Urol Res (1991) 19:207–213

Urological Research


*Original articles*

# Growth and metastasis of human bladder cancer xenografts in the bladder of nude rats*

## A model for intravesical radioimmunotherapy

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Accepted: February 1, 1991

**Summary.** A potentially useful therapeutic approach to the treatment of human bladder cancer is intravesical therapy with radiolabelled monoclonal antibodies (MAbs). We have established an animal model to study this approach. Inoculation of cloned 2B8 cells derived from the human bladder cancer cell line, UCRU-BL-17, into the bladder wall of nude rats pre-irradiated with 900 rads, resulted in local tumour growth in 39/40 (97.5%) animals, with invasion or metastases to distant organs in 25% of cases. Both the bladder tumours and the metastases were morphologically similar to the original biopsy sample from which the cell line, UCRU-BL-17, was established. The cells were of human origin, as shown by expression of HLA antigens, Alu probing, and cytogenetic analysis. Preliminary studies indicated that i.p. injection of anti-human bladder cancer monoclonal antibody (MAb), BLCA-38, radiolabelled with either iodine 131 or samarium 153 ($^{153}$Sm), resulted in tumour localisation, with tumour-to-blood ratios of 5.04 ($^{131}$I), and 4.3 and 3.1 ($^{153}$Sm) respectively. We now aim to examine the efficacy of the intravesical route for radioimmunotherapy in the nude rat model. This model will also serve for preclinical studies on the efficacy of systemically injected radioimmunoconjugates for control of metastatic growth.

**Key words:** TCC – Nude rat xenografts – Monoclonal antibodies – Radioimmunotherapy – Tumour localisation – Human bladder cancer

Urothelial tumours fall into two major groups: superficial and invasive, each with substantially different natural histories (reviewed in [22]). The traditional mainstays for management of superficial bladder cancer have been transurethral diathermy or excision biopsy [22], but the disease is characterised by frequent relapse. This may be accompanied by progression with invasion and metastases. In relapse, intravesical chemotherapy or BCG therapy are used, but both may be associated with significant morbidity when used repeatedly, and many patients ultimately require cystectomy [23].

Radioimmunotherapy with monoclonal antibodies (MAbs) provides a new therapeutic approach [34] which may be suitable for bladder cancer, because of its superficial, multifocal nature [29]. In particular, regional administration, for example by intraperitoneal injection for therapy of ovarian or colonic cancer, has promise [7, 10, 24]. Direct introduction into the bladder via a catheter (intravesical administration) could allow radioimmunoconjugates to come into direct contact with malignant urothelium [2]. In this paper, we present an animal model system that may be developed to evaluate this approach in preclinical studies.

## Material and methods

### *Patient details*

A 69-year-old female presented with a grade III, stage T4b transitional cell carcinoma (TCC) of the bladder with elements of adenocarcinomatous differentiation, as described previously [26]. She received intravenous cisplatin and radiotherapy, but sustained only a transient remission and died 4 months after presentation.

### *Cell line*

A continuous cell line, UCRU-BL-17CL (abbreviated BL-17), was established from a xenograft of the tumour biopsy [26]. A clone, 2B8, subsequently derived from this cell line [5], was maintained RPMI 1640 (Flow, North Ryde, NSW, Australia) with 10%fetal calf serum (FCS) (Commonwealth Serum Laboratories, Melbourne, Australia) in 5% oxygen, 7.5% carbon dioxide, and 87.5% nitrogen

* Supported by grants from the University of Sydney Cancer Research Fund and the Australian Kidney Foundation

at 37°C. The line was free of *Mycoplasma* as judged with the fluorochrome, Hoescht 33258 (Flow) [6]. For injection, single cell suspensions were prepared from cells harvested with 1 mM ethylene diamine tetra-acetic acid (Flow) and washed with phosphate-buffered saline, pH 7.2 (PBS).

### *Animals*

Chester Beatty-Rowett nude rats (intercrossed five times with a remote Wistar line in Australia) were obtained from the Australian Nuclear Science and Technology Organisation (ANSTO), Lucas Heights, NSW, Australia, and maintained in our animal facility in standard cages within a laminar flow rack. They were fed irradiated standard mouse diet and sterile acidulated water ad libitum. Experiments were carried out in accordance with the guidelines of the National Health and Medical Research Council, Australia.

