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BRM immunotherapy of orthotopically implanted murine bladder tumours: Treatment response monitoring by MRI

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SA KADHIM, JL CHIN, BM GARCIA, et al. BRM immunotherapy of orthotopically implanted murine bladder tumours: Treatment response monitoring by MRI. Can J Infect Dis 1992;3(Suppl B):143B-148B. The authors evaluated magnetic resonance imaging (MRI) for monitoring orthotopic bladder tumour growth and treatment response to intravesical immunotherapy with the biological response modifiers (BRMs): recombinant tumour necrosis factor alpha (TNF-α), combination of TNF-α plus interferon gamma (IFN-γ) and interleukin-2 (IL-2). MRI demonstrated detection of early superficial murine bladder tumour (MBT-2) and accurate sequential assessment of the topography and depth of intravesical tumour involvement. Response to intravesical instillations with multiple doses of BRMs was assessed against early stage MBT-2 bladder tumours (confirmed by MRI) 14 days after transurethral tumour implantation. Serial MRI scans of TNF-α treated mice revealed significant retardation of tumour growth which correlated well with corresponding histological examination of the whole mount bladder sections illustrating areas and depth of tumour regression. Intravesical instillation of combination TNF-α plus IFN-γ into tumour-bearing mice caused tumour growth inhibition up to 21 days following treatment; the results, however, were not superior to those noted with TNF-α alone. Sequential MR images of tumour-bearing bladders following intravesical treatment with IL-2 revealed tumour regression with no visible tumour from day 21 to 33 post tumour implant. Histological examination revealed foci of carcinoma in situ only. Control untreated bladders revealed deeply invasive transitional cell carcinoma. These results show that MRI offers a dependable tool for noninvasive monitoring of tumour growth and of the course of experimental bladder tumour during therapy.

Key Words: *Interferon gamma, Interleukin-2, Magnetic resonance imaging, Orthotopic murine bladder tumour, Tumour necrosis factor alpha*

Les modificateurs de ls réponse biologique dans l'immunothérapie des tumeurs vésicales murines: Survi de la réponse au traitement par résonance magnétique

RÉSUMÉ: Les auteurs ont évalué la valeur de la résonance magnétique (RM) dans la surveillance des tumeurs vésicales implantées par voie orthotopique et la réponse à l'immunothérapie intravésicale utilisant les modificateurs de la réponse biologique (BRM), incluant le facteur de nécrose tumorale alpha recombinant (TNF-alpha), ainsi que l'association de TNF-alpha, d'interféron gamma (INF-gamma) et d'interleukine-2 (IL-2). La RM a permis de déceler des tumeurs superficielles au stade initial (MBT-2) chez la souris et d'évaluer correctement la répartition topographique et la profondeur de l'atteinte tumorale. La réponse a

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des doses multiples de BRM a été mesurée au premier stade d'évolution des MBT-2 (confirmées avec RM), 14 jours après l'implantation tumorale par voie transurétrale. La RM en série des souris traitées par TNF-alpha a révélé une croissance significativement retardée qui corrélait avec l'examen histologique correspondant du montage de toutes les sections de la vessie illustrant les secteurs et la profondeur de la régression tumorale. L'injection intravésicale de TNF-alpha et d'IFN-gamma administrés en association chez les souris porteuses de tumeurs a entraîné un ralentissement de croissance qui a duré jusqu'à 21 jours après le traitement. Cependant, ces résultats ne sont pas meilleurs que ceux qu'on a notés avec le TNF-alpha administré seul. La RM en série des vessies de souris malades traitées par l'IL-2 a révélé une régression tumorale et la disparition des tumeurs visibles entre les jours 21 et 33 après l'implantation. L'examen histologique a montré des foyers de carcinome préinvasif ou in situ seulement. Dans le groupe témoin non traité, les vessies étaient atteintes de carcinome transitionnel avec tendance envahissante en profondeur. Ces résultats démontrent que la RM est un instrument fiable qui permet de surveiller la croissance des tumeurs non-invasives et l'évolution, au cours du traitement, des tumeurs vésicales expérimentales.

