R. Trott[3]

[1] Department of Urology, St. Bartholomew's Hospital, London EC1A 7BE, UK
[2] Department of Urology, Royal London Hospital, London E1 1BB, UK
[3] Department of Radiation Biology, St. Bartholomew's Hospital Medical College, London EC1M 6BQ, UK
[4] Department of Experimental Pathology, St. Bartholomew's Hospital Medical College, London EC1M 6BQ, UK

Received: 31 December 1992 / Accepted: 5 July 1993

Summary. The aim of this research was to establish a small animal model for the functional and morphological study of post-irradiation bladder dysfunction. Young adult female Wistar rats were X-irradiated with single doses of 10, 15, 20 or 25 Gy. Filling cystometry was performed to assess changes in reservoir function: the volume infused to produce a rise in intravesical pressure of 5 cmH_2O was calculated as an index of compliance. A biphasic reduction in this index was noted in animals receiving 15–25 Gy; the first reduction developed at about 4 weeks, and the second started at 3–4 months and persisted at 6 months. Bladder tissue was taken at this time (6 months post-irradiation) for morphological study. Histological examination demonstrated an increased mast cell density in the irradiated bladders, but was otherwise non-specific; fibrosis was discernible in only half of the 18 animals studied. Electron microscopy showed focal degeneration of smooth muscle cells, and in some areas there was selective degeneration of unmyelinated axon profiles. The biphasic reduction in the compliance index is consistent with the timing of the symptoms of the acute and late irradiation reactions reported by radiotherapy patients. Changes in axon profiles and mast cell density may be of functional significance.

Key words: Bladder dysfunction – Electron microscopy – Histology – X-irradiation

Radical radiotherapy is used frequently for the treatment of locally invasive transitional cell carcinoma of the bladder in the United Kingdom [2, 3]. Despite advances in technique and fractionation regimes, radiation-induced urinary symptoms may develop relatively acutely during treatment or at a much later date. The early radiation reaction is most prominent at 4–6 weeks [20], affected patients complaining of urinary frequency, nocturia, strangury and haematuria. Its incidence may be as high as 70% [10]. Urodynamic studies at the time of this early radiation reaction have demonstrated significant reductions in volume at first desire to void, cystometric capacity and bladder compliance [9]. These parameters had returned to pretreatment values by 6 months.

Symptoms associated with the late irradiation injury are probably less common, but are often progressive and intractable. In a questionnaire survey of 97 patients who had received radiotherapy for cervical carcinoma 5–11 years previously, Parkin et al. [18] reported that over half had bladder symptomatology. The commonest symptoms were urgency and urge incontinence (experienced by 45%) and frequency (in 33%). Urodynamic studies in these patients [19] demonstrated significant reductions in first desire to void and maximum cystometric capacity, and an increase in the mean maximum subtracted detrusor pressure during filling. Unstable detrusor contractions (> 15 cmH_2O) were found in a third of radiotherapy patients studied as compared with none in the control group.

Existing hypotheses for the late irradiation injury of the bladder have concentrated on urothelial injury/ulceration [7, 23, 24] and fibrosis [6, 11, 22], but evidence for each is largely empirical and such mechanisms would not account for the development of detrusor instability late after radiotherapy. The aim of this research was to develop a simple in vivo urodynamic model, and then to utilize tissue from this model for the further morphological and in vitro study of post-irradiation bladder dysfunction.

Materials and methods

Young adult female Wistar rats (age 14 weeks, weight 180–220 g) were selected as the experimental model on the grounds of availability and ease of catheterization. Prior to irradiation, cystometric measurements were performed on all animals to establish base-line readings. Animals were randomized into four equal groups of 9 rats each: a control group (no irradiation), and radiation dose groups of 10, 15, and 25 Gy. In addition, as part of a separate experiment, 7 rats from the same breeding colony received 20 Gy.