### *Xenografts*

Male or female nude rats were treated directly, or preirradiated with 900 rads total body irradiation from a cobalt source to obtain a greater level of immune suppression. The animals were anaesthetised with Pentathesin (chloral hydrate 75 mg/ml, magnesium sulphate 37 mg/ml, pentobarbitone 15 mg/ml), 0.1 ml/100 g body weight, after premedication with hyoscine hydrobromide (0.25 µg/100 g body weight) as described previously [35]. The bladder was exteriorized through a small incision made in the midline of the peritoneal wall and $10^7$ cells of the 2B8 clone were inoculated into the bladder wall (intramurally) through a 27 g needle. The bladder was then repositioned and the incision was closed with sterile sutures. Etherised rats were carefully monitored for tumour growth by palpation and were killed if there were any signs of debilitation. For tumour passage, tumours recovered from the nude rats were cultured in vitro to obtain single cell suspensions prior to reinoculation intramurally into new irradiated nude rat hosts.

### *Histology*

Specimens for light microscopy, taken from the original tumour and from xenografted passages of the 2B8 clone, were fixed in buffered 10% formalin. They were embedded in paraffin and stained with haemotoxylin and eosin, periodic acid-Schiff (PAS) plus diastase, mucicarmine and alcian blue for mucin.

### *Karyotype*

Analyses were performed on cultured cells of the 2B8 clone following passage through nude rats. Colcemid was added for 22.5 h and the monolayers were trypsinized and harvested by standard techniques. Metaphase spreads were G-banded by a modified method of Seabright [31] and cytogenetic analysis was performed according to the Paris convention [14].

### *Monoclonal antibodies*

Murine monoclonal antibodies (MAbs) used were BLCA-8 ($IgG_3$), BLCA-38 ($IgG_1$), BLCA-3 (IgM), K-1-21 ($IgG_1$), and BB7.7 ($IgG_{2b}$). BLCA-8 and BLCA-38 recognise antigens on the surface of malignant urothelial cells [36]. BLCA-3 reacts with human blood group A [36], K-1-21 with human free kappa light chains [35], and BB7.7 (American Type Culture Collection, ATCC) with HLA A, B, C and $\beta_2$ microglobulin. MAbs were used as culture supernatants or were purified from ascites by protein A column chromatography.

### *Immunofluorescent staining by an agarose assay*

Cell suspensions from xenografted 2B8 tumours were embedded in agarose before surface immunofluorescent staining, which was carried out as described previously [35]. A minimum of 100 cells was counted.

### *Flow cytometric analysis of antigen expression*

Immunofluorescent staining with BLCA-8, BLCA-38, or K-1-21 (control), followed by fluorescein isothiocyanate conjugated sheep anti-mouse immunoglobulin (FITC-SaMIg) (Silenus Laboratories Pty, Hawthorne, Victoria, Australia) followed by flow cytometric analysis (FACS 440, Becton Dickinson, Mountainview, Calif.), was used to assess the cell surface expression of antigens on cultured 2B8 cells [36].

### *Samarium-153 conjugation of MAbs*

Monoclonal antibody, BLCA-38, was labelled with iodine 131 ($^{131}I$) as described previously [34], or with samarium 153 ($^{153}Sm$) using the bifunctional chelate, benzyl-isothiocyanate-diethylene triamine penta-acetic acid (Mx-DTPA) [4]. The Mx-DTPA was kindly made available by Dr. O Gansow and Dr. M. Brechbiel, National Cancer Institute, NIH, Bethesda, Md. Mx-DTPA was freshly dissolved in ultrapure water and reacted with BLCA-38 in 0.14 M phosphate buffer, pH 8.5, at a Mx-DTPA:MAb molar ratio of 5:1. The mixture was incubated at 37°C for 2.5 h [3]. Free unconjugated Mx-DTPA was separated from Mx-DTPA:BLCA-38 by centrifugal size-exclusion chromatography on Biogel P6-DG (BioRad Richmond, Calif.). One millicurie of $^{153}Sm$-citrate was added to the Mx-DTPA-BLCA-38 conjugate (200 µg) and allowed to incubate at room temperature for 30 min. The $^{153}Sm$-BLCA-38 was further purified by size-exclusion chromatography (Biogel P6-DG) with 0.2 M citrate buffer, pH 7.0, and washed in a centricon-30 concentrator (Amicon) with 0.01 M citrate buffer, pH 7.0, to further remove loosely bound $^{153}Sm$.

