Radioimmunolocalization of Neuroblastoma Xenografts with Chimeric Antibody chCE7


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This study was performed to evaluate the tumor targeting ability of chCE7 with a view to clinical applications in neuroblastoma imaging and therapy. A chimeric (mouse/human) monoclonal antibody (chCE7) of gamma 1/kappa isotype directed against a neuroblastoma-associated cell-surface glycoprotein is described. In vitro chCE7 binds with high affinity (Kd ~ 1 x 10^-10 M) to SKN-AS human neuroblastoma cells. Binding studies with 125I-labeled chCE7 show temperature-dependent modulation of antigen binding and indicate that a proportion of the bound antibody is internalized due to rapid antigen turnover. In vivo biodistribution of radioiodinated chCE7 in nude mice bearing SKN-AS tumors shows optimal tumor uptake after 24 hr with about 30% of the injected dose per g. Optimal tumor/blood ratios (3.4:1) are reached after 4-5 days. Uptake in other organs including the reticuloendothelial system is low with tumor/organ ratios of 10 and more. Tumor uptake of chCE7 and the parent murine CE7 are found to be similar. Stability of chCE7 during and after radioiodination is good with no loss of immunoreactivity in preparations labeled with 125I up to 100 mCi/mg and 80% immunoreactivity after labeling with 13 mCi/mg of 131I. Neuroblastoma xenografts can be imaged by radioimmunoscinintigraphy with 125I- and 131I-labeled chCE7.


Radioimmunotherapy (RIT), which employs targeting of radioactivity to tumors via tumor-specific monoclonal antibodies (Mabs), has so far given good results with systemic tumors such as B cell lymphomas (1,2). It may also hold potential for some highly radiosensitive solid tumors, including neuroblastoma. Prognosis for neuroblastoma is poor and patients frequently become refractory to chemotherapy after a certain time (3). In such cases, RIT could support conventional therapies. A number of murine Mabs directed against neuroblastoma-associated antigens have undergone clinical testing (3-7). In contrast to uses in imaging, where only small amounts of Mabs (100-300 µg) are applied as a single dose, RIT has to employ repeated higher Mab doses (1 mg or more). Previous experience has shown that in such circumstances the human immune response against murine Mab can jeopardize successful application. The construction, by genetic engineering, of chimeric Mabs consisting of the original murine variable region attached to a human constant region may help to overcome this problem. Therefore, a murine Mab CE7 directed against a neuroblastoma-associated cell surface protein (8) was changed to a human/mouse chimeric form—chCE7.

Here we investigated tumor cell binding properties of chCE7, sensitivity to radioiodination and tumor localization in neuroblastoma xenografts. We found that immunocomplexes are internalized into neuroblastoma cells to some extent and that chCE7 has high affinity, high selective tumor uptake and good resistance to radioiodination.

MATERIALS AND METHODS

Cells and Antibodies

The human neuroblastoma cell line SKN-AS was kindly provided by Prof. Blaser, SIAF, Davos, Switzerland. It was grown in Eagles minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS), 1 mM glutamine, 1% nonessential amino acids, 50 U/ml penicillin and 50 mg/ml streptomycin (Amimed AG, Basel, Switzerland).

Construction and expression of chCE7 will be reported elsewhere, but it was purified from tissue culture supernatants with protein G-sepharose (Pharmacia). Iodine-125 was purchased from Nordion and 123I and 131I were purchased from the Paul Scherrer Institute, Villigen, Switzerland.

Radioiodination

chCE7 (100 µg) in 0.10 M NaCl, 0.05 M phosphate buffer pH 7.3 (PBS) was labeled in a total volume of 300 µl PBS with 0.5 mCi (18.5 MBq) of either 125I, 123I, or 131I using glass tubes coated with 20 µg of iodogen (Pierce) for 15 min on ice. In some experiments, 100 µg of antibody were labeled with higher activities of up to 19.5 mCi for 125I and 2.3 mCi for 131I, as discussed later in the text. Labeled antibody was purified on Sephadex G10 columns (PD 10, Pharmacia), which were equilibrated in PBS containing 0.5% of human serum albumin (HSA). Activities were typically 4 mCi per mg of chCE7.