Correspondence to: J. A. Vale, Department of Urology, St. Mary's Hospital, Praed Street, London W2 1NY, UK

Irradiation

Irradiation was performed on rats anaesthetized using 3% enflurane (Abbott) in a 1:1 mixture of oxygen and nitrous oxide. Anaesthesia was induced by placing each animal in a sealed box containing anaesthetic gas/vapour from a Boyle apparatus, and when the animal was unconscious it was transferred to a specially designed irradiation jig on which anaesthesia was maintained by placing the animal's snout into the gas/vapour supply tubing. The bladder was emptied using a 3Ch catheter and 0.1 ml of an iodine-containing contrast medium (Schering) was introduced. Previous studies on the ureter using the same contrast medium had shown no evidence that the contained iodine had any radiosensitizing effect [12]. The animal was secured on the irradiation jig in a supine position with the hind legs restrained, and a sheet of Polaroid film (Polapan ISO400/27) was exposed for 14 s using the 0.4 × 0.4 mm focus of the Pantak 320-kV X-ray generator but run at 45 kV and 10 mA. From this film the bladder could be accurately localized, and a lead shield 5 mm thick with a 1.5 × 1.0 cm central portal for irradiation was placed between the rat and the X-ray generator to protect structures adjacent to the bladder. To ensure correct positioning, a further film was exposed to check that the portal in the lead shield coincided with the bladder. The X-ray dose to the bladder from these planning films was less than 0.01 Gy. Without changing the position of the animal but changing to the 3 × 3 mm focus, the bladder was irradiated with 300-kV X-rays filtered by 1 mm aluminium and 0.4 mm copper at a dose rate of 1.72 Gy/min. The exposure of each animal was controlled by measuring the dose in air in a fixed position with an ionization chamber, and calculating bladder dose by comparing this dose with that measured by an ionization chamber placed in the rectum. Exposure times were varied to achieve X-ray doses of 10, 15, 20 and 25 Gy. Single doses of 20–25 Gy are equivalent, according to radiobiological models of time-fractionation-dose relationships, to the typical fractionated radiotherapy regimens which give a total dose of 60 Gy in 30 fractions in 6 weeks.

Cystometry

Following irradiation cystometry was performed weekly on all rats until 2 months post-irradiation, and thereafter once every 3 weeks. All procedures were performed under inhalational anaesthesia using 3% enflurane in a 1:1 mixture of oxygen and nitrous oxide. Urethral catheterization was performed using the tip of a ureteric catheter (Porges), the size of catheter (3–5 Ch) being varied according to the size of rat to prevent leakage around the tube. The bladder was emptied by gentle palpation, and then the catheter was connected to a three-way tap. The bladder was slowly filled at 0.05 ml/min with sterile saline using a syringe driver (Harvard Apparatus): this filling rate was chosen after preliminary studies in which it was found that more rapid filling rates of 0.1 ml/min and 0.2 ml/min resulted in rapid rises in intravesical pressure. Intravesical pressure during filling was monitored through the third limb of the three-way tap by a Sensortechnics PSC1 pressure transducer. The signal from this was displayed on a Lloyd 2002 chart recorder after digital-to-analogue conversion. Filling was discontinued when the intravesical pressure had risen by 10 cmH_2O.

Using a two-channel chart recorder and two-channel infusion pump, it was possible to perform cystometry on a control and an irradiated rat simultaneously. Cystometric measurements were discontinued at 6 months, when irradiated animals had demonstrated a sustained reduction in reservoir function when compared with control values.

To exclude endemic infection in these repeatedly catheterized animals, microscopy and urine culture were performed on catheter specimens of urine 6 months post-irradiation.

Morphological studies

Strips of bladder tissue for histological examination were placed on card with the mucosal surface uppermost, to prevent the natural tendency of the strips to curl up during fixation. The strips were then placed in 10% neutral buffered formol-saline for 1–4 weeks, prior to embedding and blocking in wax. Sections (4 μm) were taken and stained with haematoxylin and eosin, and with toluidine blue as initial observations had suggested a possible increase in mast cell numbers. Sections were examined under low-power (×40, ×100) and high-power (×250, ×400) magnification by two independent observers, and the presence or absence of features such as fibrosis determined by comparison with tissue from age-matched controls. Mast cell content was determined by counting the number of mast cells present in ten consecutive high-power fields (×250). Each high-power field has an approximate area of 0.1 mm^2.

