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Monoclonal Antibodies against Human Urinary Bladder Carcinomas: Selectivity and Utilization for Gamma Scintigraphy

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Abstract—*Mouse monoclonal antibodies to human urinary bladder carcinoma cells have been examined by indirect membrane immunofluorescence using a panel of 27 human cell lines. Two of the monoclonal antibodies, 7E9 (IgG3) and S2C6 (IgG1), were found to distinguish between urinary bladder carcinoma cells and normal urothelium. The third monoclonal antibody, T24.06.5(IgG1), discriminated among cell lines of urothelial and non-urothelial origin but did not distinguish between urinary bladder carcinoma and normal urothelial cells. None of the of the antibodies was found to be strictly selective, and occasional cross-reactions with unrelated cell types were observed. The monoclonal antibody 7E9, showing the highest degree of selectivity, was further examined by an indirect immunoperoxidase technique on frozen tissue sections from 19 patients. The antibody reacted with all (7/7) bladder carcinomas examined and gave negative results with control normal bladder mucosa (0/8) and unrelated tumor tissue (0/4) sections. The 7E9 antibody was purified by protein A affinity chromatography, labeled with ^{131}I and used for gamma-scintigraphy in nude mice xenografted with human urinary bladder carcinoma T24. The 7E9 antibody was capable of locating the T24 xenografts in nude mice; it localized preferentially in the T24 tissue compared to normal mouse tissues. The T24 xenografts could not be detected by gamma-scintigraphy with ^{131}I-labeled monoclonal antibody against human mammary carcimona cells and two other control antibodies. Likewise the ^{131}I-labeled 7E9 antibody was not capable of locating human mammary carcinoma xenografts in nude mice.*

INTRODUCTION

HUMAN urinary bladder carcinomas carry tumor-associated antigens (TAA) capable of eliciting both humoral and cell-mediated immune responses in tumor patients [1–10]. These TAA may serve as targets for specific antibodies carrying radionuclide molecules. Selective binding of the radiolabeled antibodies and a high concentration of radioactivity in tumor tissue can be detected by gamma scintigraphy and used for radioimmunolocalization of tumors.

The first attempts in the area of tumor radioimmunodetection were initiated several decades ago [11]. However, the progress has been hampered by two major difficulties: general unavailability of suitable antibodies to give sufficient target-to-non-target contrast for

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imaging, and the difficulty of reproducible preparation and purification of tumor antibodies. The required high specificity, homogeneity and purity of the immunological reagents was first provided by a more recent development by Köhler and Milstein [12] of the monoclonal antibody technique.

Since the original discovery of Köhler and Milstein, the monoclonal antibodies have been successfully used for radioimmunolocalization of tumors both in experimental animals [13–23] and in cancer patients [15, 24]. A variety of monoclonal antibodies directed against carcinoembryonic antigen and against TAA of teratocarcinomas, osteogenic sarcomas, hepatocellular carcinomas, melanomas, colon carcinomas and mammary carcinomas have been utilized [13–24].

In this communication we describe imaging of human urinary bladder carcinoma xenografts with radiolabeled monoclonal antibody showing a high degree of selectivity and directed against TAA of transitional urinary bladder carcinoma (BTCC) cells. The TAA of BTCC against which the radiolabeled monoclonal antibody (Mab) was directed had been previously characterized as a 92K glycopeptide [25].

MATERIALS AND METHODS

Cell lines

Long-term cultures of human transitional cell carcinomas of the urinary bladder T24 [26], RT4 [27], TCCSUP [28], EJ (established by Daly; see [29]), HU549, HU96lb, HU456 [8, 30, 31] and HU1703He (established by Don and Kieler; unpublished) were utilized. For comparison, the following cell lines were used: a cell line established from a squamous-cell carcinoma of the urinary bladder SCaBER [32], cell lines derived from non-malignant human urothelium HCV29 (established by Fogh; see [33]) and HU609 [8, 31] and the non-tumorigenic BTCC-derived HU1734 (established by Don and Kieler; unpublished) as well as human cell lines of non-bladder origin: MCF 7 [34] and MDA-MB-231 [35] derived from a mammary carcinoma, HeLa [36] derived from a cervical carcinoma, 2T [37] derived from an osteosarcoma, MEL-1 [38] derived from malignant melanoma, HS (Troye, unpublished) derived from prostatic carcinoma, T cell leukemia MOLT4 [39], pre-erythroid leukemia K562 [40] and Burkitt lymphoma cell lines RAJI [41], RAMOS [42], DAUDI [43], P3HR1 [44] and marmoset line B95-8 [45]. In addition, diploid human embryo fibroblasts of lung origin (LEP, obtained from the Institute of Sera and Vaccines, Prague) and human fibroblasts derived from subcutaneous connective tissue (154, established by Kieler; unpublished) were also employed.