APPLICATIONS OF MAGNETIC RESONANCE IMAGING (MRI) IN the genitourinary area have been extensive, and specialized techniques have been developed for such tissues as the bladder and prostate (1,2). MRI now is being used clinically for staging various urological malignancies at some centres (2-4). This technique has the advantage of obtaining images by a noninvasive approach in direct sagittal, coronal and transverse planes. The unique tissue contrast allows clear differentiation between normal bladder wall and neoplastic tissue revealing both mural and intraluminal tumour involvement (4,5). Comparative studies of the image quality, anatomic visualization and accuracy of diagnosis made by MRI and computed tomography (CT) show a clear advantage of MRI over CT, particularly with regard to its contributions to therapeutic strategies (6). Early in vitro studies by Damadian et al (7) demonstrated distinctive MR properties of malignant tissue compared with nonmalignant tissue in experimental animals.

Recently, the current researchers established an MRI technique for documenting intravesical tumour implants in the orthotopic murine bladder tumour (MBT-2) model (8). This method circumvents traditional reliance on late clinical signs of bladder tumours (palpable mass, hematuria, cachexia) or on final autopsy findings. The authors have used this model to assess intravesical growth of MBT-2 and the antitumour effect of several biological response modifiers (BRMs) given intravesically: tumour necrosis factor-alpha (TNF-α) alone, TNF-α in combination with interferon gamma (IFN-γ) or interleukin (IL)-2 alone. This therapeutic regimen was chosen because of the positive synergistic effect between the BRMs and their dual mode of action directly on cancer cells and indirectly through 'immune' effector cells (9).

ANIMALS AND METHODS

Tumour implantation: FANFT-induced (N[4-(5-nitro-2 furyl)-2 thiozolyl] formamide) MBT-2 transitional cell carcinoma of the bladder (originally derived by Dr M Soloway [10]) was maintained in vivo as a solid subcutaneously growing tumour by serial transplantation in syngeneic C3H/He female mice (Charles River) and in vitro in RPMI 1640 medium plus 10% fetal calf serum. Single tumour cell suspension was prepared by mincing the tumour under sterile conditions and adding 5 mL of an enzyme cocktail containing a mixture of 1% collagenase (Sigma, type II), 0.01% proteinase K (Sigma) and 0.01% DNAase (Sigma) in Dulbecco's phosphate buffered saline combining calcium and magnesium salts to the minced tissue. The cells were then washed twice with RPMI 1640 medium, resuspended to the required concentration and implanted into the bladders of female mice via the urethra according to the procedure previously described (11). For tumour implantation, the mice were anesthetized with a single dose of intraperitoneal sodium pentobarbital (130 mg/kg body weight). The bladder was catheterized via the urethra with a 24-gauge plastic intravenous cannula under sterile conditions. The bladder mucosa was then traumatized by instillation of 0.1 mL 0.1 N hydrochloric acid solution for 15 s, neutralized with 0.1 mL 0.1 N potassium hydroxide and flushed with sterile saline. A total of $5x10^5$ MBT-2 cells were instilled via the cannula and the urethra was compressed for 30 mins to prevent premature bladder evacuation. Six days later the animals were assessed by MRI to detect intravesical tumours.

Magnetic resonance imaging protocol: Anesthetized mice were placed in an 80 MHz volume imaging coil and imaged using a Bruker MS 1.9/30 MR imager (Bruker Spectrospin Canada Ltd). Whole animals were scanned transversely (four continuous, 3 mm slices) and sagitally (four 5 mm slices, 2.5 mm gap) using T_1-weighted spin echo sequences. The animals were repeatedly imaged with a 1 mL syringe containing 1:100 dilution of gadolinium-diethylenetriamine pantaacetic acid (DPTA) in saline as a fiducial marker. The syringe positioned vertically over the urethra was used as an external marker for repetitive positioning in a 35 mm inner diameter loop gap resonator. To delineate bladder tumour tissue from normal bladder and normal abdominal contents, high-contrast images were obtained by using a bladder inflation technique using neat gado-

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linium-DPTA (a paramagnetic MRI contrast agent [Magnevist; Berlex Canada Inc]) specific volume (0.1 mL) of fluid was used to inflate the bladder. Thus, the tumour in the bladder appeared as a signal-containing structure outlined against a black negative background.The animals were serially imaged from day 6 to 33 after tumour implantation. Mice with clinically palpable tumours and normal untreated mice were used as the 'tumour-bearing control' group and 'normal control' group, respectively, for MRI. Mice with demonstrable early-stage (MRI-detected) intravesical tumours were subsequently used for monitoring of treatment response to intravesical therapy.