Bladder tissue for electron microscopy was taken from the dome, lateral wall and basal regions of the bladder and cut into 1 mm^3 cubes. These were immersion-fixed for 6 h in a freshly prepared buffered solution (pH 7.3) containing 1.5% glutaraldehyde and 1.5% paraformaldehyde in cacodylate buffer (0.1 M). Paraformaldehyde was freshly made to avoid polymerization, and tissues were agitated constantly during fixation. Samples were washed in four changes of cacodylate buffer containing 3% sucrose over a 12-h period to remove the primary fixative, and then post-fixed in 2% osmium tetroxide in cacodylate buffer. Tissues were dehydrated in a graded series of ethanols and then embedded in epoxy resin using standard techniques. Sections were cut (70–90 nm thick) on a Reichert OMU2 ultramicrotome, mounted on 200 mesh copper grids, and stained with uranyl acetate and lead citrate. They were examined and photographed in a Philips EM 300 electron microscope.

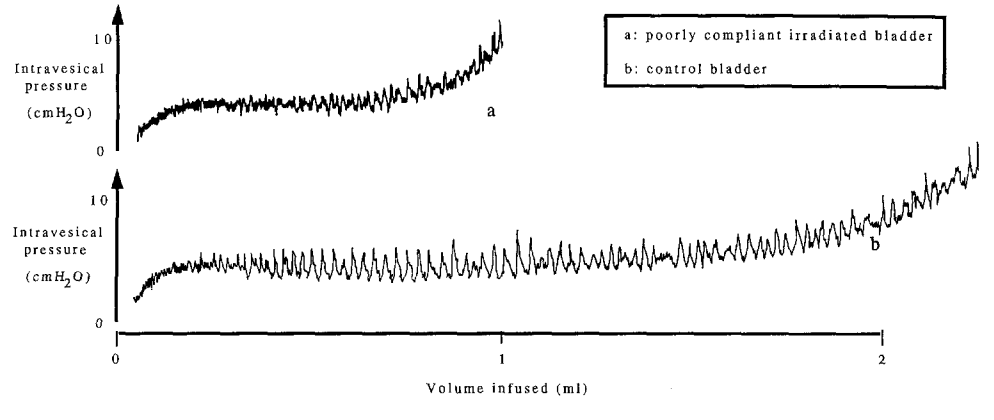

Fig. 1 a, b. Two original recordings of the volume-pressure relationship in a control rat (**b**) and an irradiated rat (**a**). The compliance index was calculated by drawing a horizontal line through the troughs (basal tonus) during the isotonic phase of bladder filling, and measuring the volume at which the basal tonus had risen 5 cmH_2O above this line

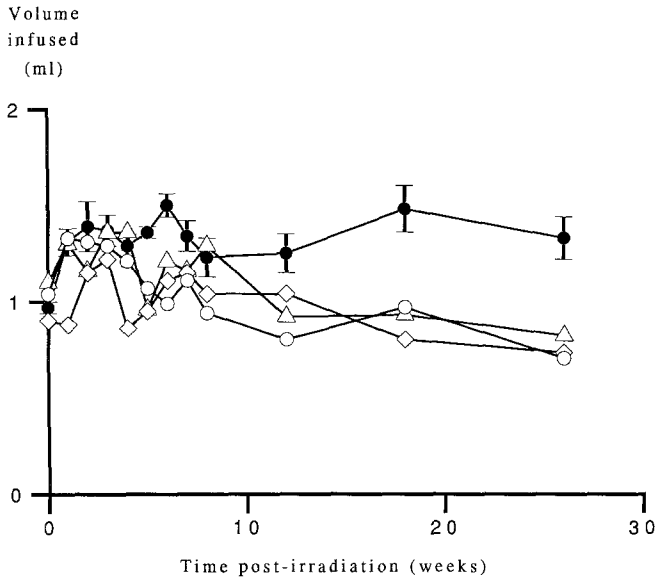

Fig. 2. Post-irradiation changes in the compliance index: all dose groups. *Points* are the mean for each dose group. *Standard error bars* are shown only for controls, but were of a similar magnitude for each group. ● Control ($n=9$); △ 15 Gy ($n=9$); ◇ 20 Gy ($n=7$); ○ 25 Gy ($n=9$)

