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Evaluation of an anti-carcinoembryonic monoclonal antibody suitable for immunoscintigraphy

D.W. Moraes[1], E.N.P. Lima[2], I.B. Prado[1,4] and C.R.W. Carneiro[1,3]

[1]Instituto Ludwig de Pesquisa sobre o Câncer, São Paulo, SP, Brasil
[2]Hospital A.C. Camargo, Fundação Antonio Prudente, São Paulo, SP, Brasil
[3]Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brasil
[4]Departamento de Gastroenterologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil

Abstract

An anti-carcinoembryonic antigen (CEA) monoclonal antibody (mAb 6D1.1) was evaluated *in vitro* and *in vivo* to determine its suitability as a tracer for immunoscintigraphy of colorectal carcinomas. Determination of mAb affinity for CEA showed a constant of association of 0.63 ± 0.11 x 10^9 M^{-1}. Binding of technetium-99m (^{99m}Tc)-6D1.1, labeled by a direct method, to human cultured lineages was highly specific. Binding to only CEA-positive LS-174T cells resulted in a saturable curve inhibited by pre-incubation with unlabeled mAb. No binding at all was observed for the human lineages MeWo (melanoma) or ZR75-30 (breast carcinoma), neither of them expressing CEA cells. Intravenous injection of ^{99m}Tc-6D1.1 into nude mice xenografted with human LS-174T tumors resulted in planar images of excellent quality. Localization of an irrelevant mAb labeled with either ^{99m}Tc or iodine-125 was never observed in tumor masses. Biodistribution studies on excised tumoral tissue showed retention of 28.48% of the injected dose per gram of LS-174T tumor. The tumor-to-blood ratio was 3.46. The same analysis performed on the other three human xenografted tumors studied demonstrated that only the CEA-producing HT-29 (colorectal adenocarcinoma) retained ^{99m}Tc-6D1.1 while the other two (ZR75-30 and MeWo) did not. These data demonstrate that this mAb is an adequate tool for targeting CEA-expressing tumors in experimental models.

Correspondence
C.R.W. Carneiro
Departamento de Microbiologia, Imunologia e Parasitologia
EPM/UNIFESP
Rua Botucatu, 862 - 4º andar
04023-900 São Paulo, SP
Brasil
Fax: +55-11-572-3328
E-mail: crwcarneiro.dmip@epm.br

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Introduction

Immunoscintigraphy is a valuable and useful approach in clinical oncology. The use of radiolabeled antibodies directed against tumor antigens can be of practical use, mainly for detection of occult, metastatic or recurrent disease. Also, there are several clinical trials with unmodified monoclonal antibodies (mAbs) or immunoconjugates being used as drugs to treat cancer (1-4).

The main targets of immunodiagnosis and immunotherapy are tumor markers. Among them, carcinoembryonic antigen (CEA) is one of the most studied markers for its ubiquity among adenocarcinomas of digestive tract origin, particularly colorectal carcinomas (CRC) (5). As a consequence, it was the

first human tumor antigen detected in CRC xenografted into nude mice (6) and thereafter in patients (7) using radiolabeled polyclonal antibodies. Soon after, mAbs replaced polyclonal antibodies (8) and are still being used today (9,10).

Imaging protocols have been performed with different radioactive isotopes but, in the nineties, technetium-99m (^{99m}Tc) became an optimal radionuclide for camera scintigraphy and single photon emission computed tomography (SPECT). The main reasons were low cost, ready availability, reduced radiation dose and better imaging (11,12).

The efficacy of mAbs in targeting tumors is dependent on the particularities of the tumor mass such as volume (13), vascularization and heterogeneity of antigen expression (14) as well as on some characteristics of the reagent, such as antigen specificity and association constant, in addition to the radiochemical purity of the mAb solution. As a consequence, the use of labeled mAbs for clinical purposes requires extensive evaluation. Here we report *in vitro* as well as *in vivo* studies that demonstrate the suitability of an anti-CEA mAb obtained in our laboratory as a tracer for immunodetection of human CRC.