### *Biodistribution studies*

Nude rats with palpable tumours in the bladder wall were anaesthetized with ether and injected with $^{131}I$- or $^{153}Sm$-BLCA-38 intraperitoneally (i.p.). Each rat received 80–120 µg protein labelled with 300–500 µCi of isotope. Seven days later, anaesthetized rats were exsanguinated by cardiac puncture and killed by anaesthetic overdose. Tissues and tumours were dissected, weighed and counted using a 1274 Reagamma counter (LKB Wallac, Stockholm, Sweden) tuned to the 360 KeV or 103 KeV gamma emissions of $^{131}I$ or $^{153}Sm$, respectively. Tissue distribution profiles of percent injected dose per gram (%ID/g) and tissue:blood (T:B) were generated using a computer biodistribution programme.

## Results

### *Establishment and characterisation of xenografts in nude rat bladder*

Intramural and subcutaneous inoculation of four nude rats with $10^7$ human bladder carcinoma cells of the 2B8 clone failed to produce tumour growth. For subsequent studies, nude rats were pre-irradiated with 900 rads whole-

**Table 1.** Growth of human bladder cancer cells of clone 2B8 in nude rat bladder following inoculation into the bladder wall

| Number inoculated (passage) | Sex | Time killed (days) | Number with tumours | Number with metastases |
|---|---|---|---|---|
| 2 (P1) | *m* | 128 | 2/2 | 0 |
| 4 (P1) | *f* | 129–140 | 4/4 | 0 |
| 2 (P2) | *m* | 109 | 2/2 | 1 |
| 6 (P2) | *m* | 115 | 6/6 | 0 |
| 8 (P3) | *f* | 121 | 8/8 | 3 |
| 8[a] (P3) | *m* | 91 | 6/6 | 1 |
| 5 (P3) | *f* | 78 | 5/5 | 0 |
| 10[a] (P3) | *f* | 35–105 | 6/7 | 5[b] |

[a] Some rats still alive
[b] In 2 cases, tumour had spread to abdominal wall

body irradiation. The prior irradiation resulted in a high take rate (to date, 39/40 rats [97.5%]) for 2B8 tumours inoculated into the bladder wall (Table 1). Tumour growth was obtained within the bladder wall in 95% of cases, and outside the bladder in two animals. Invasive outgrowth towards the peritoneal cavity was seen in some rats (Fig. 1) which, in two cases resulted in tumour spread to the abdominal wall. Tumours were also found to grow towards the bladder lumen as seen in Fig. 2b, which shows the contiguity of human bladder cancer cells with normal rat transitional urothelial cells. In some instances, the whole bladder became cancerous. In ten rats (25%), metastatic deposits were noted. These were sited in different organs, including liver (Fig. 2f), spleen, mesenteric lymph node, stomach and caecum, within one horn of the uterus, and within a seminal vesicle. In one rat, there was no apparent tumour growth within the bladder itself, but a lymph node in the peritoneal cavity contained tumour deposits, which were detected macroscopically,