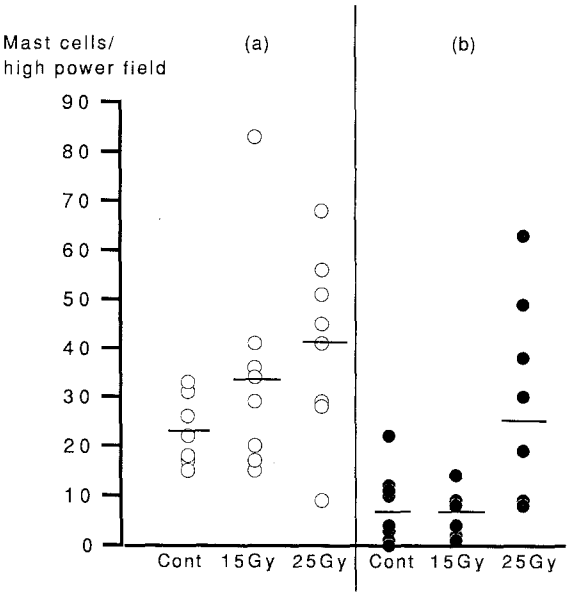

Fig. 3a, b. Mast cell density 6 months post-irradiation in urothelium and submucosa (**a**) and in the detrusor muscle layer (**b**). *Cont,* control

Results

Cystometry

Before comparing changes between control and irradiated animals it was necessary to develop a method for quantifying reservoir function. Measuring compliance per se ($\Delta V/\Delta P$ for the isotonic phase of bladder filling) was unsuitable: most irradiated rats with a reduced bladder capacity still showed a flat pressure trace during filling, albeit for a shortened period (Fig. 1). The volume infused to produce a rise in intravesical pressure of 5 cmH_2O takes this into account, and was used as an index of compliance.

Inevitably there were a number of deaths during the study period, mainly anaesthetic related. Thus at the conclusion of the study there were 8 rats remaining in the control, 10-Gy, 15-Gy and 25-Gy dose groups, and 5 in the 20-Gy dose group. A biphasic reduction in the compliance index was demonstrated following X-irradiation at doses of 15, 20 and 25 Gy (Fig. 2). The first reduction developed at 4–6 weeks post-irradiation, and was by as much as 30% in the 15-Gy group ($P<0.018$), 30% in the 20-Gy group ($P<0.10$) and 34% in the 25-Gy group ($P<0.002$). (All statistical analyses were performed using the Mann-Whitney *U*-test.) Following a transient recovery period, a second reduction in compliance started at 10–12 weeks and persisted. At 6 months the compliance index was reduced by 38% in the 15-Gy group ($P<0.0014$), 40% in the 20-Gy group ($P<0.012$) and 48% in the 25-Gy group ($P<0.0014$). Animals irradiated at 10 Gy showed no compliance changes during the 6-month study period, indicating that the threshold dose for the early radiation reaction is between 10 and 15 Gy. No conclusion can be reached as to the threshold for the late reaction as all control animals were killed at 6 months. However, a further cystometry study of 7 rats surviving 10 months after 10 Gy of X-irradiation showed that there had been no change in the compliance index as compared with their 6-month value.

Of 30 catheter specimens of urine taken 6 months post-irradiation, only that from 1 rat (25-Gy group) demonstrated a significant growth on urine culture ($>1\times10^8$ colonies/l, *Streptococcus*).