Material and Methods

Animals

Swiss *nu/nu* mice were purchased from Taconic Quality Lab. Animals and Service for Research (Germantown, NY, USA) and maintained in an adequate room in the animal house of the Ludwig Institute for Cancer Research, São Paulo, Brazil. Animals were fed germ-free food and water *ad libitum*.

Cell lines

Human colorectal carcinoma cell lines LS-174T and HT-29 as well as the breast lineage ZR75-30 were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). The human melanoma MeWo was kindly provided by Dr. Lloyd Old (Ludwig Institute for Cancer Research, New York, USA). LS-174T and HT-29 were cultivated in MEM, and the other two lineages were cultivated in RPMI-1640, both from Gibco (Grand Island, NY, USA). Media were supplemented with 10% fetal calf serum (Cultilab, Campinas, SP, Brazil) and buffered with 24 mM sodium bicarbonate and 10 mM HEPES. Gentamicin (40 mg/l) and L-glutamine (to a final concentration of 2 mM) were always added. All four human cell lines were xenografted subcutaneously in nude mice and the animals were used for biodistribution studies.

Monoclonal antibodies

The cell line 6D1.1 was obtained by recloning a hybridoma denoted 6D1 (15). Bulk production of the mAb was achieved by ascites induction in BALB/c mice. This IgG_1 antibody was purified by affinity chromatography using the Affi-gel Protein A MAPS II kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. mAb 8F9, an anti-*Staphylococcus aureus* laminin receptor mAb of the same isotype, was used as control in the immunolocalization experiments.

Immunostaining of human CRC

Paraffin-embedded tissue sections of a CRC from a patient surgically treated at the A.C. Camargo Hospital were used in order to analyze the tissue CEA reactivity of mAb 6D1.1. The immunoperoxidase reaction was performed as previously described (16) and the purified antibody was tested at a dilution of 20 µg/ml. Biotin-labeled goat anti-mouse IgG as well as the avidin-biotin-peroxidase complex were from Vector (Burlingame, CA, USA). The reaction was visualized with diaminobenzidine (Sigma Chemical Co., St. Louis, MO,

USA) in the presence of 0.05% H_2O_2.

^{125}I-labeling

Carcinoembryonic antigen (kindly provided by Dr. Jean-Pierre Mach, Institute of Biochemistry, University of Lausanne, Switzerland) and the irrelevant mAb 8F9 were labeled with iodine-125 (^{125}I) by the chloramine T method using iodo-beads (Pierce Chemical Co., Rockford, IL, USA). Labeled proteins were isolated from the free isotope by gel filtration on Sephadex G-25 columns. The specific activity was measured after precipitation with trichloroacetic acid.

Determination of mAb affinity for CEA

The anti-CEA mAb 6D1.1 affinity constant was measured using CEA radiolabeled with ^{125}I to a specific activity of 1.86 µCi/µg. Flat-bottom well strips were coated with purified mAb in increasing amounts (0.03 to 1 µg/well) diluted in 50 µl phosphate-buffered saline (PBS), for 1 h at 37°C. Twelve nanograms of ^{125}I-CEA diluted in 50 µl PBS was added to each well and incubated for 16 h at 37°C. The immunoreactive fraction was determined by linear extrapolation of the bound fraction at infinite antibody excess (17). A binding assay was then carried out by adding increasing amounts of ^{125}I-CEA (1.9 ηg to 244 ηg/well) diluted in 50 µl PBS to multi-well strips previously coated with 500 ηg of purified mAb 6D1.1. After an incubation step of 16 h at 37°C, the amount of free and bound ^{125}I-CEA was determined. Scatchard plot analysis was done after correcting the data for nonspecific binding and for the nonimmunoreactive fraction.

