Urol Res (2001) 29:13–19

ORIGINAL PAPER

Matthew S. Simms · Andrea Murray · Graeme Denton
David P. Scholfield · Michael R. Price
Alan C. Perkins · Michael C. Bishop

Production and characterisation of a C595 antibody-^{99m}Tc conjugate for immunoscintigraphy of bladder cancer

Received: 10 May 2000 / Accepted: 11 September 2000

Abstract Current radiological techniques for staging bladder cancer are inaccurate, especially in the identification of pelvic lymph node metastases. Immunoscintigraphy has the potential to offer improved staging for bladder cancer. The aim of this study was to label the anti-MUC1 monoclonal antibody C595 with ^{99m}technetium (Tc), the most widely used diagnostic radionuclide, and assess the potential of the resultant conjugate for intravenous immunoscintigraphy of bladder cancer. A direct, reduction-mediated technique was used to label the antibody. The resultant conjugate was shown to be highly immunoreactive, stable and bound specifically to MUC1. The ability of the conjugate to bind to bladder tumours was demonstrated in an ex vivo model where the mean tumour:normal urothelial uptake was 5.7:1 and by intravesical administration in patients with bladder cancer where the mean tumour:normal urothelial uptake was 20.4:1. The ability of the conjugate to localise MUC1-expressing tumours was demonstrated in a nude mouse xenograft model. A conjugate of ^{99m}Tc-C595 has been produced and characterised, and it may be suitable for intravenous immunoscintigraphy, a potential novel staging tool for bladder cancer.

Key words Bladder cancer · Staging · Immunoscintigraphy · MUC1

M. S. Simms (✉) · D. P. Scholfield · M. C. Bishop
Department of Urology, Nottingham City Hospital,
Hucknall Road, Nottingham NG5 1PB, UK
e-mail: msimms@gertrude42.freeserve.co.uk
Tel.: +44-115-9818665; Fax: +44-115-9627791

A. Murray · A. C. Perkins
Department of Medical Physics,
Queen's Medical Centre Nottingham, UK

G. Denton · M. R. Price
Cancer Research Laboratories, Pharmaceutical Sciences,
University of Nottingham, UK

Introduction

Current radiological techniques for staging bladder cancer are inaccurate. Immunoscintigraphy is the detection of a tumour after the administration of conjugates of antibody and γ-emitting radionuclides with γ-scintigraphy and has the potential to improve staging in bladder cancer.

C595 is an IgG3, murine monoclonal antibody raised against the protein core of human urinary epithelial mucin (MUC1) [8]. Epitope mapping has shown that C595 recognises a tetrapeptide motif (RPAP) within the protein core of MUC1 mucin [7]. The aim of this study was to label the monoclonal antibody C595 with ^{99m}technetium (Tc), the most widely used diagnostic radionuclide, using a direct, reduction-mediated technique and assess its suitability for the immunoscintigraphy of bladder cancer.

Materials and methods

Antibody

C595 (also known as NCRC48) was prepared using conventional hybridoma technology and purified from tissue culture supernatant by peptide epitope affinity chromatography. It was shown to be homogenous by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Antibody labelling

A direct, reduction-mediated technique was used to label the antibody [6]. The antibody was initially brought to a concentration of 10 mg/ml by centrifuge ultrafiltration in a centrifuge (Millipore Ultrafree Biomax 50 kDa membrane; Sigma). 2-Mercaptoethanol (2-ME; Sigma Chemicals) was added to the antibody at a molar ratio of 1,000:1 and the mixture was agitated at room temperature for 30 min. Excess 2-ME was eluted from the mixture using phosphate-buffered saline (PBS) by gel filtration in a PD-10 column (Amersham Pharmacia Biotech; Uppsala, Sweden) in 1-ml fractions. Aliquots of the reduced antibody were stored in 0.5-mg fractions at −80 °C until labelling. For the labelling procedure, a medronate (MDP) bone scanning kit (Nycomed Amersham, UK)

was reconstituted in 5 ml of normal saline. One-hundred microlitres of this was added to the thawed antibody aliquot and the mixture was stirred. An appropriate amount of pertechnetate was added and the mixture was agitated at room temperature for 10 min. The conjugate was separated from free pertechnetate using a PD-10 column (Pharmacia).

