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IMMUNOTARGETING WITH MONOCLONAL CYTOKERATIN 8 ANTIBODIES OF HUMAN UROTHELIAL CANCER TRANSPLANTED TO NUDE MICE

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The possibility of using cytokeratin antibodies for the radioimmunolocalization of urinary bladder cancer was studied. A monoclonal murine IgG antibody was raised against cytokeratin 8 and labelled with iodine-125; normal murine IgG was used for control purposes. The urothelial cancer cell line RT4 was transplanted into immunodeficient nude mice. The anti-cytokeratin 8 antibody was administered intraperitoneally and its uptake in the tumour and other organs was analyzed with a computerized gamma camera. Optimal scintigraphic visualization occurred 11 days after antibody administration. The tumour/blood ratio of the specific antibody was 5.64 ($\pm$5.01 SD) on day 11, compared with 0.73 ($\pm$0.35 SD) in the control. Autoradiography demonstrated antibody uptake preferentially in viable sections of the tumour. The antibody uptake is presumed to be the result mainly of binding to the released cytokeratin in and around cells lysed during natural cellular death. The monoclonal murine anti-cytokeratin antibody is of potential interest in studies aimed at improving the clinical staging of urinary bladder cancer.

The routine preoperative assessment of muscle-infiltrating urinary bladder tumours comprises a physical examination of the patient's urinary bladder and its surroundings under general anaesthesia, transurethral resection of the tumour, ultrasonography and computerized tomography (CT). These methods understage the extent of the malignancy in about 50% of cases (1). Lymph node metastases less than 10 mm in diameter cannot be detected with conventional radiological techniques (2). There is thus a need for complementary techniques in this respect. Promising results have been reported by Boeckmann et al. (3), who used monoclonal anti-CEA antibodies for the radioimmunolocalization of urinary bladder carcinomas.

In the present study, the possible use of a radiolabelled monoclonal antibody raised against cytoketarin 8 was investigated. Cytokeratins are part of the intermediate filament system of the cytoskeleton (4). Since cytokeratins are found in all epithelial cells including carcinomas (5), these antigens may serve as targets in the radioimmunodetection of malignancies. The cytokeratin group exhibits marked heterogeneity, and 20 different polypeptides have so far been identified (6). The molecular masses range from 40 to 70 kDa and the antigens occur in different combinations in different epithelia (7). They usually appear as heteropolymers built up of tetrameric subunits comprising two chains of type-I and type-II cytokeratins (8). Cytokeratin 8 is the most abundant type in both normal and malignant epithelial cells (9). A monoclonal antibody directed against this antigen has proven useful in tumour radioimmunolocalization in an experimental set-up with HeLa-cells (10).

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Cytokeratin 8 is one of the major cytokeratin subtypes expressed in urothelial cells (9), and this study was initiated to determine the potential usefulness of a monoclonal antibody raised against this antigen in the immunolocalization of urinary bladder cancer.

Materials and Methods

Monoclonal antibody A monoclonal antibody was raised against cytokeratin 8. Briefly, cytokeratin 8 present in cells from the in vitro cultured human breast cancer cell line MCF 7 (American Tissue Culture Collection. Rockville, Md.) was digested with chymotrypsin (Sigma, St. Louis, Mo.). The resulting fragments were identified by means of Western blot analysis (11) and thereafter used as the immunogen in Balb/c mice for antibody formation. Subsequently, lymphocytes drawn from the mice were fused with Sp2/0 myeloma cells (12). The hybridoma producing an anti-cytokeratin 8 antibody (6D7 IgG_1 kappa) was cloned twice. The antibody reacts extensively with intact molecules and fragments of cytokeratin 8 (Silén, Å, personal communication).

Cell culture and heterotransplantation. The human urothelial cancer cell line RT4 was obtained from the American Tissue Culture Collection. This cell line consists of well-differentiated transitional cells, possibly of papilloma origin (13). The cells were cultivated in plastic Falcon cell culture dishes in RPMI 1640 medium (Labora, Sweden) supplemented with 10% newborn calf serum and L-glutamine (Lab Systems, Sweden). The cell culture was maintained at an ambient temperature of 37°C in humidified air with 5% CO_2. The cultures were subcultivated twice a week. No supplements of antibiotics and antimycotics were used. Nude, 4-week-old male NMRI mice raised at Bomholtsgaard (Denmark) were inoculated with 20×10^6 RT4 cells subcutaneously in their right flank. The take rate exceeded 95%, and within two weeks, the tumours had grown to a diameter of 5–8 mm. The tumours investigated in this study were between 5 and 12 mm in size, the latter value being the size reached immediately before the animals were killed. The number of animals in each experimental group was 9.