Cell Binding Assays

SKN-AS cells (up to 4 x 10^6 cells/well) in EMEM medium containing 10% FCS were seeded on 24-well microtiter plates.
Plates were centrifuged briefly (2 min, 200 x g) and cells were allowed to attach for 2 hr at 37°C. Medium was then removed and cell layers were dried at room temperature for 3 days in a laminar flow hood. Cell plates could be stored at -20°C for several months.

Prior to use, plates were presaturated with 0.5 ml PBS containing 0.5% HSA for 60 min at 37°C. Binding assays were performed in duplicate wells by incubating 10^5 cpm 125I-chCE7 in 0.5 ml PBS-HSA for 16 hr at 37°C. Nonspecific binding was determined in parallel in the presence of 5 µg of unlabeled chCE7 and was subtracted. After incubations plates were washed three times with ice cold PBS-HSA, cells were dissolved in 0.5 ml 1 N NaOH and radioactivity was counted in a gamma counter.

Immunoreactivity of radiolabeled chCE7 preparations was measured by cell binding assays using 10^5 cpm 125I-chCE7 and increasing numbers of cells (0.2 x 10^6 to 4 x 10^6 cells/well). Double reciprocal plots of percentage 125I-chCE7 bound versus cell number were analyzed according to Lindmo (9), the extrapolation to infinite cell number indicating the immunoreactive fraction.

Affinity of chCE7 for its binding site on SKN-AS cells and the number of binding sites/cell was determined by incubating duplicate wells of 1 x 10^6 cells with 10^5 cpm of 125I-chCE7 and increasing concentrations of unlabeled chCE7 (1.8-1800 ng). Analysis of the saturation curves was done according to the Scatchard method.

In some experiments, binding of radiiodinated chCE7 to live cells in suspension was studied. Cells were suspended in duplicate samples in 0.4 ml PBS-HSA or in complete medium, 0.1 ml of 125I-chCE7 (1 x 10^5 cpm) was added and incubations were made on shaking platforms at 37°C or on ice. Cells were then washed with 3.0 ml PBS-HSA twice by centrifugation. In some experiments, acid stable binding was measured after incubations with 125I-chCE7 at the indicated time points. Cells were first washed twice with ice cold PBS-HSA and then kept for 10 min in 1.0 ml of 0.1 M NaCl, 0.05 M glycine-HCl pH 2.8. After centrifugation, cells were then washed once in PBS-HSA and the radioactivity associated with the cell pellets was measured.

**Measurement of chCE7 Biodistribution in Tumor-Bearing Nude Mice**

Adult female nude mice (strain ICR-ZH nu/nu), obtained from the Institute für Versuchstierforschung, University of Zürich, Switzerland, were used. Groups of two mice were anesthetized with ether and injected with 5 x 10^6 SKN-AS cells subcutaneously on each flank, resulting in tumors of 100-600 mg weight after 10-12 days. Animals received injections with 30-50 µCi of 125I-labeled chCE7 (10 ìg) into a tail vain. At the indicated time points, animals were killed. Tumors, blood samples and the major organs (heart, spleen, kidney, stomach, bowel, liver, muscle) were removed, organs were rinsed in PBS, weighed and radioactivity was measured in a gamma counter. The percent injected dose (%ID) was determined by measuring in parallel to the organ samples a diluted aliquot of the injected preparation. Results are means of four animals (two groups of two) and are expressed as %ID/g of tissue or as tumor/tissue ratios.

Whole-body clearance of chCE7 was determined by measuring activity of animals at the indicated time points in a Veenstra radioisotope detector CRC-100. In other groups of mice, uptake of 125I-labeled chCE7 in control tumors not expressing the antigen (LoVo, human colon adenocarcinoma cells) was measured.