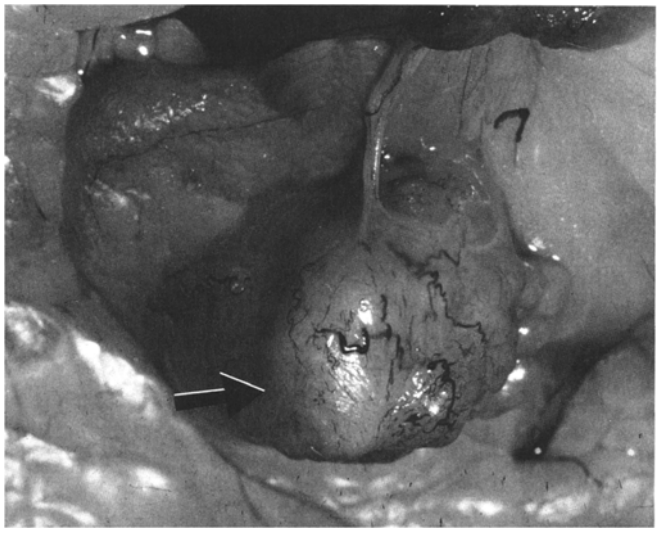

**Fig. 1.** Growth of human bladder cancer cells in the wall of the nude rat bladder

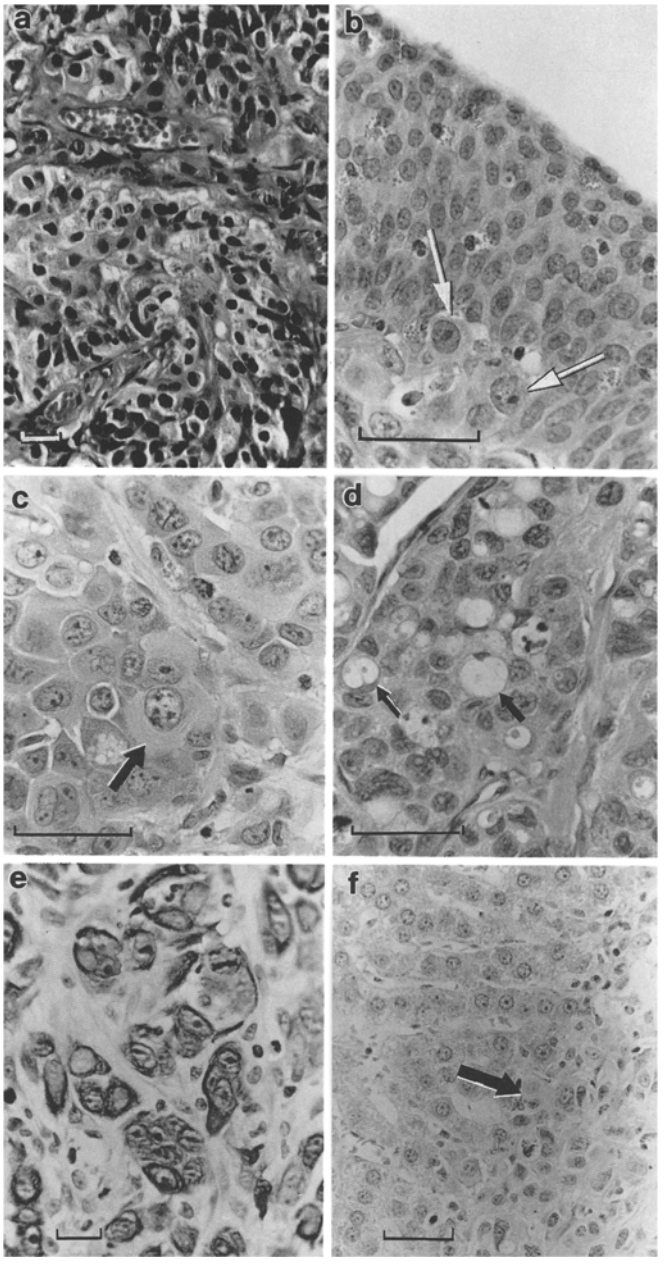


**Fig. 2a–f.** *Bars* represent 25 μM in each case. **a** Transitional cell carcinoma with squamous and adenocarcinomatous differentiation (original tumour). Haematoxylin and eosin, ×280. **b** Human bladder cancer xenograft (*arrow*) in juxtaposition to normal transitional epithelium of nude rat bladder. Haematoxylin and eosin ×860. **c** Area of predominantly squamous differentiation (*arrow*) in xenografted human bladder cancer implanted in rat bladder. Haematoxylin and eosin, ×860. **d** Area of human bladder cancer xenograft in rat bladder showing adenocarcinomatous differentiation with mucin producing cells (*arrows*). Periodic acid-Schiff plus distase, ×860. **e** Area of squamous differentiation showing keratin staining in xenografted human bladder cancer implanted in rat bladder. Callus keratin, ×860. **f** Photomicrograph of rat liver showing metastatic human bladder cancer cells (*arrow*). Haematoxylin and eosin, ×860

histologically, and in localisation studies conducted with $^{153}$Sm-labelled BLCA-38 monoclonal antibody, as described below. In this case, it seems likely that the bladder