Histology: Mice were sacrificed after completion of intravesical BRM therapy, and cystectomy was performed. Histological examination was performed with hematoxylin-phloxine-saffron staining on formalin-fixed whole bladder sections from the cystectomy specimen. The bladders were examined for tumour incidence, size, and histological grade and stage. All light microscopy sections were examined and correlated with MRI findings. Bladder washing specimens were collected for cytology from animals serially imaged by MR throughout the study.

Biological response modifiers: Recombinant human TNF-α (specific activity 4.75×10^7 units/mg) and recombinant human IFN-γ (specific activity 1.9×10^7units/mg) was donated by Genentech Inc (California). Recombinant human IL-2 (specific activity 1.8×10^6units/mg) was donated by Cetus Corporation (California).

Biological response modifiers intravesical immunotherapy: The effectiveness of intravesical BRM immunotherapy was evaluated on the growth of early-stage established MBT-2 tumours confirmed by MRI 14 days post tumour implantation. BRMs were instilled intravesically by urethral catheterization for 30 mins.

Group 1 (control untreated, n=10), bladders instilled with 0.1 mL saline; group 2 (TNF-α treated, n=13), bladders instilled with TNF-α alone (5×10^5 U every other day for a total of seven doses); and group 3 (IL-2 treated, n=11), bladders instilled with IL-2 alone (1×10^6 U every other day for a total of nine doses).

Representative serial MRI scans of one animal from each group were selected to illustrate the consistent findings of this study. Treatment response to intravesical TNF-α and IFN-γ was assessed in a separate study on mice with established orthotopic MBT-2 tumours (confirmed by MRI) 14 days after tumour implantation. Animals were instilled with TNF-α alone (5×10^5 U per mouse n=13), IFN-γ alone (5×10^5 IFN U per mouse, n=13) or TNF-α and IFN-γ (5×10^5 U plus 5×10^5 U per mouse, n=13) every other day for nine doses. Control untreated mice (n=8) were instilled with saline alone. The treated mice were sacrificed on day 21 after tumour implantation and their bladders were processed in the described manner.