^{99m}Tc labeling

Anti-CEA mAb 6D1.1 and the irrelevant mAb 8F9 were labeled with ^{99m}Tc using a direct method (18). The radioisotope in the form of sodium pertechnetate was obtained by elution with saline from molybdenium-technetium generators provided by the Nuclear Energy Research Institute (IPEN, University of São Paulo, São Paulo, Brazil). Before labeling, purified mAbs were partially reduced by treatment with 14.26 M 2-mercaptoethanol diluted 1:10 in PBS, for 30 min at room temperature. The reducing agent was removed by gel filtration on a Sephadex G-50 column (Pharmacia Fine Chemicals, Uppsala, Sweden).

For labeling, 13 µl of a mixture composed of 5 mg of methylenediphosphonic acid plus 0.75 mg of stannous chloride diluted in 5 ml saline was added to 100 µg of partially reduced mAb. Immediately after, 3 to 4 mCi of sodium pertechnetate in saline was added to a final volume of 500 µl. Labeling efficiency was always analyzed by measuring the radiochemical purity on thin layer chromatography-silica gel (ITLC-SG) (Gelman Sciences Inc., Ann Arbor, MI, USA) using saline and acetone separately as solvents.

^{99m}Tc 6D1.1 binding to cells

Binding of labeled mAb to CEA-producer and CEA-non-producer cell lines *in vitro* was done as previously described (17). Briefly, 2 x 10^5 LS-174T cells/ml were preincubated or not for 30 min at 4°C with unlabeled 6D1.1 (50 µg/ml). Cells were then incubated with 50 µl/ml of ^{99m}Tc-6D1.1 in serial dilutions starting from 5 µg/ml. After 2-h incubation at 4°C cells were washed with PBS-0.1% BSA (w/v) and bound radioactivity was measured in a gamma counter (Minigamma; LKB-Pharmacia, Bromma, Sweden). As controls, MeWo and ZR75-30 lines were similarly tested with omission of the unlabeled mAb preincubation step.

Tumor localization and tissue distribution studies with radiolabeled mAbs

Nude mice bearing tumors derived from

subcutaneous injection of human cell lines in the dorsal region were analyzed. The number of injected cells was 1 x 10^5 per animal for LS-174T and 1 x 10^7 for the other three lines (HT-29, ZR75-30 and MeWo) since the former exhibits a higher rate of tumor growth. All experiments were performed when tumors reached about 1 cm in their largest diameter.

Animals were injected with 20 µg of ^{99m}Tc-6D1.1 (500 to 700 µCi) into the tail vein. As controls, animals bearing LS-174T tumors were injected either with the irrelevant mAb ^{99m}Tc-8F9 or with ^{99m}Tc-6D1.1 plus ^{125}I-8F9. Two micrograms (10 µCi) of the iodine-labeled mAb was used per animal. Animals that received ^{125}I-8F9 had their thyroid blocked by the addition of Lugol's iodine (5%) solution to their drinking water for 3 days before mAb administration.

For the imaging studies animals were anesthetized immediately prior to scintigraphy with an intramuscular injection of 0.1 ml ketamine chlorhydrate (Ketalar®; Aché, Guarulhos, SP, Brazil) and fixed with adhesive tape to a styrofoam board in dorsal decubitus. Radiotracers were administered intravenously through the tail vein in a volume of 100 µl. Planar images were obtained 24 h after mAb administration using a Digital SPECT gamma camera coupled to a computer (Starcam 4000 XR/T, General Electric, Milwaukee, WI, USA). Image acquisition time was 10 min and a high resolution, low energy collimator was used. The photopeak was centered at 140 KeV with a 15% window. Images were enclosed with a zoom factor of 2.0 in 512 x 512 pixel word matrix. All data were stored on hard and flexible disks for further visual analyses.

Biodistribution experiments on animals bearing CEA-producing or -nonproducing lines were evaluated by counting the radiation retained in excised organs and tissues. For numerical quantification of radiotracer distribution animals were exsanguinated before removal of tissues and organs. The tumor plus heart, lungs, liver, spleen, kidneys, stomach, small and large bowels, thyroid, sternum, tail and thigh muscle were removed, washed in PBS and weighed. The amount of radioactivity in each sample was measured using a gamma-counter (Mini-gamma; LKB-Pharmacia). Results are reported as the percentage of injected dose retained per gram of tissue. The ratio between the radioactivity retained in tumors and the radioactivity in normal tissues and organs was calculated individually.