Table 2. Surface immunofluorescence reactivity of dissociated human 2B8 xenografts grown in the bladder of nude rats

| MAb | Specificity | Cells positive (%) | | | |
|---|---|---|---|---|---|
| | | Tumour bearing | | Metastatic Seminal Vesicle | Normal Seminal Vesicle |
| | | Bladder 1 | Bladder 2 | | |
| K-1-21 | Kappa light chain | 0 | 0 | 0 | 0 |
| BB7.7 | HLA A,B,C | 88.3 | 82.5 | 83.3 | 1.9 |
| BLCA-3 | Blood group A | 68.5 | 83.7 | 93.8 | 0 |
| BLCA-8 | Tumour antigen | 97.3 | 70.6 | 77.4 | 0 |
| BLCA-38 | Tumour antigen | 59.6 | 13.9 | 0 | 0 |

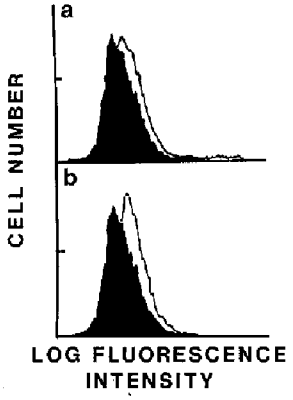


Fig. 3a, b. Surface expression of BLCA-8 and BLCA-38 antigens on the surface of cloned 2B8 cells grown in vitro. The *solid* histogram indicates staining with control MAb, K-1-21, and the *open* histogram represent staining with a BLCA-8 and b BLCA-38, followed by FITC-SaMIg. Surface immunofluorescence staining was analysed by flow cytometry

cancer cells leaked back out of the bladder wall following inoculation.

Histologically, the tumours were transitional cell carcinomas with evidence of both squamous and glandular differentiation (Fig. 2). Thus large cells containing keratin ringing were observed (Fig. 2c), and these were positive for keratin staining (Fig. 2e). In addition, cells containing mucin droplets were widely distributed throughout the tumours, with positive staining for PAS + D, Alcian blue and mucicarmine (Fig. 2d). The biopsy from which the cell line, UCRU-BL-17 CL was established [25, 26] also showed an admixture of TCC, squamous and adenocarcinoma (Fig. 2a), and this pattern was reflected in subcutaneous deposits of 2B8 in nude mice [5]. The 2B8 tumours which grew in the nude rats were confirmed to be of human origin by positive probing of both bladder and metastatic tumour DNA with the human repetitive element Alu, using the plasmid pBLUR8 (Amersham, Little Chalfont, UK) (data not shown). In addition, cells prepared from tumours in the rat bladder and in a seminal vesicle were shown to express HLA class I antigens (see Table 2). Moreover, metaphase spreads of cells cultured after recovery from the rat bladder confirmed that the chromosomes were not acrocentric, indicative of rodent morphology, but were of human origin (data not shown).

As the 2B8 tumours were very hard in texture, passage from one nude rat to another was accomplished by growing tumour cells recovered from the rat bladder in culture to obtain healthy, single cell suspensions for further inoculation. The time taken from inoculation until rats were killed with palpable tumours decreased with increasing passage number through the rats and culture (Table 1).

### *Expression of antigens on 2B8 cells in vitro and in vivo*

The expression of BLCA-8 and BLCA-38 antigens was examined by flow cytometry on clone 2B8 cells maintained in tissue culture (Fig. 3). The expression of both antigens was very low. In contrast, cells dissociated from 2B8 xenografts grown in the bladder wall of nude rats showed strong reactivity with MAbs BLCA-8 and BLCA-38, and expressed both human blood group A antigen and HLA class I (Table 2). A metastasis to a seminal vesicle exhibited a similar profile but failed to express the BLCA-38 antigen. Tissue from a normal seminal vesicle gave negative staining.

### *Localisation of radiolabelled BLCA-38 to xenografts in the bladder wall of nude rats*

Biodistribution studies were carried out on nude rats bearing palpable 2B8 tumours in the bladder wall, 7 days after injection of $^{131}$I- or $^{153}$Sm-BLCA-38. Tissue-to-blood ratios are shown in Fig. 4. In one animal (Fig. 4a), which received $^{131}$I-BLCA-38, two tumour deposits were found to be growing towards the peritoneum from the bladder wall. The larger tumour showed a xenograft-to-blood ratio of 5.04 while the normal bladder-to-blood ratio was only 1.10. The highest uptake in normal tissues occurred in the lung (lung-to-blood; 3.42).