Immunoreactivity

The immunoreactivity of the resultant conjugate was assessed using the following technique. Labelled antibody was diluted to 100 ng/ml in PBS containing 1% bovine serum albumin (BSA); 0.5-ml aliquots of this were added to increasing concentrations of Sepharose (Pharmacia) beads coated with a synthetic peptide epitope domain APDTRPAPG. The amounts of coated beads were 25, 50, 100 and 200 µl, mixed correspondingly with 175, 150, 100 and 0 µl of uncoated Sepharose and made up to a total volume of 1 ml with PBS-1% BSA in a screw-cap Ependorf tube. The tubes were incubated at 4 °C for 4 h. The antigen Sepharose was then separated by centrifugation in a microcentrifuge. Count rates were then measured in 0.8 ml of supernatant and pellet and in 0.2 ml of supernatant using a γ counter (LKB Wallac 1282). The percentage of activity bound in each tube was calculated using the equation:

$$\text{Bound activity} = [\text{counts per minute (cpm) of 0.8 ml of pellet/supernatant}] - [4 \times \text{cpm in 0.2 ml of supernatant}].$$

The proportion of labelled antibody counts that bound to antigen, assuming antigen excess, was determined from a reciprocal plot of (1/fraction bound) against (1/antigen concentration); an extrapolation of binding to (1/antigen concentration) was made according to the method of Lindmo et al. [4].

Stability

The stability of the conjugate was assessed by serial instant thin-layer chromatography (ITLC) over a 24-h period. The labelled antibody was left in PBS over this period. ITLC was performed on 6-cm silica gel strips using 0.9% sodium chloride. In addition, the immunoreactivity was assessed immediately and at 24-h post-labelling. The antibody was diluted in PBS-1% BSA during this time.

Gel chromatography

A 90 × 1.6-cm column was packed with Sephacryl S-300. The column was pre-equilibrated with PBS at 4 °C. Initially, a molecular weight (MW) marker of 150 kDa (alcohol dehydrogenase; Sigma Chemicals) was passed through the column in order to estimate the elution volume for whole antibody (MW = 150 kD). The column was eluted in 5-ml fractions. Gel filtration chromatography was performed on labelled antibody (1 mg labelled with 25 MBq ^{99m}Tc) and on ^{99m}Tc-labelled MDP. Individual fractions were counted using a γ counter (LKB Wallac) to determine the location of any radioactive peaks.

Ex vivo model

The ability of the conjugate to target bladder tumours was tested in an ex vivo model using fresh cystectomy specimens ($n = 5$), which were obtained from patients undergoing radical cystectomy for bladder cancer. A solution of labelled antibody [0.5 mg labelled with 88–121 MBq (mean = 104 MBq)] in 50 ml of normal saline was instilled into the bladder via a urethral catheter. This was left for 1 h. During this time the position of the specimen was changed in such a way as to ensure that the solution came into contact with all of the bladder mucosa. This was followed by washout of the bladder with 3 × 50-ml volumes of normal saline. Immunoscintigraphy was performed using an IGE Starport γ camera (IGE, Slough, UK). The bladder was then opened. The position of the tumour within the bladder was noted and biopsies were taken from three of the bladders, along with separate biopsies of normal urothelium using a scalpel. Mucosa included in the biopsy was teased from any underlying muscle and the specimens were blot-dried and weighed. Biopsies were counted in a γ counter (LKB Wallac) to assess tumour:normal urothelial uptake. Biopsies were finally fixed in formalin for histopathological examination. A positive result for ex vivo studies and later on in the patient studies was defined as an area of increased uptake at the macroscopic site of the tumour, as visualised by γ scintigraphy; this was confirmed by the biopsy counts. A negative result was defined as a study where no uptake was seen on scintigraphy at the macroscopic tumour site.