Histology

Histological specimens were examined from a total of 18 rats 6 months after irradiation; 9 had received 15 Gy and 9 had received 25 Gy. They were compared with sections from 8 age-matched controls. Half of the irradiated bladders demonstrated marked fibrosis, with fibrotic infiltration of muscle bundles. However, the remaining bladders showed no discernible fibrosis, and there was no association between the presence of fibrosis and the magnitude of any reduction in compliance. The mean 6-month compliance index for rats showing fibrosis was 0.82 ± 0.14 ml (for a 5 cmH_2O rise in pressure) as compared with 0.85 ± 0.11 ml for rats showing no fibrosis. There was no association between fibrosis and dose, 4 of 9 bladders showing fibrosis after 15 Gy and 5 of 9 after 25 Gy.

Mast cells were more abundant in irradiated bladders than in controls (Fig. 3) and in the 25-Gy dose group this

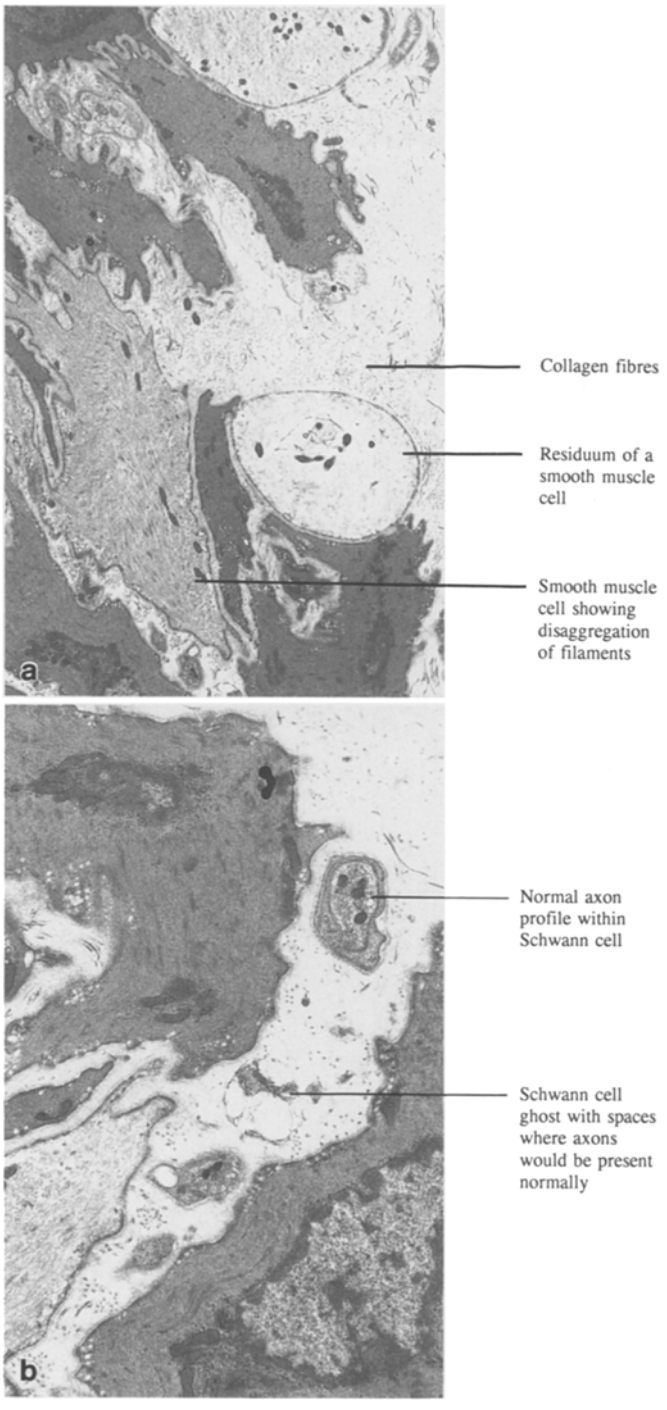

Fig. 4a. Various degrees of smooth muscle disruption are visible in this electron microscopy preparation, as well as abundant intercellular collagen (×8000). **b** A degenerate axon profile (×18000)

reached statistical significance for the detrusor mast cell sub-population ($p < 0.05$ by the sign test). In addition, 4 of the 18 specimens showed a marked inflammatory cell infiltrate predominantly comprising polymorphonuclear cells and plasma cells. These changes were evenly distributed between the radiation dose groups: 2 of 4 had received 15 Gy and 2 of 4 had received 25 Gy.