**Gamma Scintigraphy**

Tumor-bearing mice were anesthetized and injected intravenously with 40-50 µCi of 125I-chCE7 or 131I-chCE7. Mice were imaged with a Siemens Basicam camera and Microdelta computing system. A pinhole collimator positioned 10 cm from the animal was used and pictures generated from 50,000 counts were taken.

**RESULTS**

**Binding of chCE7 to SKN-AS Cells: Effect of Temperature**

Binding kinetics of 125I-labeled chCE7 to live SKN-AS cells was studied at 37°C and 0°C (on ice). Figure 1 shows the results, binding at 0°C (open circles) increasing over a time period of 6 hr and leveling off between 6 and 24 hr. In contrast, binding at 37°C (closed circles) increases more rapidly, levels between 1 and 2 hr and drops between 4 and 24 hr, with two-thirds of the maximal binding lost after a 24-hr incubation.

In order to find out if the decrease in 125I-chCE7 binding observed at 37°C is due to internalization of the antibody followed by degradation or to shedding of the antigen, a number of experiments were performed. First, cells were preincubated for the indicated time at 37°C and at 0°C and then incubated further for 1 hr at 0°C with 125I-chCE7 to detect the remaining binding sites. Figure 2A shows that binding sites for chCE7 are lost upon incubation at 37°C. The presence of unlabeled chCE7 does not significantly affect this process (data not shown). The effect was observed when cells were incubated either in PBS-HSA buffer or in the complete culture medium. In order to test if this effect was due to shedding of the antigen, supernatants from cells kept for 6 hr either at 37°C or at 0°C were tested for their activity in blocking the binding of 125I-chCE7 to dried monolayers of SKN-AS cells, which were prepared as described in Materials and Methods. No effect was found. Similarly, when culture media from cells cultivated

**FIGURE 1.** Time course of chCE7 binding to SKN-AS cells. Iodine-labeled chCE7 (1 x 10^5 cpm) was incubated at 37°C (closed circles) or 0°C (open circles) for the indicated times with live SKN-AS cells in suspension and cell binding was measured. Data are means ± s.d. of two experiments performed in duplicates and are expressed as percentage of maximum binding of 125I-chCE7 per 1 x 10^6 cells.
can be dissociated from the cells by acidic buffer (13) at 37°C or at 0°C. Figure 2B shows that at 0°C only a small portion of the total binding is not removed by an acid wash. In contrast, at 37°C, acid stable binding increases rapidly with time and after a 2-hr incubation time 35%–40% of the total binding of 125I-chCE7 is acid stable. This value corresponds roughly to the loss of binding sites from the cell surface observed after 2 hr at 37°C (Fig. 2A). Taken together, these results suggest that the rapid initial loss of binding sites at 37°C is due to internalization of the antigen and the acid stable binding of 125I-chCE7 observed at 37°C represents internalization of the chCE7 antibody-antigen complex.

Binding of chCE7 to SKN-AS Cells: Determination of B\textsubscript{max} and K\textsubscript{D}

chCE7 was labeled with 125I and cell binding to SKN-AS cells (which were used as the target tumor xenograft) was measured. Twenty-four well microtiter plates with cells dried to wells were prepared and used as described in Materials and Methods. Iodine-125-chCE7 shows slower binding to dried cell monolayers than to suspensions of live SKN-AS cells and equilibrium binding equivalent to the level with live cells in suspension incubated at 4°C was reached only after an overnight (16-hr) incubation period at 37°C. Because dried cells show no modulation of binding by temperature and can be prepared in advance and stored at −20°C for several months, they were used for all subsequent in vitro binding experiments.

Immunoreactivity of iodinated chCE7 preparations was measured by cell binding to increasing numbers of cells. A typical saturation curve is shown in Figure 3A. Nonspecific binding of 125I-chCE7, which is not displaced in the presence of 5 μg of unlabeled chMab, ranges between 1% and 3% of total binding (lowest trace, Fig. 3A) and was subtracted. Figure 3B shows a double reciprocal plot of the data according to the method of Lindmo (9). Extrapolation to the y-axis indicates an immunoreactivity of 100%.