Xenograft studies

The ability of the labelled antibody to bind to target tumours after intravenous administration was tested using an animal model. Athymic, nude mice were given subcutaneous injections of the human cancer cell line MCF7 BIM, which has been shown to stain strongly for MUC1 in indirect immunohistochemistry using C595. Tumours were allowed to grow on the mice for approximately 15 days prior to the experiment. Three of the mice were given tail vein injections of 100 µg of the antibody labelled with 5 MBq ^{99m}Tc. Imaging with a γ camera was performed at 2, 4, 6 and 24 h, after which time the mice were killed. Individual organs and the tumour were dissected, weighed and counted in order to estimate the uptake of labelled antibody per gram of tissue.

Patient studies

In order to test the ability of the conjugate to bind tumours in vivo, the conjugate was administered intravesically to patients. This study was approved by ARSAC and the Nottingham City Hospital Ethics Committee. Informed consent was obtained from patients prior to participation. Patients with newly diagnosed bladder tumours ($n = 6$) from flexible cystoscopic or radiological examination were recruited. Studies took place on the day of operation (transurethral resection of tumour). The patients were catheterised and a solution of 0.5 mg of antibody labelled with between 170 and 231 MBq (mean = 206 MBq) of ^{99m}Tc in 50 ml of normal saline was instilled into the bladder. The catheter was spigotted and the solution left in the bladder for 1 h. During this time, the patients lay on a couch but were asked to change position every 15 min. Prior to washout, a planar pelvic antero-posterior (AP) scan was obtained on a γ camera (Starport) to image the bladder outline. Washout was performed with 3 × 50 ml volumes of 0.9% saline; the bladder was then refilled with 50 ml of 0.9% saline prior to a further AP planar pelvic γ scan to assess tumour localisation. Transurethral resection was performed approximately 2 h after washout. During the operation, loop biopsies of both the tumour and the normal urothelium were obtained in four patients. The mucosa from the biopsies was separated from any underlying muscle, and the biopsies were weighed and counted in order to assess tumour:normal urothelial uptake.

Results

Labelling

The mean labelling efficiency of the procedure was 75% [$n = 10$, standard deviation (SD) = 8.9].

Immunoreactivity

The mean immunoreactivity was 89.7% ($n = 6$, SD = 7.12).

Stability

Serial ITLC at 0, 1, 2, 5, 6 and 24 h showed 85% incorporation of ^{99m}Tc after 24 h (see Fig. 1). The immunoreactivity remained high over 24 h, being 97% immediately post-labelling and 84% after 24 h.

Gel chromatography

S-300 gel chromatography of the alcohol dehydrogenase (MW = 150 kDa) revealed a single peak at the same elution volume as the labelled antibody. This was easily distinguishable from the ^{99m}Tc-labelled MDP peak.

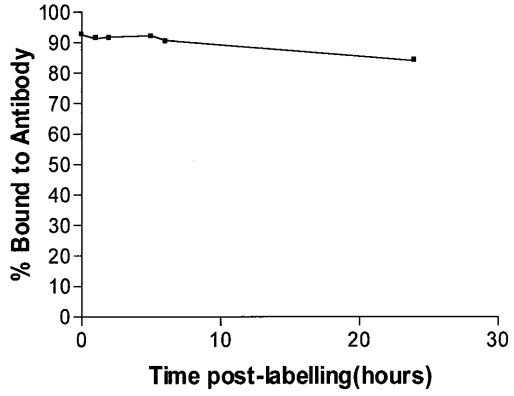

Fig. 1 Graph showing stability of conjugate in PBS over 24 h

Ex vivo studies

A positive result (i.e. increased uptake at the site of the tumour) was observed in four of the five bladders with a mean tumour:normal uptake of 5.6:1. Examples of positive results are shown in Figs. 2 and 3. The results are summarised in Table 1.

Xenograft studies

Tumour xenografts were visualised in all three mice (see Fig. 4). The biodistribution of the labelled antibody is summarised in Table 2.

Patient studies

Intravesical administration of the conjugate showed tumour localisation in five of the six patients. This was demonstrated by increased uptake on AP pelvic γ scintigraphy (see Fig. 5), which corresponded to the position of the tumour. The mean tumour:normal uptake was 20.4:1. The results are summarised in Table 3.