Monoclonal antibodies

The construction of mouse hybridomas that secrete antibodies to human urinary bladder carcinoma cells is described in detail elsewhere [46]. Briefly, a modification of the method of Fazekas de St. Groth and Scheidegger [47] was used. Immune spleen cells were obtained from BALB/c mice immunized intraperitoneally with viable cells from transitional bladder carcinoma cell lines TCCSUP and EJ (monoclonal antibodies 7E9 and S2C6) or T24 (monoclonal antibody T24.06.5). The immune spleen cells were fused with Sp2/0Ag14 myeloma cells (monoclonal antibodies 7E9 and S2C6) or P3-X63-Ag8-653 myeloma cells (monoclonal antibody T24.06.5) and the resulting hybridoma cells were selected in the HAT medium and cloned. Hybridoma supernatants from wells with colonies originating from single cells were screened using a cell-ELISA test, and selected cell clones were expanded. Immunoglobulin isotypes were determined either by immunodiffusion in 1% agarose gels with class- and subclass-specific rabbit anti-mouse antisera from Bionetics, Kensington, MD, U.S.A. (monoclonal antibodies 7E9 and S2C6 [46]) or by a modification of enzymoimmunological assay using nitrocellulose carrier membranes and rabbit antibodies specific for subclasses of murine immunoglobulins from Miles, Vienna, Austria (monoclonal antibody T24.06.5 [48]). The isoelectric point of the T24.06.5 monoclonal antibody (pI 5.9–6.0) was determined by isoelectric focusing in the agarose gel (Agarose IEF, Pharmacia, Uppsala), blotting of the separated protein into the nitrocellulose membrane, visualization of the antibody band by immunoperoxidase staining and comparison of its position with those of monoclonal antibodies of known isoelectric points.

Immunofluorescence

Indirect immunofluorescence for detection of membrane epitopes was performed with monoclonal antibodies directed against human urinary bladder carinomas (7E9, S2C6, T24.06.5) or control monoclonal antibody against human growth hormone (anti-HGH, a generous gift from Dr. I. Jónsdóttir, University of Stockholm) and FITC-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Copenhagen, Denmark, or a generous gift from Dr P Mančal, Institute of Sera and Vaccines, Prague, Czechoslovakia). A modification [49] of the method first described by Möller [50] for the demonstration of mouse cell-surface isoantigens was used.

Immunoperoxidase technique

An indirect immunoperoxidase method [51] was utilized. In the staining procedure the frozen tissue sections were dried, fixed with 1% formaldehyde for 5 min at room temperature, exposed to the respective monoclonal antibody (30 min at 37°C) and washed twice for 5 min with Tris-saline. The slides were then reacted with peroxidase-coupled, rabbit anti-mouse IgG serum (Dako, Copenhagen, Denmark), washed again and developed with a solution of 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, U.S.A.) and hydrogen peroxide. The tissue sections were counterstained with Mayer's hematoxylin to facilitate visualization of the nuclei and cytoplasm. The characteristic dark reddish-brown reaction present in the majority of cells was considered positive. The preparations were read independently by two pathologists.