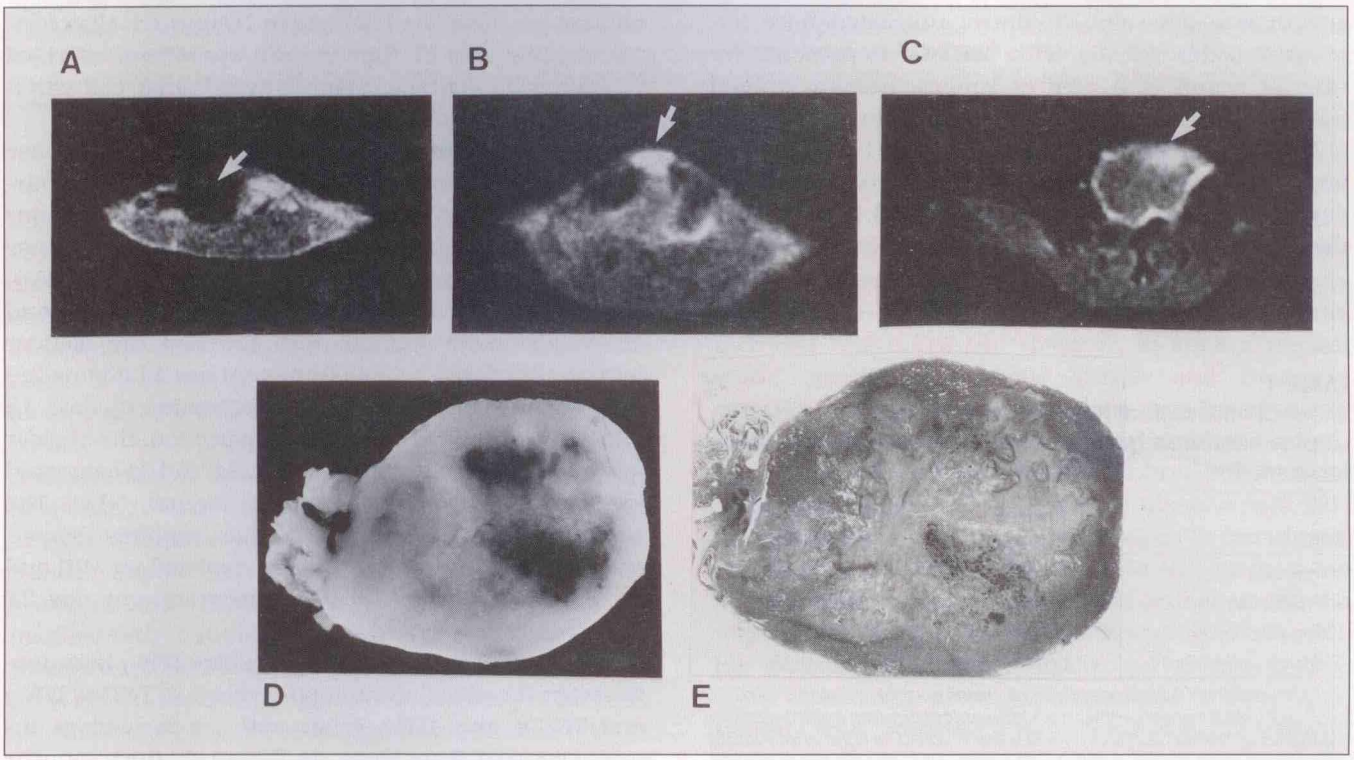

Figure 1) *Serial magnetic resonance imaging (MRI) and histopathology examination of control untreated bladder from mouse 1.* **A** *MRI bladder six days post tumour implant, no visible tumour (arrow);* **B** *MRI Bladder partially filled with tumour (arrow) 18 days post tumour implant;* **C** *MRI bladder completely filled with tumour (arrow) 21 days post tumour implant;* **D** *Corresponding gross mount on day 21;* **E** *Corresponding light microscopy on day 21*

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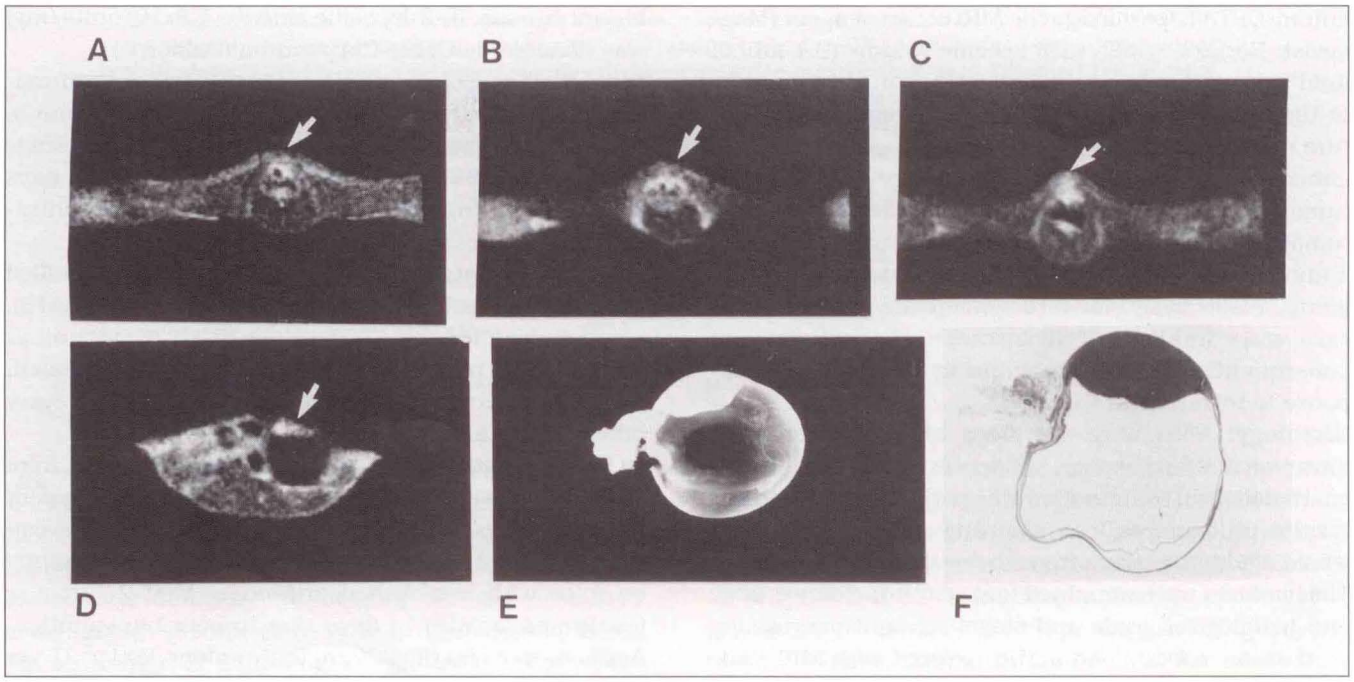