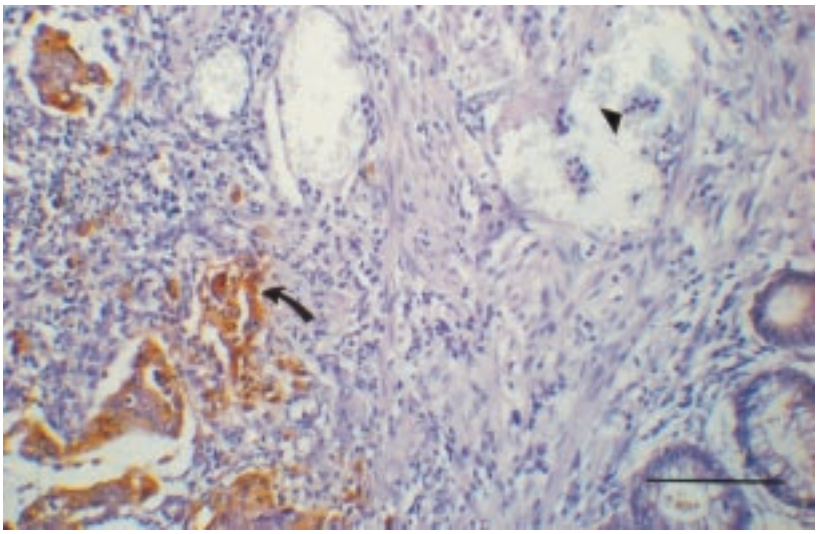

Figure 1 - Immunoperoxidase staining of a colorectal carcinoma tissue section with mAb 6D1.1. Tumor tissue presents positive staining (arrow), whereas neutrophils do not (arrowhead). Bar corresponds to 100 µm. Magnification, 400 X.

Results and Discussion

The literature is rich in reports concerning the utilization of anti-CEA mAbs in experimental models and clinical trials to localize or treat cancer. With the same objective in mind we have generated anti-CEA mAbs that have already proved to be adequate for serological diagnostic purposes (15,19).

One of our mAbs called 6D1 is an IgG_1 antibody with the property of specific recognition of CEA-expressing neoplastic cells in CRC tissue sections by the immunoperoxidase method. Thus, in the present study we

performed a complete evaluation of mAb 6D1 for *in vivo* tumor targeting. For this purpose, the hybridoma was recloned to assure monoclonality and a subclone was designated 6D1.1.

IgG_1 mAb 6D1.1 was also shown to be CEA specific in immunoperoxidase reactions on CRC tissue sections (Figure 1) since it binds only to tumor tissue and not to neutrophils, rich in nonspecific cross reacting antigen (NCA) (20).

Before starting the *in vivo* experimental studies, we measured mAb 6D1.1 affinity for CEA. The association constant (*Ka*) was $0.63 \pm 0.11 \times 10^9$ M^{-1} derived from a linear Scatchard plot as depicted in Figure 2. The percentage of ^{125}I-CEA binding to mAb-coated strips was 40%, indicating a non-immunoreactive fraction of 60% radiolabeled CEA, while nonspecific binding was 2%. The affinity constant order of magnitude suggested adequacy for immunolocalization when compared to literature data (21).

For further study of mAb 6D1.1, ^{99m}Tc labeling was standardized using a direct method (18). Labeling mAb 6D1.1 with ^{99m}Tc was always highly efficient, resulting in 97% radiochemical purity as evaluated by ITLC-SG. This result is in absolute accordance with others (22) and reflects an excellent labeling efficiency.