Discussion

Computed tomography (CT) scanning has limitations when used for radiological staging. It is unreliable for estimating the depth of tumour penetration. Furthermore, with benign perivesical alterations such as oedema and scarring, which may occur after a previous resection, CT scans can often overestimate the tumour stage.

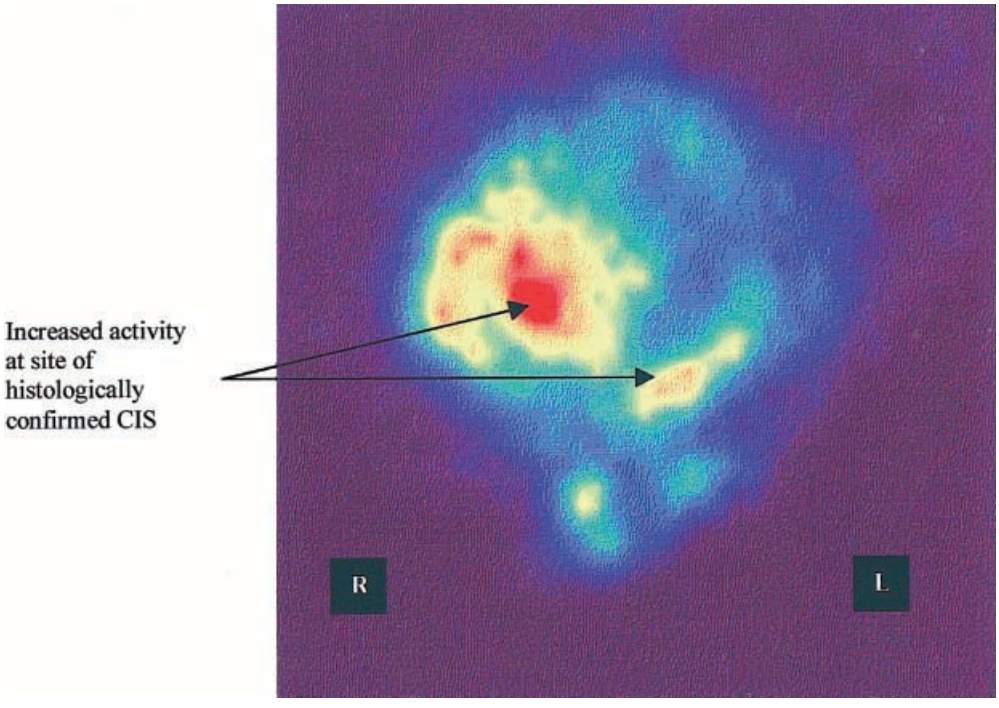

Fig. 2 γ scan from ex vivo bladder number 1 showing hot spots in regions of histologically confirmed CIS

In addition, it cannot detect microscopic perivesical infiltration. Metastatic lymph nodes are not reliably seen on CT scans. In a study by Voges et al. [10], it detected only 2 out of 19 true positive nodes in 164 patients undergoing radical cystectomy. CT scans can only detect enlarged nodes and cannot distinguish between reactive and metastatic nodal enlargement. In addition, many lymph node metastases are microscopic and will escape detection. It is generally accepted that magnetic resonance imaging (MRI) and CT scans give similar results in the detection of abnormal pelvic nodes [9].

In patients with prostate cancer, immunoscintigraphy using indium (In)-111-capromab pendetide has been shown to be superior to conventional radiological techniques in the detection of pelvic lymph node metastases [5]. Experience with immunoscintigraphy for staging is limited in bladder cancer and only one large series has been reported. That study used an anti-carcino-embryonic antigen (CEA) antibody labelled

Table 1 Summary of results for the ex vivo model. *N/D* not determined

Ex vivo number	Histology	Result	Tumour:normal uptake
1	CIS	Positive	N/D
2	G3pT3	Positive	4.8
3	G3pT3	Positive	6.1
4	G3pT1 + CIS	Positive	6.0
5	G3Pt3	Negative	N/D