Figure 2) *Serial magnetic resonance imaging (MRI) and histopathology examination of mouse 2 treated with seven doses of tumour necrosis factor-alpha intravesically.* **A** *MRI bladder partially filled with tumour (arrow) 14 days post tumour implant;* **B** *MRI bladder at 18 days post tumour implant;* **C** *MRI bladder at 21 days post tumour implant showing limited tumour;* **D** *MRI bladder at 21 days post tumour implant at a different transverse level showing limited tumour;* **E** *Corresponding gross pathology at day 21;* **F** *Corresponding light microscopy on day 21, note significant growth retardation compared with control (mouse 1)*

RESULTS

Assessment of intravesical tumour growth in control untreated mice: MRI monitoring of intravesical tumour growth was done on 10 control untreated mice implanted orthotopically with MBT-2. Representative sagittal scans of a control tumour-bearing animal (mouse 1) are shown in Figure 1 which illustrates serial MR images of mouse 1 at day 6, 18 and 21 post tumour implant. No visible tumour was detectable on MRI at day 6 (Figure 1A). When imaged at day 18, MRI revealed the bladder partially filled with tumour [Figure 1B]. By day 21, the bladder was completely occupied with tumour (Figure 1C). The corresponding gross mount and light microscopy for mouse 1 at day 21 post tumour implant are illustrated in Figures 1D and 1E. Histological examination of the tumour specimens revealed tumour cells characteristic of transitional cell carcinoma of the bladder.

Assessment of intravesical TNF-α therapy: Response to TNF-α was monitored by serial MRI of 13 tumour-bearing mice at 14, 18 and 21 days after tumour implantation. Serial MR images of representative mouse 2 following seven intravesical instillations of TNF-α, ($5x10^5$ U) are shown in Figure 2. Imaged at days 14 and 18 post tumour implant, MRI revealed the bladder partially filled with a small tumour at day 14 (Figure 2A) with evidence of partial tumour regression by days 18 and 21 (Figures 2B-D). By visual inspection, the bladder was less extensively involved at day 18 and 21 compared with untreated controls (mouse 1, Figures 1B,C). The corresponding whole mount bladder sections demonstrated limited tumour growth as depicted on MRI and correlated well with the MRI appearance at day 21 (Figures 2E,F).

Assessment of intravesical TNF-α plus IFN-γ immunotherapy: Results illustrating the effects of TNF-α, IFN-γ and TNF-α plus IFN-γ treatments on early-stage intravesical MBT-2 are shown in Table 1. In the treatment groups of mice instilled with multiple doses of either TNF-α alone ($5x10^5$ U) or TNF-α plus IFN-γ ($5x10^5$ U plus $5x10^5$ U), a significant difference in bladder tumour

TABLE 1
Effect of intravesical tumour necrosis factor alpha (TNF-α) plus interferon gamma (IFN-γ) on established orthotopic murine bladder tumour-2

BRMs[§]	Tumour incidence**	Tumour weights** (mean±SD)
TNF-α alone	4 (13)[†]	0.104±0.02[†]
IFN-γ alone	5 (13)	0.468±0.34
TNF-α plus IFN-γ	2 (13)[†]	0.182±0.02*
Control untreated[¶]	5/8	0.457±0.35

**P<0.05 and [†]P<0.01 by Student's t test versus control untreated; [†]P<0.05 by χ^2 test versus control untreated; [§] Intravesical bladder instillations with biological response modifiers (BRMs) were initiated 14 days after tumour implantation. Mice were instilled with either TNF-α ($5x10^5$ U), IFN-γ alone ($5x10^5$U) or TNF-α plus IFN-γ ($5x10^5$ U plus $5x10^5$ U) every other day for a total of nine doses. Control mice instilled with 0.1 mL saline alone. ** Tumour incidence and weights determined at day 21 post tumour implant, expressed as number of mice with tumours (total number of mice)*