Sections were not ideal for the assessment of urothelial changes, as the fixation technique permitted urothelial shedding. In those regions in which the urothelium was well preserved, it appeared generally normal with focal areas of hyperplasia.

Electron microscopy

Multiple blocks from a total of 25 rats were studied by electron microscopy: 9 control, 8 from rats which had received 15 Gy and 8 from rats which had received 25 Gy 6 months previously. Within the irradiated bladders the most striking feature was the presence of areas showing focal degeneration of smooth muscle cells (Fig. 4a). Muscle cells exhibiting disaggregation of filaments were interspersed between muscle cells of normal morphology. In some regions of the bladder wall, cytoplasmic organelles were observed free in the intercellular space, indicative of cellular disruption. There were occasional macrophages between these damaged muscle cells, presumably to remove this debris.

In comparison with controls there was a considerable increase in the deposition of collagen between the muscle cells in some sections. However, electron microscopy is not a suitable method for quantification due to the randomness of sampling. In scattered foci, selective degeneration of unmyelinated axon profiles was noted. In its most marked form, empty spaces were apparent within Schwann cells where axons would normally be present (Fig. 4b). Lesser degrees of axonal injury were discernible as a loss of intraneuronal ultrastructure.

These findings were consistent regardless of the site of sampling. Thus blocks from the dome, lateral walls and base of the bladder showed similar changes.

Discussion

The aim of this research was to develop a simple in vivo model for the study of radiation reactions affecting the urinary bladder. The essential requirement of any such model is that it should demonstrate measurable, reproducible changes following irradiation which are consistent with the clinical picture observed in patients after radiotherapy. The biphasic reduction in compliance reported in this study is consistent with the early and late symptoms reported by patients, and also with the cystometric findings in the limited number of patient series published [9, 13, 19].

There have been other models proposed for the assessment of radiation injury, mainly based upon the mouse. The first, reported in 1978 [23], measured urinary frequency by placing individual mice in metabolic cages and counting the number and size of wet patches on a sheet of blotting paper moving slowly beneath the cages. A late radiation injury was demonstrated, with a maximal increase in urinary frequency by 1 year after irradiation with doses up to 40 Gy. However, no early radiation reaction was detected, probably because measurements were performed only once a month. Lundbeck et al. [15, 16] developed a further mouse model and performed

cystometry following irradiation; their findings were similar to ours, with an acute reversible response developing within 28 days of irradiation (20 Gy) and a later irreversible response starting 4 months post-irradiation. They used individual bladder volumes before irradiation as their controls, and their end-point for evaluation was a 50% decrease in bladder volume relative to this pre-irradiation volume for a given rat at an intravesical pressure of 20 mmHg. This overcomes the problem of natural variation between animals, but was unsuitable for the evaluation of the data obtained in our study; there was a trend towards a sustained increase in compliance in control animals in the first 2 months of study, which made comparison between different times in the same rat impossible. The reason for this change in the controls may have been growth-related, with an increase in body weight from a mean of 199 g at 14 weeks of age to 224 g 6 months later. However, an alternative explanation is that the repeated cystometry measurements may have produced some increase in capacity by a stretching mechanism. If this was the case the effect should have been similar in control and irradiated animals and minimized by limiting the rise in intravesical pressure to less than 10 cmH_2O.

A criticism of both this study and that of Lundbeck et al. is that, of necessity, all cystometry measurements were performed under general anaesthesia. The use of anaesthetic agents in urodynamic studies has been criticized, and some – halothane in particular – have been shown to diminish the rise in intravesical pressure during filling and may abolish unstable contractions [8]. Thus anaesthesia limits the interpretation of absolute values of compliance. In this study, however, a standardized anaesthetic technique was used throughout and results have been used for comparative purposes only. Techniques have been developed for the investigation of voiding function in conscious animals. However, these all involve the use of indwelling catheters, which can be left in place for a maximum of 6 weeks only. After that time the loss of animals due to infection and the prevalence of stone formation become unacceptably high [25]. Such models are therefore inappropriate for the study of the late irradiation injury.