Peroxidase labelling of antibody. The monoclonal anti-cytokeratin 8 antibody was labelled with peroxidase according to Tijssen & Kurstak (14) with certain minor modifications. Briefly, 10 mg of horseradish peroxidase (Ezymatix, UK) at a concentration of 4 mg/ml were oxidized with 100 mM sodium periodate at a volume ratio of 5:1 for 20 min at room temperature. The oxidized enzyme was then isolated by gel chromatography on a PD 10 column (LKB-Pharmacia). Absorbance of the harvested fractions at 280 nm was plotted and the first peak to contain the oxidized enzyme was pooled. One milligram of the antibody in a solution of 0.2 M sodium bicarbonate was made to react with 0.53 mg of oxidized enzyme at 2–8°C for 17 h. Reduction was achieved by adding 12.5 μl of $NaBH_4$ (4 mg/ml) and permitting the reaction to continue for 5 min at 2–8°C. The peroxidase-labelled antibody was then separated from the remaining peroxidase which had not reacted on a Sephacryl S-300 column (LKB-Pharmacia, Sweden).

Immunohistochemistry. The RT4 tumours grown on nude mice were freeze-sectioned and immunostained using a one-step technique developed at the ImmunoDevelop Laboratories, Borlänge, Sweden. Briefly, the tissue sections were incubated with a peroxidase-labelled anti-cytokeratin 8 monoclonal antibody (MoAb) for 30 min at room temperature and then washed twice with PBS. Three-amino-9-ethylcarbazole was used as the chromogenic substrate. The specimens were counterstained with hematoxylin and mounted in glycerol-gelatin. The specificity of the anti-cytokeratin 8 antibody was verified with Western blot analysis, according to a method described by Towbin et al. (11).

Radiolabelling of the monoclonal antibodies. The anti-cytokeratin 8 and normal mouse IgG antibodies were labelled with iodine-125 (^{125}I) according to the chloramine-T technique (17). Briefly, 1 mg of antibody was allowed to react with 370–400 MBq of ^{125}I (Nordion Int., Ontario, Canada) in the presence of 20 μl chloramine-T (2 mg/ml) in 200 μl of 0.2 M sodium phosphate buffer at pH 7.5. The reaction was terminated after 2 min by the addition of 20 μl of sodium thiosulphate (4 mg/ml). The antibodies were then separated from free iodine and the reagents on a PD 10 column (LKB-Pharmacia). The specific radioactivity of the radiolabelled antibodyes was, as a rule, 150–200 MBq/mg. The immunoreactivity of the radiolabelled anti-cytokeratin antibody was tested against cytokeratin-coated beads (11) and resulted in a B/T >90%. The corresponding value of radiolabelled normal murine IgG antibodies (Sigma, St. Louis, Mo.) was <2% B/T when tested against the cytokeratin-coated beads.

Immunoscintigraphy. One hundred microlitres of an iodine-125-labelled monoclonal anti-cytokeratin 8 MAb solution (25 μg/ml; 185 kBq/μg), corresponding to 470 kBq, were injected intraperitoneally. Scintigraphy was then done after 1, 3, 5, 8 and 11 days using a gamma camera (Philips Tomognost) equipped with a low-energy general purpose collimator. Data of frontal planar images were collected on a 64 × 64 matrix with an energy window of 24 keV ±60% (10–40 keV). Approximately 100 000 counts were acquired for each image. The mice were given potassium iodide in their drinking water to block the uptake of ^{125}I in the thyroid gland. The collected images were digitalized and analyzed with a PDP Gamma 11 system (Nuclear Diagnostics, Sweden). Three regions of interest (ROIs) were chosen; one over the tumour in the right thigh, one over an area in the left flank which served as the control, and one over the spleen, chosen to represent the reticuloendothelial system (RES). The tumours and

other organs of interest were excised after the animals were killed 11 days after antibody injection. The tumours and normal organs were weighed and analyzed for radioactivity using a gamma counter (Searle).