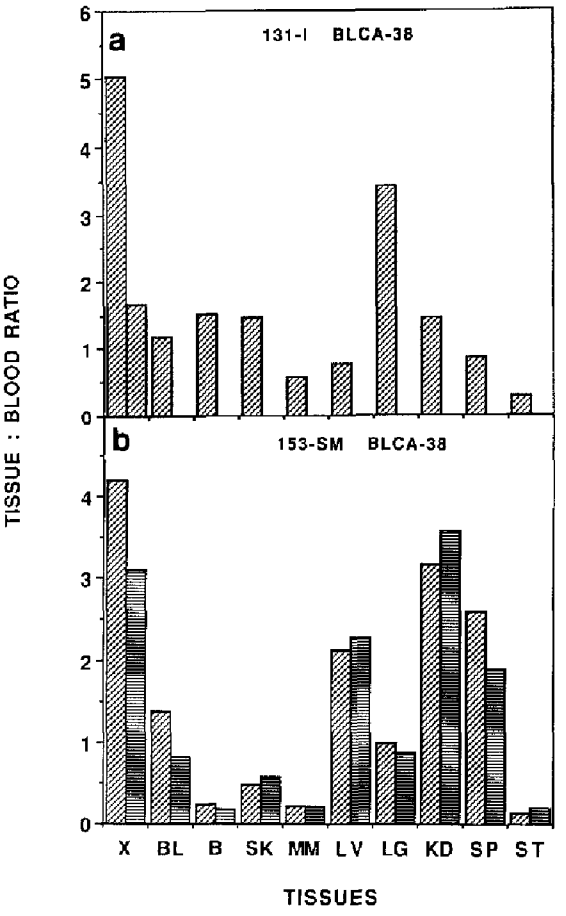


Fig. 4a, b. Biodistribution of BLCA-38 immunoconjugates in nude rats bearing human bladder cancer xenografts in the bladder wall. Nude rats were killed 7 days after i.p. injection of a $^{131}$BLCA-38 (one rat with two tumour deposits in the bladder) or b $^{153}$Sm-Mx-DTPA-BLCA-38 (two rats, one with a tumour in the bladder, one with a tumour in nearby lymph node, but not in the bladder). Tumour: Blood ratios are shown for each organ. *X*, Xenograft; *BL*, bladder, *B*, bone; *SK*, skin; *MM*, muscle; *LV*, liver; *LG*, lung; *KD*, kidney; *SP*, spleen, *ST*, stomach

Two rats were injected with $^{153}$Sm-BLCA-38 (Fig. 4b). One was found to have a tumour in the bladder wall, while the other had a small metastatic deposit in the peritoneum, although no visible tumour mass was apparent within the bladder wall itself. Tumour-to-blood ratios were 4.3 and 3.1, respectively, with tumour uptakes of 3.9 and 2.1 %ID/g. In contrast to the results with $^{131}$I-BLCA-38, there was appreciable uptake of label in the moncyte phagocyte system (liver and spleen), as well as high kidney activity.

## Discussion

Our aim is to develop immunoconjugates suitable for intravesical treatment of human bladder cancer. In this study, we describe a new animal model in which the therapeutic efficacy of this approach can be assessed.

The growth of TCC of the bladder as subcutaneous xenografts in nude or immune-deprived mice has previously been validated for studies of tumour biology [15, 28], including examination of chromosomal patterns and ploidy changes [27], radiobiology of bladder cancer [32] and drug-radiation interactions [17]. Such a model may also be used for examining the tumour localisation, biodistribution and therapeutic efficacy of systemically administered antibody conjugates ([18]; Walker KZ, Boniface GR, Lightfoot DV, Ormsby S, Izard ME, Parkes SL, Weedon A, Russell PJ, unpublished results). However, it is not suitable for a study of intravesical administration, which must be the route of choice in patients with superficial bladder cancer.

A rat model has evident advantages in terms of size for such an approach [29]. Rat bladder carcinomas can be induced with carcinogens such as methylnitrosourea [32], benzidine derivates [33] or N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) [8], but human tumours are more appropriate targets for immunoconjugates designed for clinical use. We thus followed the approach of Ahlering and colleagues [1], who had shown that intravesical inoculation of human bladder cancer cell lines into the bladder wall of nude mice resulted in tumour growth. Intramural inoculation of 2B8 cells into the bladder wall of irradiated nude rats was found to produce local tumour growth and invasion and metastasis to different organs (Table 1).