In vitro assays of ^{99m}Tc-6D1.1 binding to the CEA-producing CRC cell line LS-174T demonstrated that immunoreactivity was maintained after mAb labeling, as demonstrated in Figure 3, where a saturable binding curve of ^{99m}Tc-6D1.1 to LS-174T cells is shown. Specificity was confirmed by the binding inhibition obtained by pre-incubation with unlabeled 6D1.1. No binding was observed to cell lines that do not produce CEA, i.e., the melanoma MeWo line and the breast carcinoma ZR75-30 line. These results were important for planning further 6D1.1 studies *in vivo.*

Imaging experiments were then performed on nude mice individually xenografted with either LS-174T or ZR75-30 cell lines as shown in Figure 4. ^{99m}Tc-6D1.1 was retained in the LS-174T tumor, the CEA-producing cell line (animal B). Also, the unrelated mAb 8F9, identically labeled, did not bind to the LS-174T tumor (animal A), showing that radioactive retention was not a consequence of tumor vascularization. This is one of the possible factors affecting tumor targeting by radiolabeled mAbs. The confirmation of uptake specificity of mAb 6D1.1

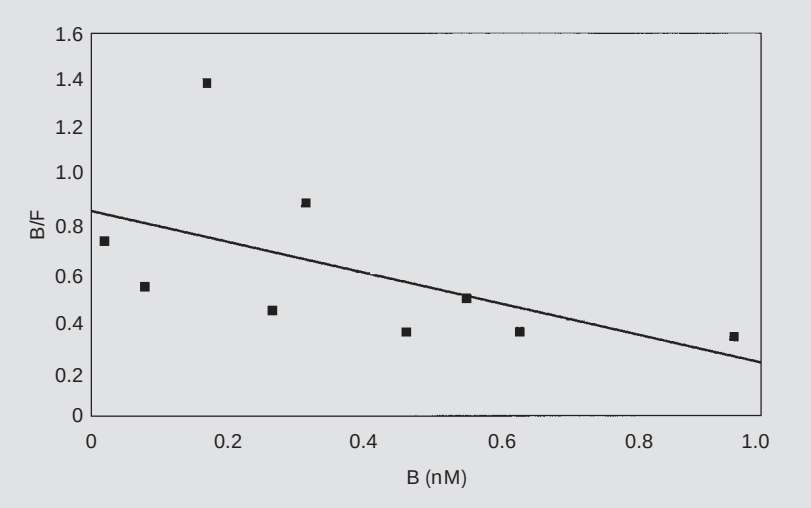

Figure 2 - Determination of mAb 6D1.1 affinity for CEA. Scatchard plot analysis obtained from binding of increasing amounts of ^{125}I-CEA to mAb 6D1.1-coated wells. The calculated affinity constant (*Ka*) was $0.63 \pm 0.11 \times 10^9$ M^{-1}.

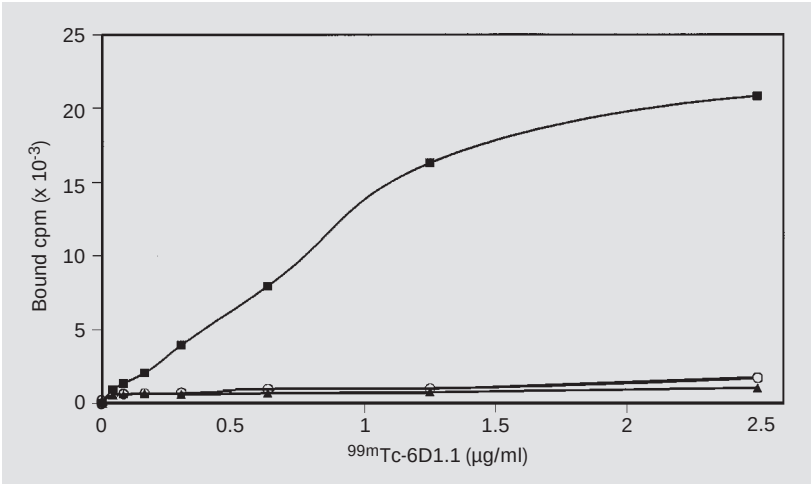

Figure 3 - ^{99m}Tc-6D1.1 binding curves to tumor cell lines: LS-174T (CEA positive), without (squares) and with (circles) prior to incubation with unlabeled antibody, and MeWo (CEA negative; triangles).

by CEA-producing tumors is the non-visualization of non-CEA-producing ZR75-30 tumor (animal C).