Cryosectioning, autoradiography and densitometry. Cryosectioning was carried out according to Gillberg et al. (16). Mice intended for whole-body autoradiography were immediately after sacrifice allowed to cool for 5 h and then frozen in a −20°C compartment for 24 h. They were then stored at −70°C pending further use. Before actual cryosectioning, the frozen animals were first left at −20°C for 24 h and then embedded in a semiliquid gel of carboxymethyl-cellulose (CMC, Kebo). They were then rapidly immersed into hexane (Kebo) at −80°C for 15 min and then allowed to reach a steady temperature of −20°C for a minimum of 24 h before cryosectioning. Laterally directed 100 μm sections of the mice were obtained; they encompassed representative areas of the subcutaneous tumours. These sections were collected on paper tapes, transferred and thaw-mounted on chrome-gelatinized glass plates. The sections were dried at 4°C for 12 h and stored over a desiccant at −20°C for a minimum of 7 days. The dried sections were then left at room temperature for 4 h to allow moisture to evaporate. Subsequently, the dried sections were opposed with Hyperfilm (Amersham, UK), exposed for 8 days at 4°C, developed and fixed (16). The whole body autoradiographs were digitalized with the help of a video camera system (Philips LDH400), and analyzed by measuring the optical density on Cromemco SDD computer equipment (17). Relative radioactivity was assessed in two different areas of the tumours: the centre and the outer 'viable' part. An area over muscle was used as background.

Quantitation of cytokeratin 8 and 18 in serum. Cytokeratin 8 and 18 were quantitated in serum with the help of an ELISA technique. Briefly, the assay was of the 'sandwich' type with MoAbs directed against cytokeratin 8 and 18 bound covalently to the wells of a microtitre plate. The standard used in the assay was purified cytokeratin 8 and 18. One hundred microlitres of the solution to be tested (standard, control or specimen) and 100 μl of a horseradish peroxidase tracer antibody solution were added to the wells and left to incubate at 37°C for 2 h. The plates were rinsed and an o-phenylene-diamine dihydrochloride (OPD) substrate was added; after 30 min at room temperature the reaction was stopped with sulphuric acid. Absorbance at 490 nm was then read. This quantitation technique technique has proven highly sensitive and reproducible (Wiklund B, personal communication).

Analysis of serum radioactivity. Serum samples obtained from the mice at the moment of killing were subjected to gel chromatography using Sephadex G-10 and Sephadex G-200 columns (LKB-Pharmacia, Sweden) in PBS, pH 7.4. Radioactivity in the eluted fractions was measured with a gamma counter (Searle). In a separate set of experiments, 20 μl of the serum samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10–20% gradient gels, according to Laemmli (18). The serum samples were mixed with equal volumes of Laemmli's sample buffer solution containing beta-mercaptoethanol, and boiled for 5 min. The proteins separated in this way were transferred from the gel to a nitrocellulose membrane (Bio-Rad, Richmond, Ca.) according to the method of Towbin et al. (11). Molecular weight standard proteins (LKB-Pharmacia, Sweden) were stained with amido black and autoradiography of the blotted proteins was done with Kodak diagnostic film (XAR 5).

Statistical analysis. For comparisons between groups the Mann-Whitney U-test was used (19). A p-value of less than 0.05 was considered statistically significant.

Results

Tumour model. Cells from the human urinary bladder cancer cell line RT4 were inoculated subcutaneously into the right flank of nude mice. In an initial experiment, the expression of cytokeratin 8 in tumour cells was verified with immunohistochemical techniques using the anti-cytokeratin 8 MoAb. With immunohistochemical staining, the RT4 cells were positive for the antibody (Fig. 1.). The tumours generally developed a central area of necrosis