Patients

Tissue samples from patients with transitional urinary bladder carcinomas and from control patients were obtained from the Department of Urology, Hvidovre Hospital, Hvidovre; Department of Urology, Københavns Amtssygehus, Herlev and Department of Pathology, Finsen Institute, Copenhagen, Denmark. Samples of urinary bladder carcinoma tissue from three patients with non-invasive, grade II BTCC and four patients with invasive, grade III BTCC were examined. Control samples of normal bladder mucosa were obtained from four patients with BTCC, two patients with prostatic hypertrophy and two patients with urolithiasis. In addition, tumor samples from two patients with mammary adenocarcinoma and one patient with leiomyosarcoma and embryonal carcinoma of the testis were used. The diagnosis of bladder carcinoma was based in all cases on cystoscopy and on histological examination of tumor specimens.

Animals and tumors

Two-month-old athymic nude mice (BALB/c, females, Institute of Biophysics and Nuclear Medicine, Faculty of General Medicine, Charles University, Prague) served as recipients of human carcinoma T24 [26] and MDA-MB-231 [35] xenografts. The xenografts were grown by serial transplantation and routinely transferred when 1-2 cm in diameter.

Gamma scintigraphy

Monoclonal antibodies were purified by affinity chromatography on a protein A-Sepharose 4B matrix according to Ey *et al.* [52]. Purified monoclonal antibodies were labeled with ^{131}I (148-185 MBq/mg protein) using the chloramine-T method [53]. Free iodine was removed by gel filtration on a Sephadex G10 column. Nude mice whose thyroids were blocked by the addition of iodine (Lugol) solution to drinking water and who were bearing tumors 1.0-2.0 cm in diameter were given i.p. or i.v. injections of 0.1 ml of buffered phosphate solution containing 20 μg of the respective ^{131}I-labeled monoclonal antibody (3.7 MBq). Mice were anesthetized with 3 mg of pentobarbital per 100 g body wt and placed prone on the collimator face. Images were obtained using a parallel-pinhole collimator (360 keV) and a Pho Gamma V camera (Nuclear Chicago Siemens Gamma Sonics, Chicago, IL, U.S.A.) linked to a PDP 11/34A computer (Digital Equipment Corporation, MA, U.S.A.) with data display.

RESULTS

Selectivity of monoclonal antibodies 7E9, S2C6 and T24.06.5 is illustrated in Table 1. The 7E9 antibody reacted with seven of the nine bladder carcinoma cell lines; it did not react with any of three different urothelial cell lines of normal origin or with two fibroblast cell lines. Nor did it react with eleven non-bladder tumor cell lines. However, the 7E9 antibody gave a positive reaction with HeLa cells and a marginal reaction with MEL-1 cells.

The S2C6 antibody reacted with eight out of nine bladder carcinoma cell lines. It showed a marginal reaction with one of the three cell lines of normal urothelium origin and it reacted with three out of five Burkitt lymphoma cell lines including one line of EBV-transformed marmoset leukocytes.

The T24.06.5 antibody reacted with all cell lines of urothelial origin, irrespective of whether or not they were malignant. It did not react with human fibroblasts and with tumor cell lines of non-bladder origin, except HeLa cells. It also gave a marginal reaction with RAMOS cells.

The monoclonal antibody 7E9, showing the highest degree of selectivity, was further examined by an indirect immunoperoxidase technique on frozen tissue sections from 19 patients; it reacted with all bladder carcinomas examined and gave negative results with normal bladder mucosa samples (Table 2). The characteristic dark reddish-brown staining was present in tumor sections from three patients with BTCC grade II and from four patients with BTCC grade III which were stained with the 7E9 Mab and immunoperoxidase (Fig. 1c, d). No staining was seen in the sections from the same tumors that were reacted with anti-HGH Mab and immunoperoxidase (Fig. 1e, f). No positive reactions were

Table 1. Monoclonal antibodies to human urinary bladder carcinomas: indirect cell membrane immunofluorescence using a panel of human cell lines