Early accounts of bladder radiation reactions concentrated on urothelial changes and ulceration [7, 24] and fibrosis [6, 11]. Although the method of sample fixation was not ideal for the preservation of the mucosa, urothelial desquamation and ulceration were not a feature in the 18 irradiated bladders studied during this research. Most cases were characterized by a tendency to urothelial hyperplasia, as has been previously reported in the Fischer rat [1]. Likewise, in the 18 post-irradiation specimens studied during this research, only half showed discernible fibrosis and the changes in compliance were not limited to this more fibrotic group. However, the subjective assessment of minor degrees of fibrosis is difficult, and ideally a more objective method of quantifying fibrosis should be used, such as selective collagen staining and morphometric analysis. The latter is fraught with difficulties when applied to a distensible organ such as the bladder and analysis would have to be performed at a standard degree of distension, which could be achieved only by infusing fixative until a given intravesical pressure is reached. This was not feasible during this research as the whole viscus was not available for study. Our finding of a reduction in the compliance index of up to 50% in the absence of microscopic evidence of increased collagen deposition suggests that fibrosis is not an essential requirement for post-irradiation bladder dysfunction, although clearly it may be contributory where it is present.

Mast cell numbers are increased in some inflammatory conditions of the bladder, including interstitial cystitis [14, 17], parasitic infections of the bladder and chronic bacterial cystitis [5]. Bacterial infection could not have been responsible for the mast cell changes in this research; only 1 rat demonstrated a significant growth on culture of a catheter specimen of urine 6 months after irradiation. In addition, the mast cell changes did not appear to represent part of a general inflammatory cell infiltrate. The increase in mast cell numbers affected both the detrusor and the more superficial layers, which is similar to the distribution seen in interstitial cystitis [4, 17]. Mast cells release highly potent inflammatory mediators including histamine, leukotrienes and prostaglandins, and their presence may explain the symptoms of frequency, nocturia and urgency which are so characteristic of interstitial cystitis [17]. The presence of increased mast cells following irradiation may also be significant, although detrusor mast cells were only significantly elevated in one radiation dose group (25 Gy).

The ultrastructural finding of focal degeneration of smooth muscle cells is consistent with previous studies [1], and suggests that either smooth muscle may be less radioresistant than some workers have reported [21], or smooth muscle degeneration is occurring secondary to denervation. It is unlikely that the foci of selective degeneration of unmyelinated axon profiles were artefacts resulting from poor tissue fixation; multiple blocks were sampled and great care was taken to select small blocks which were well-fixed, well-penetrated by plastic and devoid of artefact.

In conclusion, a technique of cystometry has been developed for the female Wistar rat, and following X-irradiation at doses of 15 Gy or more a biphasic reduction in compliance has been demonstrated. Morphological studies have failed to support the hypotheses previously put forward to explain post-irradiation bladder dysfunction, i.e. a predominant role of tissue fibrosis, but have demonstrated other changes that might be of functional significance. These include the presence of neural degeneration, which might lead to changes in detrusor activation mechanisms, and an increase in mast cell numbers in the detrusor layer of some animals. Further elucidation of underlying mechanisms may come from pharmacological studies on muscle strip preparations in vitro.

Acknowledgements. We are grateful to the Imperial Cancer Research Fund for financing this project.