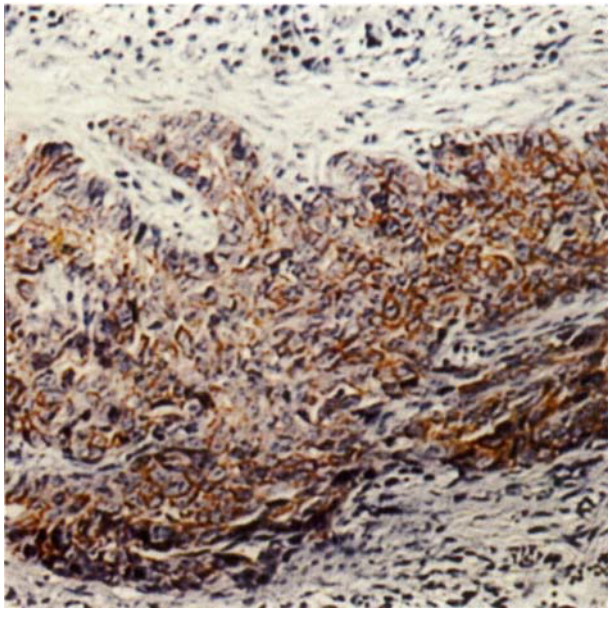


Fig. 1. Immunostaining of a freeze-sectioned tumour obtained from the heterotransplantation of urothelial cancer cells (RT4) in a nude male mouse. The peroxidase-labelled anti-cytokeratin 8 monoclonal antibody was used in a one-step procedure. The staining reaction was developed with 3-amino-9-ethylcarbazole as the substrate. After immunostaining, the tissue section was counterstained with hematoxylin. Note the positive immunostaining in the outer part of the tumour and the essentially negative staining in its necrotic central part. Magnification × 250.

Fig. 2. Immunoscintigraphy of a nude mouse with a subcutaneous urothelial cell cancer (RT4) heterotransplant in the right flank. The images were obtained 11 days after the intraperitoneal injection of the ^{125}I-labellec anti-cytokeratin 8 monoclonal antibody. Region of interest (ROI) areas indicated, corresponding to tumour (1), background (2) and RES (3).

after having reached a diameter of 10 mm or more. Immunostaining for cytokeratin 8 was mainly localized to the cytoplasm of the tumour cells in the peripheral 'viable' part of the tumour. The necrotic central area was generally negative or only faintly immunostained.

Immunoscintigraphy. Groups of 9 mice with RT4 tumours were injected intraperitoneally with either the ^{125}I-labelled monoclonal anti-cytokeratin 8 antibody or ^{125}I-labelled normal mouse IgG. The distribution of radioactivity was recorded with a gamma camera after 1, 3, 5, 8 and 11 days after injection. Tumour uptake was assessed repeatedly during this period using a computer-based gamma camera. The regions of interest (ROIs) were the tumour in the right thigh, a control area in the left thigh and the spleen, chosen to represent the reticulo-endothelial system (RES), (Fig. 2). The specific antibody accumulated mainly in the tumour and highest activity was measured in the centre of the tumour. No accumulation of the non-specific murine IgG was seen in the tumours of the control group.

Distribution and measurements of radioactivity. The tumour/background ratio, assessed with the ROI technique, increased steadily during the 11-day period after the injection of the specific antibody. The tumour/background ratios calculated in the specific and non-specific groups were 3.33 ($\pm$1.7 SD) and 1.09 ($\pm$0.27 SD) respectively ($p < 0.0001$). The results from the experiments are shown in Fig. 3a. Non-specific antibodies seemed to dominate the uptake in the spleen and lungs, which were regarded as representative of RES. The tumour/RES ratios calculated in the specific and non-specific groups were 2.10 ($\pm$1.28 SD) and 1.09 ($\pm$0.18 SD) respectively ($p < 0.002$). The results are shown in Fig. 3b. The mice were killed on day 11 and the tumours and individual organs identified and excised after blood had been drained. The radioactivity present in the different tissues was then measured, and tissue-to-blood quotes calculated. The tumour/blood ratio of the radioactivity emanating from the specific antibody was 5.64 ($\pm$5.01 SD) on day 11, compared with 0.73 ($\pm$0.35 SD) in the control ($p < 0.015$). The results from