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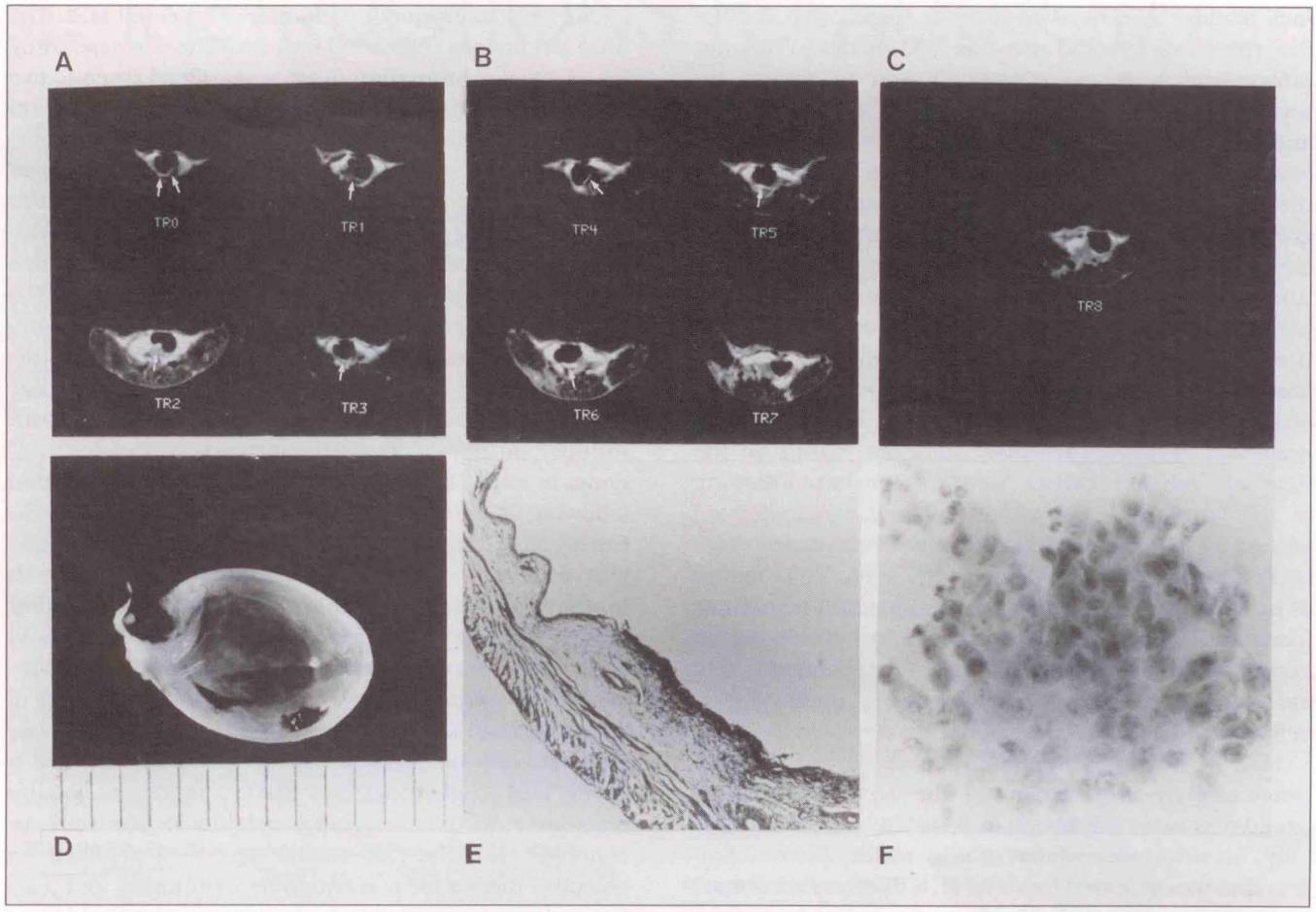