These results were further corroborated by experiments where both ^{99m}Tc-6D1.1 and ^{125}I-8F9 mAbs were injected simultaneously into the same animals. Only the anti-CEA mAb was concentrated in the CEA-expressing tumor as demonstrated in Figure 5. Taken together, these *in vivo* data are compatible with the *in vitro* results, indicating specificity and adequate labeling yield. Concerning the latter, it is worth mentioning that thyroid and/or stomach uptake, which was not observed, would indicate an excess of free ^{99m}Tc.

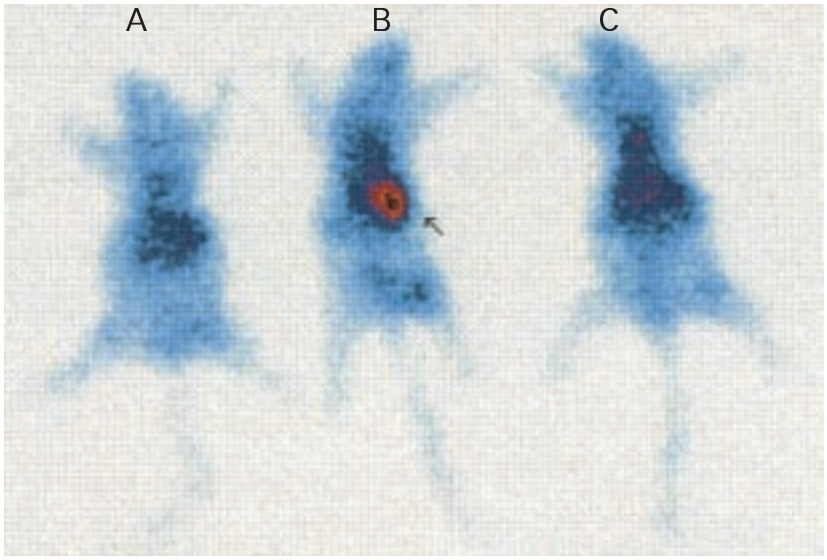

Figure 4 - Images of nude mice bearing tumors of 1 cm in their largest diameter treated with ^{99m}Tc-labeled mAbs 24 h after injection. Animals: A) isotype control 8F9 mAb in a CEA-positive tumor (LS-174T); B) anti-CEA 6D1.1 mAb in a CEA-positive tumor (LS-174T) indicated by the arrow, and C) anti-CEA 6D1.1 mAb in a CEA-negative tumor (ZR75-30).