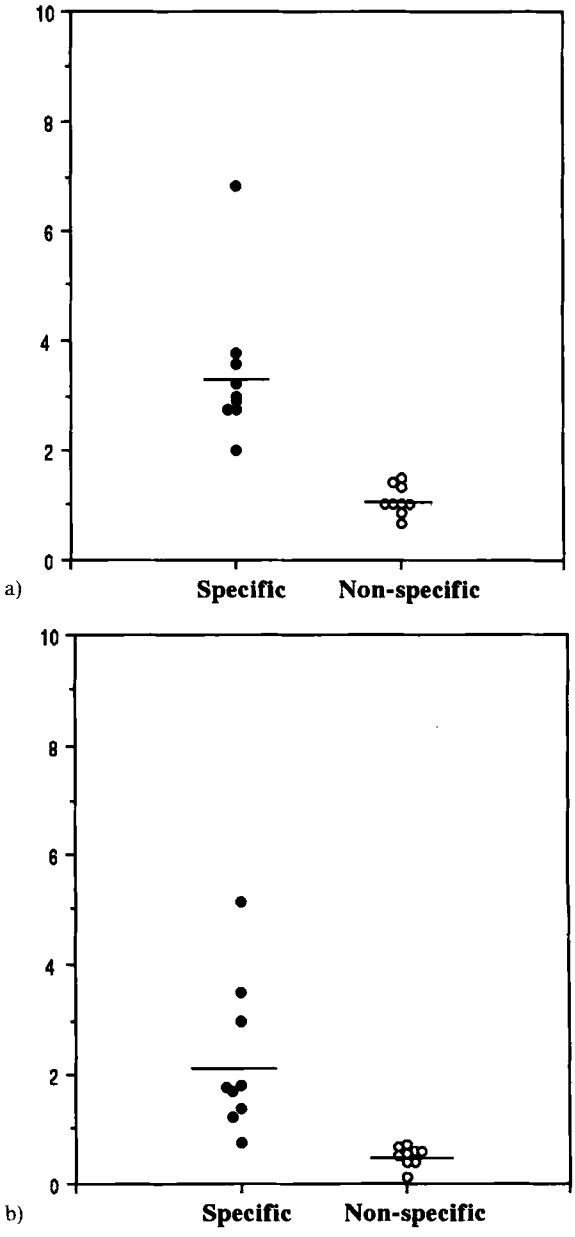


Fig. 3. Calculated tumour/background (a) and tumour/RES (b) ratios from immunoscintigraphic acquisitions 11 days after antibody injection. The terms 'specific' and 'non-specific' denote anti-cytokeratin 8 antibody and normal mouse IgG respectively. The uptake values were obtained from computerized ROI analysis of images of 9 mice in each of the two groups.

the experiments are shown in Fig. 4. Table 1 gives the results of the measurements of radioactivity in tumours and selected tissues, related to the radioactivity in whole blood.

Autoradiography and densitometric analysis. Fig. 5a and b shows two typical autoradiographies of tumour-hetero-transplanted mice, one of which (Fig. 5a) was injected with specific antibody and the other (Fig. 5b) with non-specific antibody. The higher activity was seen in the tumour of the mouse given specific antibody. The image obtained after

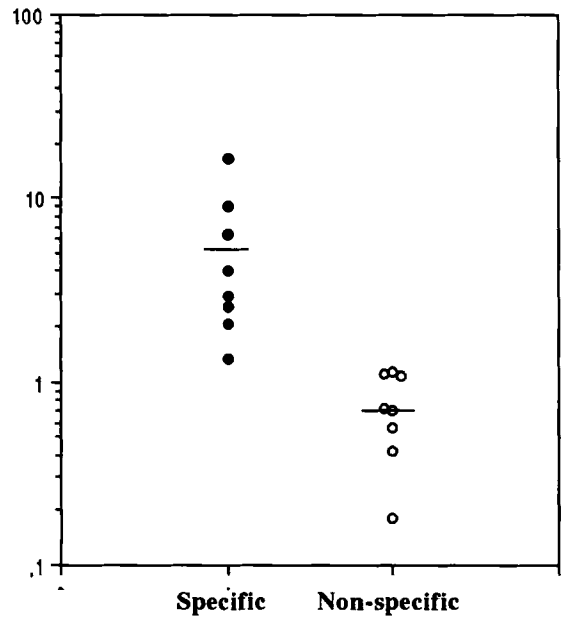


Fig. 4. Tumour/blood ratios obtained from the measurement of radioactivity 11 days after the injection of antibodies in nude mice hetertramsplanted with urothelial cancer cells (RT4). The terms "specific" denote anti-cytokeratin 8 antibody and normal mouse IgG respectively.