Figure 3) *Serial magnetic resonance imaging (MRI), histopathology and cytology examination of mouse 3 treated with nine doses of interleukin-2 intravesically.* **A-C** *MRI bladder partially filled with tumour (arrow) from treatment 1 (TR0) at day 0 (14 days post tumour implant) to treatment 9 (TR8) at day 8 (33 days post tumour implant). No evident tumour by MRI at TR7 and TR8;* **D** *Corresponding gross pathology at day 33;* **E** *Histology showing normal epithelium with foci of carcinoma in situ;* **F** *Cytology of the bladder washings collected at day 33 showing massive eosinophil infiltration*

incidence and tumour weights ($P<0.05$ to $P<0.01$) was observed in comparison with control untreated mice at day 21 after implantation. However, combined TNF-α and IFN-γ treatment did not result in an enhanced antitumour effect compared with TNF-α treatment alone. Instillation of tumour-bearing mice with IFN-γ alone ($5x10^5$ U) had no effect on tumour growth retardation.

Assessment of intravesical IL-2 immunotherapy: Response to intravesical IL-2 was monitored by serial MRI of 11 tumour-bearing mice from day 14 to 33 after tumour implantation (Figures 3A-C). Sequential MRI scans of IL-2 treated mice (representative mouse 3 instilled with nine sequential doses of $1x10^6$ U) revealed tumour regression with no visible tumour between day 31 to 33 post tumour implant (Figures 3B,C). This was confirmed by gross morphological examination (Figure 3D). Histological examination revealed mostly normal epithelium with foci of carcinoma situ only (Figure 3E). Cytology of the bladder washings collected at day 33 showed massive eosinophil infiltration (Figure 3F).

DISCUSSION

The orthotopic MBT-2 model has been used to evaluate intravesical therapy with a number of immunoadjuvants and BRMs, eg, Bacillus Calmette Guerin, IL-2 and TNF-α (12-14). However, in such a tumour model monitoring tumour growth and treatment response to intravesical therapy has been based mainly on the final endpoints, ie, examination of cystectomy specimens at autopsy or on crude signs such as diminution of palpable suprapubic mass, weight gain and improvement in animal well-being. To circumvent the reliance on clinical symptoms an MRI method was established for accurate detection of orthotopic tumour implants in the experimental orthotopic MBT-2 model (8). This technique is noninvasive and allows long term serial assessment of treatment response to intravesical therapy with potential anticancer agents. The noninvasive approach has the advantage of obtaining images in direct sagittal, coronal and transverse planes in the same animal over an extended period. The unique tissue contrast allows clear differentiation between nor-

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mal bladder wall and neoplastic tissue (4,5,7). The described experiments use this MRI model to monitor intravesical tumour progression and to assess intravesical BRM therapy with TNF-α, TNF-α plus IFN-γ and IL-2 against early-stage orthotopic MBT-2. Bladder carcinomas may respond well to intravesical agents if the treatment is initiated at an early stage of tumour growth. Experimental immunotherapy theoretically should be more effective on early superficial bladder tumours than on advanced tumours because of a higher chance of complete tumour regression when the tumour burden is smaller. Experimental therapy studies have been conducted either on animals with obvious tumours (usually advanced) or on animals presumed to have superficial tumours (based on the time interval after transurethral tumour implantation). In the latter case, the validity of any conclusion on efficacy of experimental agents is doubtful since there is no accurate documentation of the presence or extent of tumour prior to therapy. The mouse MRI orthotopic model has a close analogy to the clinical situation and allows accurate detection of early stage tumours and assessment of therapeutic responses noninvasively before the onset of clinical signs.

MRI 14 days post implantation confirmed the presence of early-stage tumours – the detection of which would otherwise have been impossible by clinical signs only. Accurate assessment of intravesical tumour progression was achieved by serial MRI that was confirmed by gross pathology and histological examination of the corresponding whole mount bladder sections. Effects of BRMs were subsequently monitored by serial MRI. The final MR images correlated well with the corresponding histological examination of mice sacrificed three to five weeks post implant. The topography and depth of tumour invasion were shown on MRI. The animals tolerated well repeated anesthesia and imaging, and had no apparent side effects from the intravesical BRM therapy. TNF-α treated mice showed tumour retardation up to day 21 compared to control untreated mice which had deeply invasive tumours. TNF-α plus IFN-γ had similar effect to TNF-α alone. IFN-γ had no significant antitumour effect. Daily IL-2 treatment resulted in complete tumour regression with 'normal' bladders on MRI between days 21 and 33 post implant. Autopsy on day 33 revealed microscopic foci of carcinoma in situ. The observed tumour growth retardation following BRM therapy was significant but transient. Intravesical instillation with multiple doses of BRMs was required to sustain continuous tumour growth inhibition. Neither tumour growth impedance nor other survival benefits were realized after longer periods of observation upon therapy withdrawal. In the researchers' earlier study of the antitumour effects of TNF-α against MBT-2, accelerated tumour growth was reported despite administration of additional TNF-α doses (15), indicating that MBT-2 tends to acquire resistance to the exogenous cytokines. The authors conclude that the MRI model described provides an objective means for noninvasively monitoring and testing antitumour agents which may eventually lead to clinical trials.