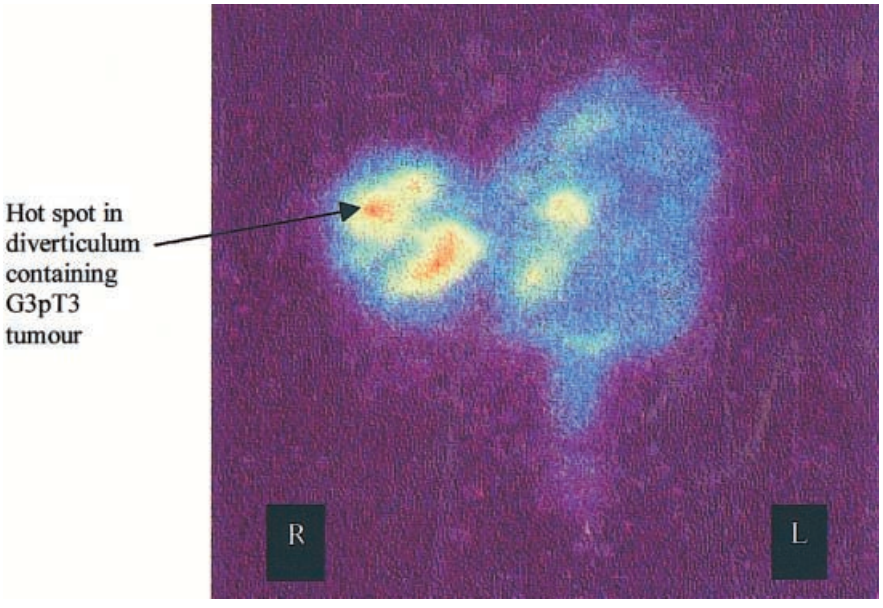

Fig. 3 γ scan of ex vivo bladder number 3 showing hot spot within diverticulum

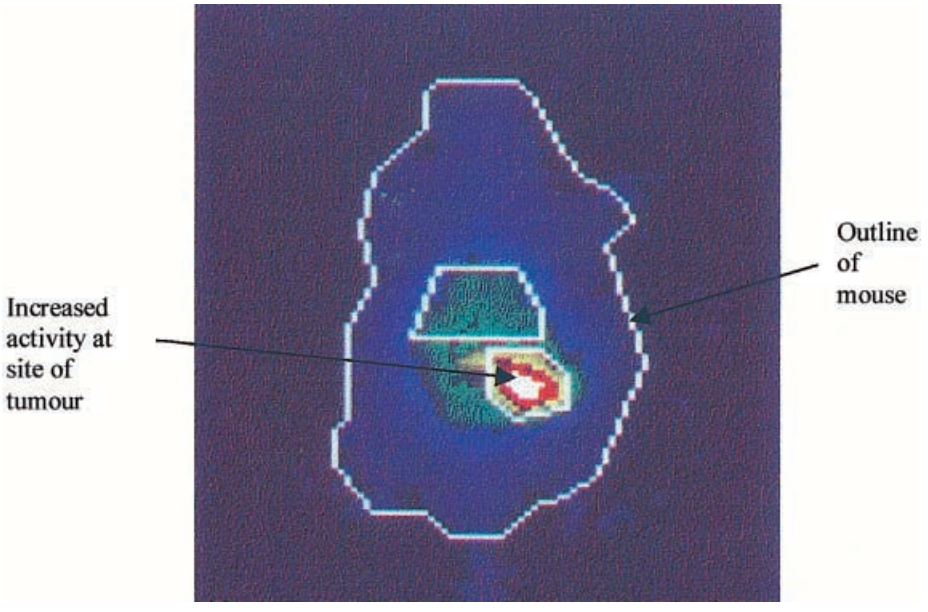

Fig. 4 γ scan of nude mouse bearing tumour xenograft showing hot spot at the site of tumour

Table 2 Mean tissue biodistribution of ^{99m}Tc-C595 from xenograft study

Tissue	Weight (g)	Counts	Counts (g)	Tissue/blood
Blood	0.39	30.57	78.31	1.00
Tumour	0.10	20.57	195.87	2.56
Spleen	0.11	18.57	196.18	2.37
Pancreas	0.18	24.90	145.86	1.89
Gut	2.75	32.90	12.10	0.15
Kidneys	0.45	47.57	93.41	1.09
Liver	1.60	53.57	33.82	0.38
Heart	0.10	32.57	312.15	4.28
Lung	0.17	21.90	128.36	1.71
Carcass	18.14	128.53	7.24	0.09
Bladder	0.06	15.90	295.55	3.96