Both the metastatic deposits and the primary tumours in the bladder wall (Fig. 2) showed an admixture of cells with adenocarcinomatous and squamous patterns of differentiation (Fig. 2). This appearance was also characteristic of both the original human biopsy (Fig. 2a) and the UCRU-BL-17CL line [27] which was initially established in vitro, possibly from a pluripotent epithelial stem cell [26].

The tumourigenic conversion of host cells has been described as a rare occurrence following the introduction of human tumour cells into immune-deficient hosts [12, 29]. However, the tumours produced in the nude rats were shown to be of human origin by Alu probing, HLA expression (Table 2) and chromosomal analysis.

The appearance of human blader tumour metastases described here in nude rats after introduction of tumour cells into the bladder supports the "seed and soil" hypothesis [11, 22] that both host and tumour cell factors are important in determining metastatic spread. Our results are thus in agreement with findings for other tumour types – that implantation into the organ of origin of the tumour is more likely to result in the development of metastases [20, 21]. In contrast, human tumours of seven different origins implanted subcutaneously into immunodeficient Wistar rats [13], and 2B8 cells implanted s.c. in nude mice [5] did not induce metastases.

A number of MAbs reactive with antigens on the cell surface of human bladder cancer have been described (for review, see [19]). Recently, we have made two murine MAbs, BLCA-38 ($IgG_1$), and BLCA-8 ($IgG_3$) [36], which react with urothelial cells shed into the urine of patients with TCC of the bladder. Both antigens recognised by these antibodies are weakly expressed by 2B8 cells grown in vitro (Fig. 3), yet strongly expressed in 2B8 xenografts in nude rats (Table 2). The reason for antigen induction in

vivo is not yet known and is the subject of further study in our laboratory.

BLCA-38 immunoconjugates, radiolabelled with either $^{131}I$ or $^{153}Sm$ were found to localise to tumours after intraperitoneal injection into nude rats (Fig. 4). The $^{153}Sm$ was chelated to the BLCA-38 antibody with Mx-DTPA, a chelating agent of high metal-binding affinity which minimises label deposition in osseous bone and consequent bone marrow toxicity [16]. Both $^{131}I$ and $^{153}Sm$ are radionuclides with concomitant gamma and beta emission, which will allow radiotherapy to be monitored by scintigraphy [3]. In earlier studies we found that both $^{131}I$-BLCA-38 and $^{153}Sm$-BLCA-38 localised to and had therapeutic effects on subcutaneous UCRU-BL-17 xenografts grown in nude mice ([18]; Walker et al., unpublished data). The present study has indicated that tumour localisation will occur after intraperitoneal administration of these immunoconjugates into nude rats bearing intramural xenografts. Given that the tissue penetration from $^{153}Sm$ is approximately 2.0 mm [9], it is possible that, despite the submucosal site of the tumour, topical uptake of the antibody will also occur. This would thus create a useful model for the study of intravesical therapy. Alternatively, further manipulation of the model, such as scarification of the urothelium under anaesthesia to allow tumour take following intravesical inoculation of tumour cells could facilitate these studies. Whatever nuances of experimental study are required, this model will have useful application to the study of the biology of bladder cancer. As the implantation of human bladder cancer cells in the nude rat bladder is associated with the development of metastases, the model will also serve for preclinical studies on the efficacy of systemically injected radioimmunoconjugates for control of metastatic growth.

*Acknowledgements.* We thank Dr. Gladstone McDowell, III, Department of Tumor Biology, M. D. Anderson Cancer Center, Houston, Texas, for demonstrating the technique for intramural inoculation, Ms. Joanne Brown, Ms. Janelle Hanley, Ms. Zambela Palavidis, and Mr. Sean Grimmond, Urological Cancer Research Unit, Royal Prince Alfred Hospital, for providing us with the 2B8 clone, assistance with the nude rats, assistance with cell culture, and for Alu probing, respectively, and Ms Robyn Lukeis, Cytogenetics Department, St. Vincents' Hospital, Victoria, Australia, for cytogenetic analysis. We also thank Dr. M. Brechbiel and Dr. O. Gansow, National Cancer Institute, NIH, Bethesda, Md., for providing the bifunctional chelate, benzyl-isothiocyanate-diethylene triamine penta-acetic acid for these studies, and Dr. R. Garsia, Department of Clinical Immunology, Royal Prince Alfred Hospital, for helpful discussions.

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