Cell line	Monoclonal antibody* 7E9	S2C6	T24.06.5	anti HGH
Bladder carcinoma				
T24	50†	60	35	5
RT4	20	35	40	0
TCCSUP	100	100	35	0
EJ	50	80	40	0
SCaBER	25	15	85	0
HU 549	20	100	55	0
HU 961b	5	60	100	5
HU 456	100	100	100	0
HU 1703He	10	25	100	5
Non-malignant urothelium				
HCV 29	15	5	100	0‡
HU 609	15	20	100	0
HU 1734	10	0	100	0
Non-bladder tumors				
MCF 7	0	0	5	0
MDA-MB-231	0	5	NT	0
2T	15	10	0	0
HeLa	85	0	80	0
HS	10	0	15	5
MEL-1	20	10	5	0
RAJI	0	95	10	0
RAMOS	0	80	20	0
P3HR1	0	10	5	0
B95-8	0	85	0	0
MOLT 4	0	0	0	0
DAUDI	0	10	0	0
K562	0	0	0	0
Normal fibroblasts				
LEP	10‡	0‡	0‡	0
154	0‡	0‡	0‡	0

*150–200 cells were examined in each cell sample; samples containing more than 15% of IF^+ cells were considered positive; antibodies were tested in dilutions 1:2, 1:5 and 1:10; results obtained with the 1:5 dilution are shown in this table.
†Percentage of IF^+ cells; mean values from 3–5 experiments.
‡Diffuse, non-specific IF.
NT, not tested.

observed with the 7E9 Mab in normal bladder mucosa sections from four patients with BTCC and in normal bladder mucosa sections from four patients with non-neoplastic diseases. Similarly, no positive reactions were detected with the 7E9 Mab in tumor sections from two patients with mammary carcinoma or in leiomyosarcoma and embryonic carcinoma sections (Table 2). However, an elevated background staining was observed in endothelial cells or muscle tissue of four out of 19 patients and in normal bladder mucosa from one control patient (Table 2).

The 7E9 antibody purified by protein A affinity chromatography and labeled with ^{131}I was used for gamma-scintigraphy. Seven BALB/c nu/nu mice xenografted with T24 bladder carcinoma cells and four control BALB/c nu/nu mice bearing xenografts of mammary carcinoma MDA-MB-231 were injected with 20 μg of the ^{131}I-labeled 7E9 antibody. The T24 xenografts could be located by whole-body gamma-scintigraphy without background subtraction. Clear tumor localization was obtained 72–144 hr following 7E9 antibody administration (Fig. 2a, b). An average of 42.3 (±2.6)% of the total body burden of radiolabeled 7E9 antibody was present per g of tumor 144 hr after injection. The inflammation induced in tumor-bearing mice by subcutaneous injection of 20 μl turpentine oil did not increase localization of the 7E9 antibody at the site of injection. The MDA-MB-231 mammary carcinoma xenografts could not be located with the [^{137}I]7E9 antibody (Fig. 3). The ^{131}I-labeled monoclonal antibody HBCA12 directed against human mammary carcinoma MDA-MB-231, which has been found to be capable of locating the MDA-MB-231 xenografts by gamma-scintigraphy in nude mice ([54]; Kopřivová, unpublished), could not visualize the T24 xenografts. Only 7.2% of the HBCA12 total-body radiolabel was localized per g of tumor 144 hr after injection. The

Table 2. The 7E9 monoclonal antibody: indirect immunoperoxidase staining of frozen tissue sections

Patients (diagnosis)	Tissue	No. positive/total No.
BTCC II°	BTCC*	3/3
BTCC III°	BTCC	4/4
BTCC II°–III°	normal bladder†	0/4
Prostatic hypertrophy	normal bladder	0/2
Urolithiasis	normal bladder‡	0/2
Mammary carcinoma	mammary carcinoma	0/2
Leiomyosarcoma	leiomyosarcoma	0/1
Teratocarcinoma	teratocarcinoma	0/1

*High background staining was observed in endothelial cells of the vessels of two patients.
†High background staining was observed in muscle tissue from two patients.
‡High background staining of normal urothelial cells from one of the two patients was observed.