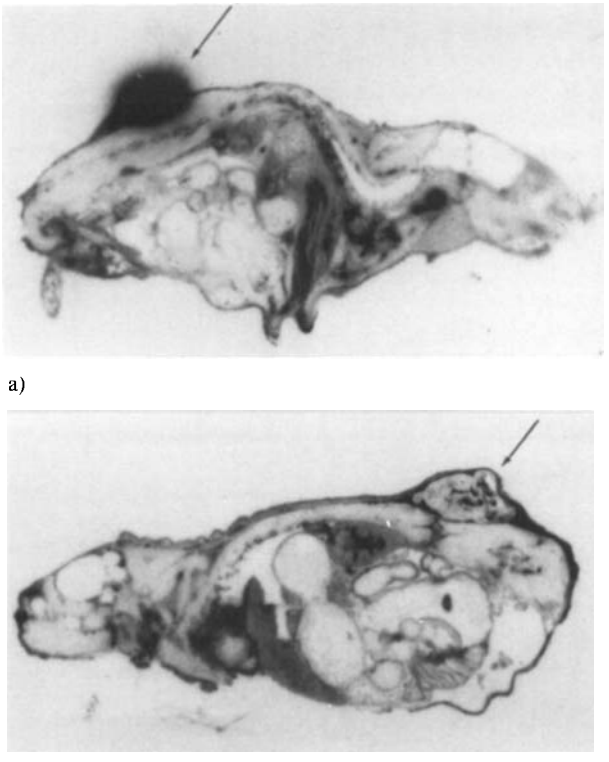
Table 1

Tissue/blood ratios calculated from mice injected with specific (anti-cytokeratin 8) and non-specific (normal mouse IgG) radiolabelled antibodies. The tissues were obtained at sacrifice, 11 days post-injection. The results represent data from 8 animals in each group

	Specific antibody	Non-specific antibody
Tumour	5.64 (1.41 – 10.8)	0.73 (0.18 – 1.22)
Liver	0.47 (0.46 – 0.51)	0.59 (0.44 – 0.82)
Kidney	0.37 (0.15 – 0.51)	0.44 (0.04 – 0.63)
Spleen	0.46 (0.25 – 0.84)	1.33 (0.78 – 1.78)
Lung	0.86 (0.70 – 1.00)	1.31 (0.76 – 1.59)
Muscle	0.19 (0.15 – 0.26)	0.29 (0.22 – 0.36)
Urine	0.58 (0.31 – 0.72)	1.11 (0.41 – 1.53)
Urinary bladder	0.69 (0.29 – 0.92)	0.86 (0.21 – 2.06)

computerized densitometry of the radiographs is shown in Fig. 6a and b is an enlargement of the tumour area, where the relative radioactivity was much higher in the peripheral part of the tumour than in its central parts and in background tissue (muscle) (Table 2).

Analysis of serum radioactivity. Radioactivity was still present in serum 11 days after the administration of radiolabelled specific or non-specific antibody. To determine whether this activity was antibody-bound, serum samples were analyzed on gel chromatography on Sephadex G-10. The radioactivity eluted with the void volume of the gel represented 99–100% of the activity in the sample. When subjected to gel chromatography on a Sephadex G-200 gel, the radioactivity was co-eluted with the peak representing



a)

b)

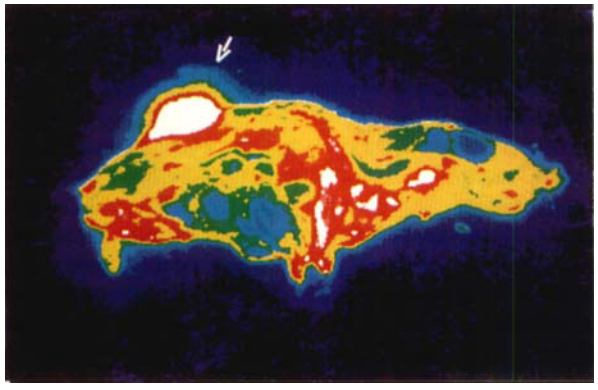
Fig. 5. Autoradiographs from whole body freeze-sectioned nude mice heterotransplanted with uorthelial cancer cells (RT4). The mice were killed 11 days after injection with anti-cytokeratin 8 antibody (a) or normal mouse IgG (b). The arrow points out the tumour.