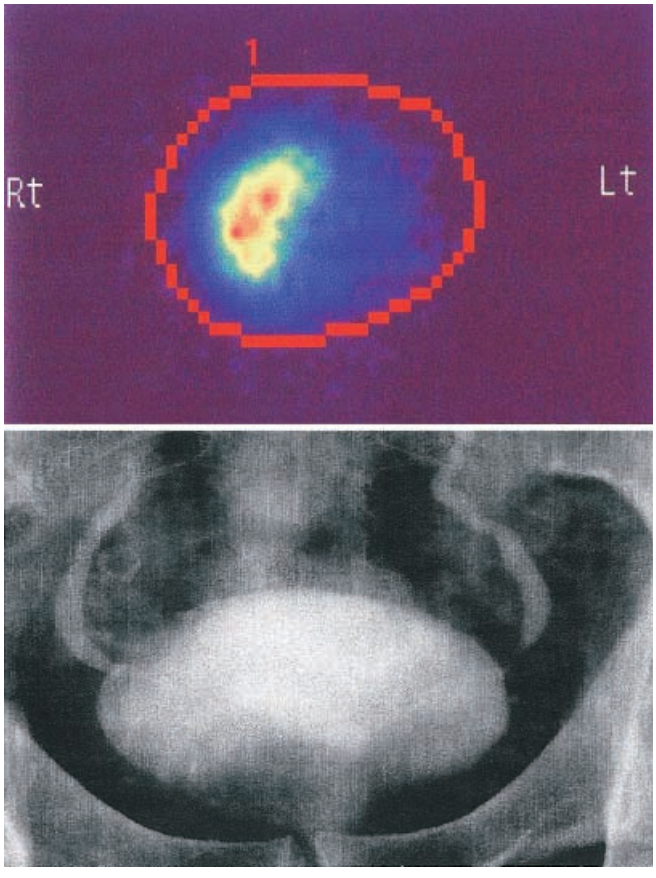

Fig. 5 AP γ scan in patient number 4 showing hot spot in the top right-hand corner of the bladder, corresponding to filling defect on IVU

with ^{111}In to stage patients with bladder cancer and the authors reported that immunoscintigraphy was more successful than CT scanning in the detection of pelvic nodal metastases [1].

Mucins are a group of high MW glycoproteins that are found on a variety of epithelial surfaces, including the bladder. Essentially, they act as biological lubricants. In the bladder, the mucin layer may shield cells from lethal concentrations of hydrogen ions and may help prevent bacterial and crystal adhesion [11]. Up to 12

Table 3 Summary of patient studies. *CIS* carcinoma in situ, *N/D* not determined

Patient number	Histology	Result	Tumour:normal uptake
1	G3PT1, CIS	Positive	7.8
2	G3pT2	Positive	N/D
3	G3pT2	Positive	3.7
4	G3pT1, CIS	Positive	44.5
5	G3pT2	Negative	N/D
6	G3pTa	Positive	25.7

mucin genes have been identified [12], but the most widely known mucin is MUC1. It consists of a protein core comprising highly variable numbers of the 20 amino acid "variable number tandem repeat" (VTNR) unit, bristling with oligosaccharide (O-glycan) chains. MUC1 is found to be frequently upregulated and abnormally glycosylated in a number of common malignancies, including breast, bladder, colon, ovarian and gastric cancer. Cancer-associated MUC1 is structurally different to normal MUC1 in that the former has shorter and less dense O-glycan chains, which exposes novel regions of the protein core [2]. MUC1 can therefore act as a tumour-related antigen and can be used as a target for cancer diagnosis and therapy. Immunohistochemical studies with C595 have shown that MUC1 is abnormally expressed in around 95% of invasive bladder tumours and that metastatic lymph nodes stain strongly with this monoclonal antibody (O.D.M. Hughes, personal communication). C595 does not bind specifically to granulocytes and does not stain normal lymphoreticular tissue [8].