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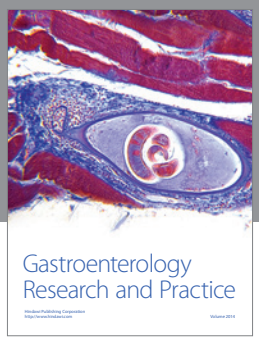

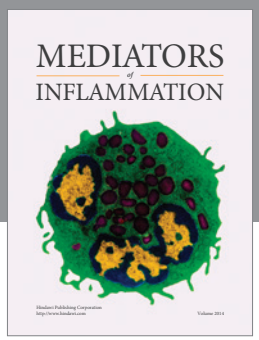

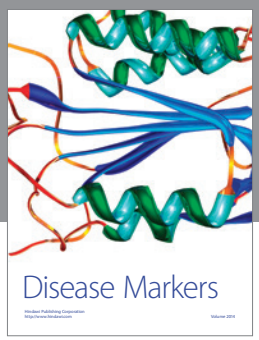

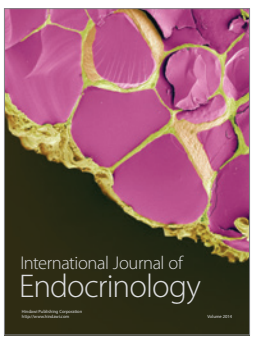

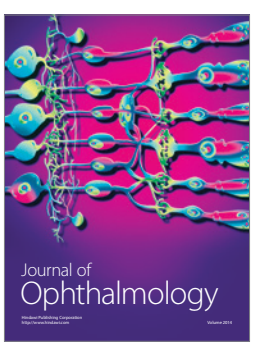

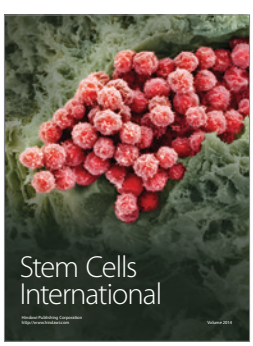

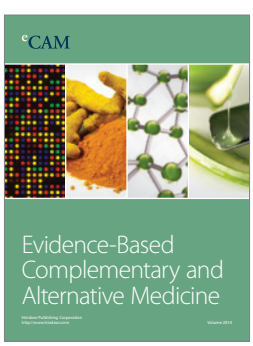

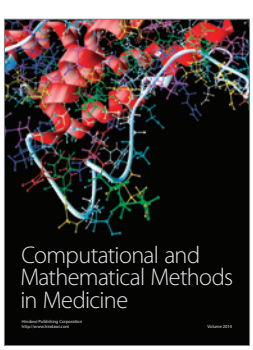

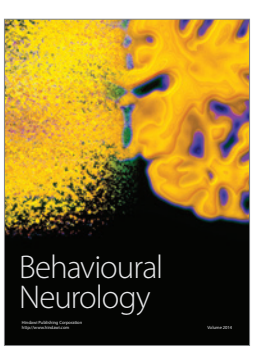

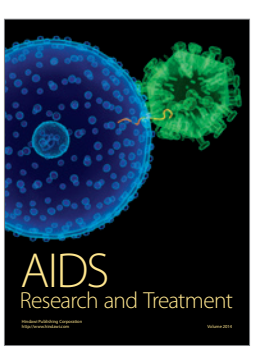

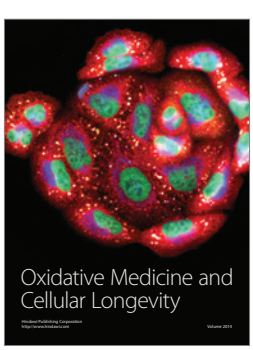

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