IgG (data not shown). Aliquots of the seum obtained on day 11 were separated on SDS-PAGE under reducing conditions, trans-blotted onto a nitrocellulose membrane and subjected to autoradiography (Fig. 7). The radioactivity was bound to polypeptides with molecular masses of 25 kDa and 53 kDa, corresponding to the heavy and light chains of the IgG molecule. The data thus confirm that the radioactivity in serum at the time of sacrifice represents circulating intact antibodies.

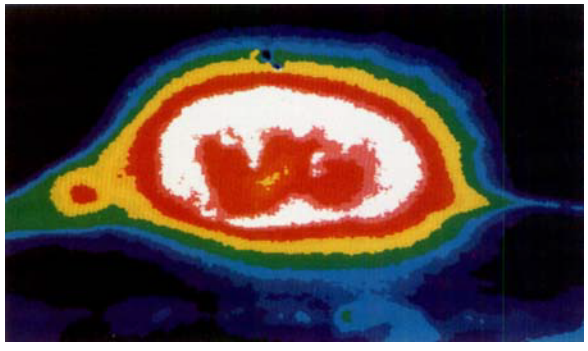
Quantitation of cytokeratin 8 and 18 in serum. Serum samples obtained on day 11 were analyzed for the occurrence of circulating cytokeratin 8 and 18, using an ELISA assay. The mean concentration of the antigen was 5.51 (1.48 – 7.22) ng/ml in mice injected with the anticytokeratin 8 antibody, whereas the corresponding value was 1.55 (0.52 – 6.37) ng/ml in mice injected with normal mouse IgG. The difference was, however, not statistically significant.

Discussion

Immunoscintigraphy with monoclonal antibodies raised against tumour-associated antigens has been successfully used in ovarian cancer (20) and colorectal adenocarcinoma



a)



b)

Fig. 6. Whole-body autoradiographs shown in Fig. 5 were digitalized with a video camera system. Fig. 6a shows a mouse injected with anti-cytokeratin 8 antibody, where the arrow points out the tumour. Fig. 6b is an enlargement of the tumour area. The greatest accumulation of activity is seen in peripheral areas of the tumour. Activity diminishes towards the necrotic centre of the tumour. Optical density in the outer part, the centre and the background is shown in Table 2.

Table 2

Densitometric analysis of a representative autoradiograph presented in Fig. 6b. Five different areas (regions of interest, ROIs) were randomly chosen for radioactivity measurement within the 'viable' outer part, the necrotic cnetre of the tumour and adjacent muscle

Antibody	Tumour (peripheral ROIs)	Tumour (central ROIs)	Muscle (ROIs)
Anti-cytokeratin	111 ± 6	63 ± 7	19 ± 6
Non-specific	19 ± 5	19 ± 6	10 ± 3

(21). A similar technique with radiolabelled anti-CEA has been used for the in vivo detection of urinary bladder cancer (3). However, many tumour-associated antigens, including CEA, are often heterogeneously expressed within one and the same tumour, and also vary between tumours of the same morphological type and grades of differentiation (22, 23). Therefore, a more universally expressed antigen was thought to be preferable for the immunoscintigraphic study of urothelial cancer. Representative of such 'universal' antigens are intermediate filaments, such as the