References

1. Antonakopoulos GN, Hicks RM, Berry RJ (1984) The subcellular basis of damage to the human bladder induced by irradiation. J Pathol 143:103–116

2. Blandy JP (1985) Primary radiotherapy approach. In: Zingg EJ, Wallace DMA (eds) Bladder cancer. Springer, Berlin Heidelberg New York, pp 225–229
3. Blandy JP, England HR, Evans SJW, Hope-Stone HF, Mair GMM, Mantell BS, Oliver RTD, Paris AMI, Risdon RA (1980) T3 bladder cancer: the case for salvage cystectomy. Br J Urol 52:506–510
4. Christmas TJ, Rode J (1991) Mast cell characteristics in normal bladder, bacterial cystitis and interstitial cystitis. Br J Urol 68:473–478
5. Cornish J, Vanderwee MA, Ormrod DJ, Miller TE (1986) Mucosal mast cells as a component of the inflammatory response to lower-urinary tract infection. Int Arch Allergy Appl Immunol 81:337–342
6. Dean AL (1927) Ulceration of the urinary bladder as a late effect of radium application to the uterus. JAMA 89:1121–1123
7. Dean AL, Slaughter DP (1941) Bladder injury subsequent to irradiation of the uterus. J Urol 46:917–924
8. Doyle PT, Briscoe CE (1976) The effects of drugs and anaesthetic agents on the urinary bladder and sphincters. Br J Urol 48:329–335
9. Farquharson DIM, Shingleton HM, Sanford SP, Soong SJ, Varner RE, Hester S (1987) The short-term effect of pelvic irradiation for gynaecologic malignancies on bladder function. Obstet Gynecol 70:81–84
10. Goffinet DR, Schneider MJ, Glatstein EJ, Ludwig H, Ray GR, Dunnick NR, Bagshaw MA (1975) Bladder cancer: results of radiation therapy in 384 patients. Radiology 117:149–153
11. Gowing NFC (1960) Pathological changes in the bladder following irradiation. Br J Radiol 33:484–487
12. Knowles JF, Trott KR (1987) Experimental irradiation of the rat ureter: the effects of field size and the presence of contrast medium on incidence and latency of hydronephrosis. Radiother Oncol 10:59–66
13. Kümper HJ (1983) Postaktinische Reaktion des Harntraktes. In: Gynakologische Urologie. Thieme, Stuttgart, p 87–97
14. Larsen S, Thompson SA, Hald T, Barnard RJ, Gilpin CJ, Dixon JS, Gosling JA (1982) Mast cells in interstitial cystitis. Br J Urol 54:283–286
15. Lundbeck F, Djurhuus JC, Vaeth M (1989) Bladder filling in mice: an experimental in vivo model to evaluate the reservoir function of the urinary bladder in a long term study. J Urol 141:1245–1249
16. Lundbeck F, Ulso N, Overgaard J (1989) Cystometric evaluation of early and late irradiation damage to the mouse urinary bladder. Radiother Oncol 15:383–392
17. Lynes WL, Flynn SD, Shortliffe LD, Lemmers M, Zipser R, Roberts LJ, Stamey TA (1987) Mast cell involvement in interstitial cystitis. J Urol 138:746–752
18. Parkin DE, Davis JA, Symonds RP (1987) Long-term bladder symptomatology following radiotherapy for cervical carcinoma. Radiother Oncol 9:195–199
19. Parkin DE, Davis JA, Symonds RP (1988) Urodynamic findings following radiotherapy for cervical carcinoma. Br J Urol 61:213–217
20. Rubin P, Casarett GW (1968) Urinary tract: the bladder and ureters. In: Clinical radiation biology. Saunders, Philadelphia, pp 334–373
21. Rubin P, Casarett GW (1968) Clinical radiation pathology as applied to curative radiotherapy. Cancer 22:767–778
22. Stewart FA (1986) Mechanism of bladder damage and repair after treatment with radiation and cytostatic drugs. Br J Cancer 53 [Suppl VII]:280–291
23. Stewart FA, Michael BD, Denekamp J (1978) Late radiation damage in the mouse bladder as measured by increased urination frequency. Radiat Res 75:649–659
24. Watson EM, Herger CC, Sauer HR (1947) Irradiation reactions in the bladder: their occurrence and clinical course following the use of X-ray and radium in the treatment of female pelvic disease. J Urol 57:1038–1050
25. Yaksh TL, Durant PAC, Brunt C (1986) Micturition in rats: a chronic model for study of bladder function and effect of anaesthetics. Am J Physiol 251:R1177 R1185