A number of radioisotopes have been used for the purposes of immunoscintigraphy. To date, most experience has been with ^{111}In. Indeed, we have previously shown that a conjugate of ^{111}In and C595 will target bladder tumours after intravesical administration [3]. The most widely used isotope in general nuclear medicine is ^{99m}Tc. It is available in the majority of nuclear medicine departments from a simple bench-top generator. The half-life of ^{99m}Tc, with its almost pure γ-emission of 140 KeV, along with its high photon flux, make it ideal for imaging and means that the radiation dose to both patients and staff is kept to a minimum if this isotope is used. Despite its general use for routine nuclear medicine procedures, ^{99m}Tc has only relatively recently achieved widespread use as a radiolabel for monoclonal antibodies. This stemmed from uncertainty as to whether the short half-life of ^{99m}Tc was appropriate for systemic administration with monoclonal antibodies and also from difficulties in labelling, which now seem to have been overcome.

This study shows that the C595 monoclonal antibody can be successfully labelled with ^{99m}Tc to produce a conjugate that may be suitable for intravenous immunoscintigraphy of bladder cancer. This conjugate has been shown to be both stable for 24 h, which would allow for adequate imaging to take place, and highly

immunoreactive. The gel chromatography experiment proves that the labelled species is indeed whole antibody and that there is no fragmentation of antibody during reduction. The conjugate has been shown to target bladder tumours after intravesical administration in both ex vivo and patient studies. The ex vivo model provides a cheap and reproducible test bed that could be used for assessing potential conjugates for both radioimmunotherapy and radioimmunoscintigraphy.

There was no tumour "hot spot" from uptake of the conjugate in bladder number 5; this may have been due to the fact that the tumour was focally calcified, which may have prevented uptake of the conjugate. However, uptake of the conjugate did seem to be slightly better for the patient studies, indicating that the ex vivo model may not behave exactly in the same way as the bladder in vivo.

In the intravesical patient studies, five of the six tumours were visualised. In the patient in whom no tumour uptake was demonstrated, the tumour was located over the trigone of the bladder where the catheter balloon would have been sitting, and this may have prevented adequate contact between tumour and conjugate.

It is of significance that the conjugate was able to detect carcinoma in situ (CIS). Immunohistochemical studies using C595 have shown that CIS cells stain with a similar proportion to tumours with an invasive element (O.D.M. Hughes, personal communication). In ex vivo bladder number 1, it was possible to visualise areas of CIS by γ scintigraphy (Fig. 2). In ex vivo bladder number 4, an area of increased uptake well away from the solid tumour was seen at immunoscintigraphy and this was confirmed histologically as CIS. In addition, in patients 1 and 4, CIS was found in the urothelium adjacent to the tumours. However, it is difficult to say what contribution the area of CIS made to the hot spot seen on γ scintigraphy. Nevertheless, this may be of considerable clinical significance. It is possible that the conjugate may be able to detect areas of CIS in bladders after intravesical administration. In bladders with multifocal tumours, however, it might prove difficult to differentiate patches of CIS from tumours themselves. Such patches can be difficult to detect cystoscopically. This supposition needs to be addressed in a separate study.

The xenograft study shows that this conjugate will target an MUC1-expressing tumour after intravenous administration in nude mice. The mean tumour:blood uptake in the three xenografts was 2.56:1 and tumours were visualised with γ scintigraphy. The renal metabolism of the ^{99m}Tc-labelled antibody is reflected in the relatively high renal and bladder tissue:blood ratio. In the clinical setting, a catheter could be inserted into the bladder to wash out the urine, preventing the high amount of urinary activity in the bladder from obscuring uptake by the tumour and pelvic metastases. However, the nude mouse model does have drawbacks. In it, mice would not be expected to produce a marked antibody response to the monoclonal antibody, which is the opposite to the situation in humans where the human anti-mouse antibody (HAMA) response may have a significant effect on the biodistribution of antibodies. In any case, the biodistribution of a labelled antibody in mice will be different to that in man. In addition, the successful localisation of tumours by scintigraphy in humans is dependent on other factors, such as blood supply and size. These are optimal in the nude mouse model. Therefore, although successful immunolocalisation in our xenograft model has shown that our conjugate has the ability to image tumours in mice, this may not necessarily equate to the successful visualisation of tumour in man.