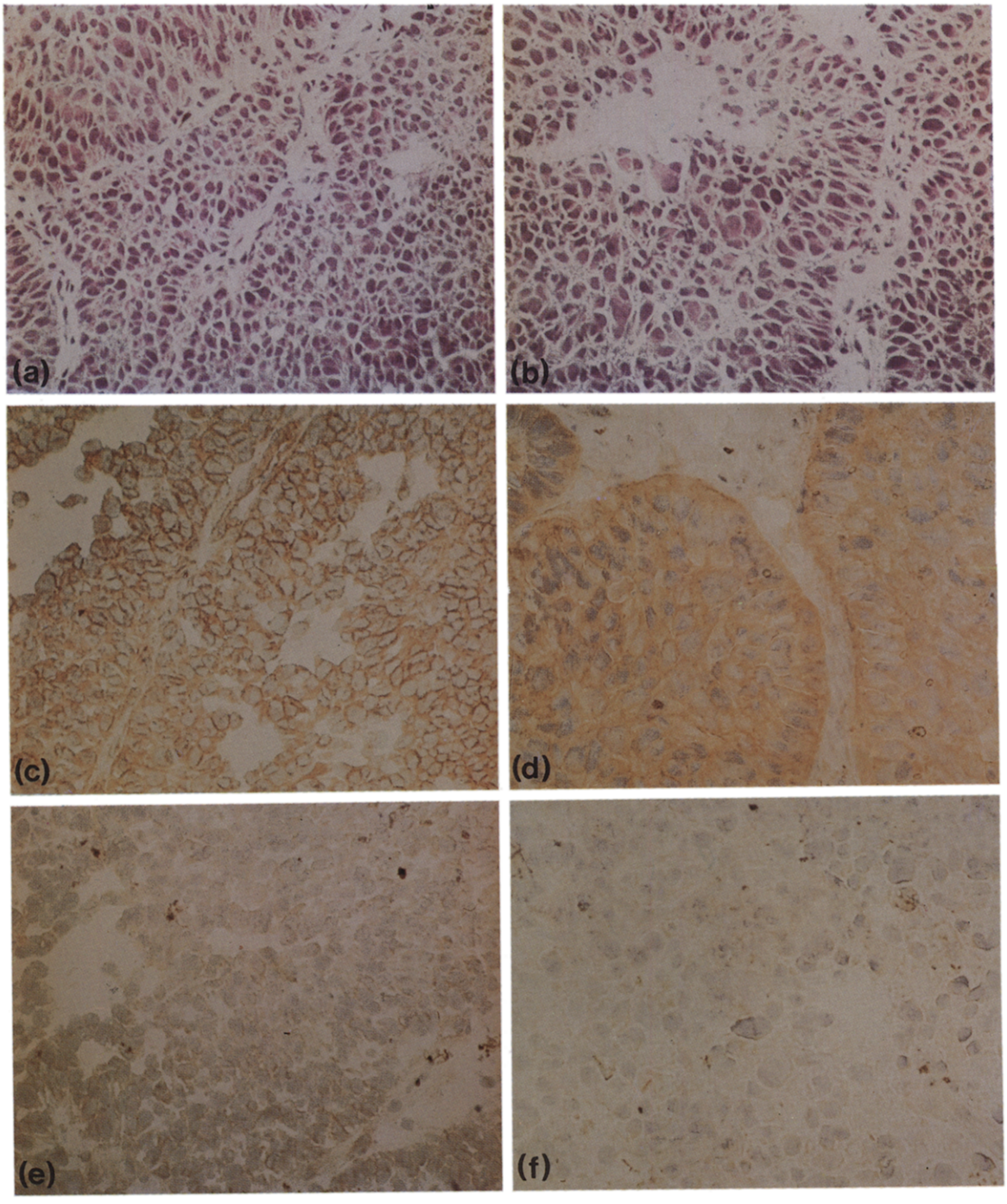

Fig. 1. Frozen tissue sections stained by 7E9 monoclonal antibody and immunoperoxidase. Papillary BTCC grade II, non-invasive (a, c, e). (a) Hematoxylin–eosin; (c) 7E9 Mab, counterstained with Mayer's hematoxylin; (e) HGH Mab, counterstained with Mayer's hematoxylin (×170). *Papillary BTCC grade III with squamous metaplasia, invasive (b, d, f). (b) Hematoxylin–*eosin; *(d) 7E9 Mab, counterstained with Mayer's hematoxylin; (f) HGH Mab, counterstained with Mayer's hematoxylin* (×170). *The tumor cells of both BTCC show strong cytoplasmic staining with 7E9 Mab (c, d); note the broad rim of 7E9 deposits surrounding the centrally located nuclei.*