The number of binding sites and their affinity for chCE7 on SKN-AS cells was determined by incubating 1 × 10⁶

FIGURE 3. Binding of chCE7 to increasing numbers of SKN-AS cells (A) and double-reciprocal plot of the saturation curve (B). Iodine-labeled chCE7 was incubated at 37°C for 16 hr with 0.2 × 10⁶ to 4 × 10⁶ SKN-AS cells (dried cells) and cell binding was measured. Data are from a typical experiment performed in duplicates. (A) Open circles represent specific binding, closed circles show corresponding nonspecific binding which is not displaced by 5 μg/well of unlabeled chCE7. (B) Extrapolation of the double-reciprocal plot of the data from Figure 2A to the y-axis (cpm 125I-chCE7 added/cpm 125I-chCE7 specifically bound) results in an immunoreactivity of 100%.
cells with 100,000 cpm $^{125}$I-chCE7 and increasing amounts (1.8–1800 ng) of unlabeled chCE7 for 16 hr at 37°C. Saturation curves (inset in Fig. 4) were analyzed according to the Scatchard method and gave typical values of 44,000 sites per cell and a $K_D$ of $1 \times 10^{-10}$ M ($B_{max}$ values of four experiments were 53,200, 40,400, 43,000, 40,000).

**Biodistribution of $^{125}$I-chCE7 in Nude Mice Bearing SKN-AS Tumors**

Iodine-125-chCE7 (30–50 µCi) was injected into a tail vein of mice bearing SKN-AS tumors weighing between 100–600 mg. Whole-body clearance as well as organ distributions were measured over a period of 7 days as described in Materials and Methods. Figure 5 shows clearance of chCE7, about 40% of the injected dose remaining in the animal after 5–7 days.

Table 1 shows chCE7 levels as %ID/g in tumors and various organs after 1–7 days. A maximal tumor uptake of about 32% ID/g is visible after 1 day, when blood levels are about 12% ID/g and all other organs except the heart (4.2% ID/g) 2.8% ID/g or less. At later time points, both blood and tumor levels of $^{125}$I-chCE7 decreased to about 15% ID/g in tumors and 6.2% ID/g in blood after 7 days. The best tumor/blood ratio was obtained at Day 5 (3.4:1). Throughout the measured time, levels in liver, spleen, kidneys and the other measured organs were low (2% ID/g or less).

The main focus of this study was to describe cell binding and tumor targeting properties of the chimeric CE7 Mab. However, it was also of interest to compare tumor uptake of the parent murine CE7 with chCE7. The two Mabs labeled with $^{131}$I were compared at a single time point (3 days postinjection) by measuring uptake in SKN-AS xenografts and some major organs. Table 2 shows the data, indicating essentially similar tumor and tissue uptake.

**Radiolabeling of chCE7 with $^{125}$I and $^{131}$I: Effects of Increasing Specific Activity on Immunoreactivity**

Labeling of Mabs with $^{125}$I and $^{131}$I for clinical uses in imaging and RIT requires high-specific activity preparations. We therefore investigated the sensitivity to radiolabeling of chCE7 over a range of specific activities. chCE7 (100 µg) was iodinated with the indicated amounts of $^{125}$I or $^{131}$I and immunoreactivity of the resulting preparations was measured as indicated in Materials and Methods.

Table 3 shows that chCE7 can be labeled with $^{125}$I, an appropriate isotope for diagnostic purposes, up to a specific activity of 100 mCi/mg without affecting immunoreactivity. This Mab is more sensitive to $^{125}$I where labeling with 13 mCi/mg results in 80% immunoreactivity and a specific activity of 23 mCi/mg leads to impaired (28%) immunoreactivity.