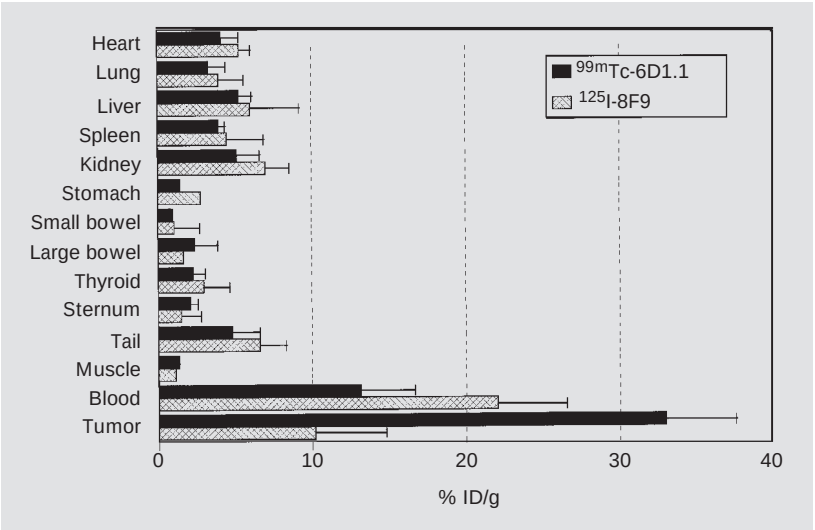

Figure 5 - Biodistribution of radiolabeled mAbs injected simultaneously in mice xenografted with the LS-174T line and analyzed 24 h after injection. Black bars represent ^{99m}Tc-6D1.1 anti-CEA mAb and grid bars, unrelated ^{125}I-labeled mAb 8F9. Results are reported as % of injected dose per gram of tissue (% ID/g) obtained as the mean values ± SD from three animals.

To quantitate antibody capture in different tumor masses in comparison to normal tissues and organs and to further evaluate the efficacy of ^{99m}Tc-6D1.1 biodistribution, experiments were performed in animals xenografted with four cell lines expressing or not CEA. For comparison we used two lines that express CEA in different amounts and two lines that do not. Table 1 shows that the obtained tumor-to-blood ratio for the lines that do not express CEA (MeWo and ZR75-30) was lower than 1.0, while the CRC presented ratios of 3.46 for LS-174T and 2.6 for HT-29. These results once again confirm CEA recognition by ^{99m}Tc-6D1.1 and also demonstrate that different CEA-producing tumors can be targeted. It is necessary to mention that we have shown elsewhere (23) that the HT-29 line expresses approximately three and a half times less CEA than LS-174T when CEA is measured in membrane-enriched cell extracts. The tumor-to-blood ratios obtained are as good as those described by others who studied anti-CEA mAbs (21,24).

Taken together, the presented results demonstrate that mAb 6D1.1, apart from being a good reagent for detecting CEA in neoplastic tissue sections, is a highly suitable tool for experimental immunoscintigraphy in human CRC tumor models.

The obvious next step should be the utilization of this mAb in CRC patients to locate metastatic lesions. Nevertheless, more stringent tests to confirm CEA specificity are required before human trials are started. Thus, using binding assays to CEA-family transfectants and to isolated human granulocytes

it was demonstrated that mAb 6D1.1 probably recognizes non-specific cross-reacting antigen, NCA-95 (personal communication, Dr. Fritz Grunert, Institute of Immunobiology, Albert-Ludwigs University, Freiburg, Germany). The apparent contradiction between this last result and the immunoperoxidase reaction (Figure 1) may be explained either by the possibility that the epitope recognized by this mAb was damaged by the tissue section fixation procedure which resulted in neutrophil nonstaining or by the lower sensitivity of this reaction. In view of these last results, we are not yet authorized to use this mAb in patients.

On the other hand, the importance of this report is also that it provides the complete sequence of experiments necessary for previous evaluation of any mAb potentially useful for targeting human CRC *in vivo.*

Table 1 – Tumor (T)-to-normal-tissue ratios in mouse tissues obtained 24 h after ^{99m}Tc-6D1.1 mAb injection.

Tumor-to-normal tissue ratios were obtained by dividing the concentration of radioactivity in tumors by those of each normal tissue indicated. LS-174T and HT-29 are CEA-producing cells while ZR75-30 and MeWo are not. Results for the two groups composed of more than two animals are presented as means ± SD.

	ZR75-30	MeWo	LS-174T	HT-29
T/blood	0.65 ± 0.16	0.35	3.46 ± 0.88	2.61
T/liver	1.18 ± 0.10	0.62	7.86 ± 2.68	6.64
T/kidney	0.94 ± 0.29	0.61	4.57 ± 1.01	4.79
T/lung	1.64 ± 0.38	2.61	9.28 ± 2.35	5.38
T/spleen	1.78 ± 0.48	0.98	13.77 ± 2.93	6.40
Number of animals	4	2	6	2

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