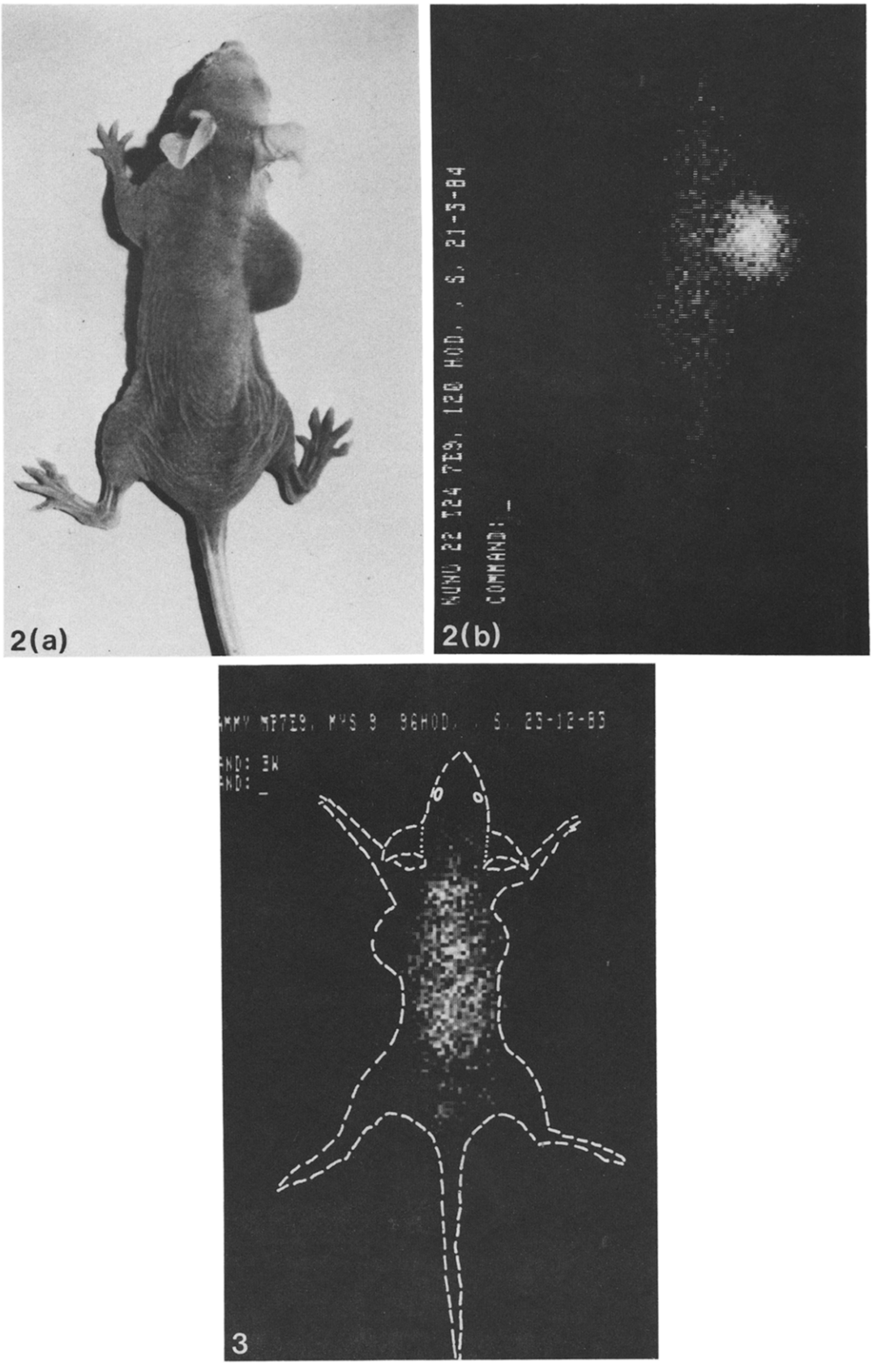

Fig. 2. BALB/c nu/nu mouse with xenograft of human urinary bladder carcinoma T24. (a) Subcutaneously growing tumor: (b) Gamma-scintigraphy with ^{131}I-labeled monoclonal antibody 7E9 given 120 hr before examination.