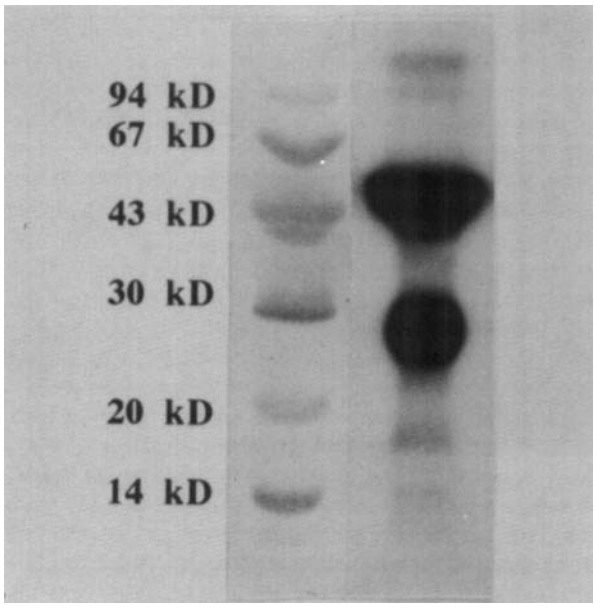


Fig. 7. SDS-PAGE, under reducing conditions, of serum obtained from a nudde mouse heterotransplanted with the human urothelial cancer cell line RT4. The mouse was given ^{125}I-labelled anti-cytokeratin 8 antibody 11 days earlier as described in 'Materials and Methods'. After electrophoresis, the proteins were transblotted onto a nitrocellulose membrane. Left lane: amido black stailning of molecular weight marker proteins. Right lane: autoradiography showing two major protein bands, corresponding to the heavy and light chains of IgG. The results show that radiolabelling occurred in both the polypeptide chains of the IgG molecule, and that both chains retained their major physicochemical characteristics in serum throughout the entire experiment.

cytokeratin group. These are the main components of the cytoskeleton in epithelial cells that line the inner cavities of the body (5). Sundström et al. (10) investigated a monoclonal antibody raised against cytokeratins and demonstrated in an animal system that HeLa cells could be detected by immunoscintigraphy in vivo.

In the present study, a MoAb was raised against cytokeratin 8, an antigen commonly expressed in urothelial cell cancer. Antibody accumulated in urothelial cancer cell heterotransplants. The antibody binding to tumour cytokeratin was regarded as specific since normal IgG did not accumulate in the tumour. However, cytokeratins are intracellular antigens, and the mechanism responsible for the accumulation of the antibody has yet to be clarified. It is tempting to assume that the antibody binds to cytokeratin molecules that have been released from dead or dying tumour cells. The highest uptake was observed in tumours, probably due to the fact that they undergo a greater degree of cell death, during which cell membrane disruption occurs. The uptake profile over the tumour was monophasic and no conclusions as regards preferential uptake could be drawn. However, the densitometric analysis of the autodiographs indicated a biphasic uptake with higher levels peripherally, suggesting active uptake in areas with

viable cells. This discrepancy can be explained by the lower resolution of the scintigraphic technique. Radioactivity uptake was observed mainly in the 'viable' part of the tumour, which corresponded well to the immunohistochemical staining pattern. The antibody could, however, also be identified in necrotic areas of the tumour. One reason for the lower levels of accumulation within these necrotic areas may be that the vascularization in necrotic areas is deficient in comparison with that in other areas of the tumour. The preferential uptake in the 'viable' part of the tumour contrasts with findings in previously reported experiments on HeLa cells (24). The uptake of anti-cytokeratin 8 antibodies in these cells was found mainly in the necrotic parts of the tumour (26). The reason for the discrepancy between these two studies is not known. One possible explanation is that the growth of urothelial cells differs from that of HeLa cells in vivo, especially as regards vascularization and/or cellular turnover. The intracellularly located cytokeratins are high molecular weight structures. Successive degradation of the cytokeratin probably occurs in the immediate vicinity of the tumour, as the antigen recognized in serum has a considerably lower molecular weight. In the present study, cytokeratins could be quantified in serum. The antibodies used in the serum assay were directed against cytokeratins 8 and 18. Interestingly, the highest concentrations of cytokeratins 8 and 18 at the time of sacrifice were 5–6 times greater than the upper normal level seen in healthy humans (Wiklund B, personal communication). The range of the cytokeratin levels was large and the mean concentrations of the antigens in the two groups of mice were not statistically different. Of interest are the pronounced targeting capabilities of the specific antibody for the tumour cells, despite the high concentrations of circulating antigens; this indicates that complex-binding in serum is of little importance. The characterization of the radioactivity still present in serum after 11 days demonstrated that the activity represented intact monoclonal antibodies.

The data obtained in this study show that the monoclonal antibody raised against cytokeratin 8 can be used for the in vivo detection of urothelial cancer in an experimental set-up. To investigate whether the antibody can be used for clinical immunoscintigraphic purposes, a phase 2 study is being initiated at our department.

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