Technetium has many advantages over other radioisotopes for the purposes of immunoscintigraphy and the direct labelling technique has been applied to many other antibodies. Certainly both the intravesical administration of conjugates and the ex vivo model enable novel conjugates to be tested prior to intravenous administration in bladder cancer.

The bladder is perhaps the ideal organ for targeted radioimmunotherapy of tumours since radioactive conjugates can be directly instilled into it. ^{188}Rhenium (Re) is an isotope that is readily available and whose chemical properties are similar to those of ^{99m}Tc. It has ideal properties for use as a therapeutic isotope in bladder cancer in terms of its half-life (17 h) and particle range in tissue (11 mm). The direct, reduction-mediated labelling technique could, therefore, be applied to ^{188}Re to produce a conjugate for targeted radioimmunotherapy of superficial bladder cancer.

This method of labelling allows the production of a useful, stable ^{99m}Tc-antibody conjugate in 10 min and, therefore, is highly practical. As a result of these studies, we have embarked upon a clinical study of intravenous immunoscintigraphy for staging bladder cancer.

References

1. Boeckmann W, Baum RP, Schuldes H, Kramer W, Hertel A, Baew-Christow T, Hanke P, Jonas D, Hor G (1990) Tumour imaging of bladder carcinomas and their metastases with ^{111}In-labelled monoclonal anti-CEA antibody BW 431/26. Br J Cancer 62 [Supp 10]: 81
2. Brockhausen I, Yang J, Burchell J, Whitehouse C, Taylor-Papdimitrou J (1995) Mechanisms underlying aberrant glycosylation of MUC1 mucin in breast cancer cells. Eur J Biochem 233: 607
3. Kunkler RB, Bishop MC, Green DJ, Pimm MV, Price MR, Frier M (1995) Targeting of bladder cancer with monoclonal antibody NCRC48 – a possible approach for intravesical therapy. Br J Urol 76: 81
4. Lindmo T, Boven E, Cutitta F, Fedorko J, Bunn PA Jr (1984) Determination of the immunoreactive fraction of radiolabelled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. J Immunol Methods 72: 77
5. Manyak MJ, Hinkle GH, Olsen JO, Chiaccherini RP, Partin AW, Piantadosi S, Burgers JK, Texter JH, Neal CE, Libertino JA, Wright GL Jr, Maguire RT (1999) Immunoscintigraphy with indium-111-capromab pendetide: evaluation before definitive therapy in patients with prostate cancer. Urology 54: 1058

6. Mather SJ, Ellison D (1990) Reduction-mediated technetium-99m labelling of monoclonal antibodies. J Nucl Med 31: 692
7. Petrakou E, Murray A, Price MR (1998) Epitope mapping of anti-MUC1 protein core monoclonal antibodies. Tumour Biol [Suppl] 19: 21
8. Price MR, Pugh JA, Hudecz F, Griffiths W, Jacobs E, Symonds IM, Clarke AJ, Chan WC, Baldwin RW (1990) C595 – a monoclonal antibody against the protein core of human urinary epithelial mucin commonly expressed in breast carcinomas. Br J Cancer 61: 681
9. Roy C, Lebras Y, Mangold L, Saussine C, Tuchmann C, Pfleger D, Jacqmin D (1997) Small pelvic lymph node metastases: evaluation with MR imaging. Clin Rad 52: 437
10. Voges G, Tauschke E, Stockle M, Alken P, Hohenfellner R (1989) CT an unreliable method for accurate staging of bladder tumours in patients who are candidates for radical cystectomy. J Urol 142: 972
11. Walsh MD, Hohn BG, Thong W, Devine PC, Gardiner RA, Samaratunga ML, McGuckin MA (1994) Mucin expression by transitional cell carcinomas of the bladder. Br J Urol 71: 256
12. Williams SJ, McGukin MA, Gotley DC, Eyre HJ, Sutherland GR, Antalis TM (1999) Two novel mucin genes down-regulated in colorectal cancer identified by differential display. Cancer Res 59: 4083