Fig. 3. Gamma-scintigraphy in BALB/c nu/nu mouse bearing s.c. two large paravertebral xenografts of human mammary carcinoma MDA-MB-231 and given ^{131}I-labeled monoclonal antibody 7E9 96 hr before examination.

T24 xenografts could not be located with the ^{131}I-labeled swine anti-human globulin (SwAHu, Institute of Sera and Vaccines, Prague) or with ^{131}I-labeled antibody to human ferritin.

The concentration of radioactivity in T24 tumor xenografts and in normal mouse organs was compared 144 hr after the inoculation of 20 μg of ^{131}I-labeled 7E9 monoclonal antibody (Table 3). The mice were killed and the tissues removed, washed, blotted dry and weighed. Tissue-specific activities were measured using a gamma counter and the binding indices were calculated. The tissue/blood ratio of radioactivity was found to be the highest in T24 carcinoma xenografts; the concentration of radioactivity was on average 5–10 times higher in the tumor than in the normal organs of the mouse (Table 3).

DISCUSSION

The objective of these studies was to determine the selectivity of mouse monoclonal antibodies directed against human urinary bladder carcinoma cells and to use the monoclonal antibodies for imaging of human urinary bladder carcinoma xenografts growing in nude mice. A variety of mouse monoclonal antibodies have been previously used for the radioimmunolocalization of teratocarcinomas, colon carcinomas, osteogenic sarcomas, hepatocellular carcinomas, mammary carcinomas and melanomas [13–24]; however, imaging of human urinary bladder carcinomas with radiolabeled monoclonal antibodies has not been previously described. Our data demonstrate that the 7E9 Mab, despite its binding to only one epitope [25], can be useful for tumor radiolocalization. Any clinical use of the monoclonal antibody is critically dependent on its selectivity. One of our antibodies, S2C6, was shown to react not only with bladder carcinoma cells but also with EBV-transformed cells, even of marmoset origin. The T24.06.5 antibody reacted with all cell lines derived from human urothelium and did not discriminate between malignant and non-malignant cells; it gave higher positivity in reaction with normal urothelium than with most bladder carcinoma cell lines. The 7E9 Mab was shown in both immunofluorescence tests with tumor cell lines and immunoperoxidase staining of frozen tumor sections to discriminate between malignant and non-malignant urothelium, although occasional reactions with tumor cells of non-urothelial origin were also observed. It is possible that the reactivity of the 7E9 monoclonal antibody with HeLa cells in the immunofluorescence tests may be related to a common ontogenetic origin of the urinary bladder and cervix and that the embryonal epitopes present during the development of the urinary bladder and uterus can be re-expressed after malignant conversion of their epithelium. This question remains to be examined. Similarly, the significance of the elevated background immunoperoxidase staining which has been occasionally observed in the endothelial cells and muscle cells from the bladder wall as well as in the epithelial cells from one of the eight specimens of normal bladder mucosa remains to be elucidated. However, occasional reactions of the 7E9 monoclonal antibody with bladder carcinoma-unrelated cell types should not prevent the use of this antibody for immunodiagnosis, radioimmunolocalization and monitoring of bladder carcinoma patients. The errors caused by the reactions with the unrelated cell types can be prevented and excluded by using a panel or a mixture of several monoclonal antibodies, each recognizing a different epitope belonging to the same or different molecules.

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Table 3. Distribution of ^{131}I-labeled 7E9 monoclonal antibody in mice with urinary bladder carcinoma T24 xenografts

Tissue	Tissue:blood ratio of radioactivity mean (range)*
T24 carcinoma xenograft	2.68 (2.37–2.89)
Lung	0.56 (0.52–0.60)
Heart	0.36 (0.33–0.39)
Liver	0.27 (0.17–0.36)
Spleen	0.25 (0.20–0.31)
Kidney	0.46 (0.41–0.51)
Intestine	0.29 (0.19–0.42)
Carcass	0.23 (0.18–0.29)

*Calculated from ^{131}I cmp/g tissue (wet wt); 3 mice/group; repeated in triplicate with similar results.

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