**Imaging of SKN-AS Tumors with $^{125}$I**

Figure 6 shows gamma scintigraphy of tumor-bearing mice injected intravenously with 40 µCi of $^{125}$I-labeled

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**Figure 4.** Binding of $^{125}$I-chCE7 to SKN-AS cells: Scatchard plot of the saturation curve (inset). Iodine-125-labeled chCE7 was incubated at 37°C for 16 hr with $1 \times 10^6$ SKN-AS cells (dried cells) in the presence of increasing amounts of unlabeled chCE7 ranging between 1.8 ng and 1800 ng. Cell binding was measured as described in Materials and Methods. Data (nonspecific binding in presence of 5 µg of unlabeled chCE7 subtracted) are from a typical experiment performed in duplicate resulting in a $B_{max}$ of 44,000 sites/cell and a $K_D$ of $1 \times 10^{-10}$ M.

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**Figure 5.** Whole-body clearance of $^{125}$I-chCE7 from tumor-bearing nude mice. Thirty to 50 µCi of $^{125}$I-chCE7 were injected intravenously into mice bearing SKN-AS tumors and whole-body activity was measured at the indicated time points as described. Data (% of initial activity) are means ± s.e.m. of four animals.

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**Table 1**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Days after injection</th>
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<tr>
<td></td>
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<tr>
<td>Tumor</td>
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<tr>
<td>Blood</td>
<td>12.1 ± 2.0</td>
</tr>
<tr>
<td>Heart</td>
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<tr>
<td>Liver</td>
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<td>Spleen</td>
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<td>Kidney</td>
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</tr>
<tr>
<td>Stomach</td>
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</tr>
<tr>
<td>Bowel</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.3 ± 0.04</td>
</tr>
</tbody>
</table>

Data are means ± s.d. of four animals and are expressed in %ID/g tissue.
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chCE7. Images were obtained after 2 hr (Fig. 6A) when activity is still circulating in the blood, after 24 hr (Fig. 6B) with the tumors clearly visible and after 48 hr (Fig. 6C) when background is further reduced. Similar imaging can be performed with 131I-labeled chCE7 (data not shown).

Table 3 compares tumor/organ ratios after injection of 123I-labeled chCE7 in mice bearing neuroblastoma (SKN-AS) tumors and in separate animals with control colon adenocarcinoma (LoVo) tumors. Whereas tumor/tissue ratios in neuroblastoma-bearing mice increase over a time period of 48 hr, the same values remain low in colon carcinoma-bearing animals, indicating neuroblastoma-specific uptake of chCE7.

DISCUSSION

The chimeric Mab chCE7 shows high and selective tumor uptake in neuroblastoma xenografts (Table 1, Table 3, Fig. 6). The absolute amounts of radioactivity delivered to such tumors (about 30% ID/g tumor after 24 hr) and the low uptake in other organs, such as liver, spleen or kidney, are comparable to the antiangiogloside (G02) Mab 3F8 described previously (10). Interestingly, in contrast to similar biodistributions in tumor-bearing mice, the in vitro binding of these two Mabs is different in several aspects.

**TABLE 3**

Effect of Increasing Specific Activity on Immunoreactivity of chCE7

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Activity (mCi)</th>
<th>Immunoreactivity (%)</th>
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<tr>
<td>123I</td>
<td>0.5</td>
<td>100</td>
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<tr>
<td></td>
<td>1.3</td>
<td>100</td>
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<tr>
<td></td>
<td>1.7</td>
<td>100</td>
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<tr>
<td></td>
<td>10.0</td>
<td>100</td>
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<td></td>
<td>19.5</td>
<td>50</td>
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<tr>
<td>131I</td>
<td>0.8</td>
<td>100</td>
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<tr>
<td></td>
<td>1.3</td>
<td>80</td>
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<tr>
<td></td>
<td>1.7</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>28</td>
</tr>
</tbody>
</table>

chCE7 (100 μg) was labeled with the indicated amounts of 123I or 131I and immunoreactivity was measured as described in Materials and Methods.

chCE7 shows saturable, high affinity binding to SKN-AS cells (Fig. 4). Its affinity (Kᵦ ≈ 1 × 10⁻¹⁰ M) is about 10-fold higher than the affinity reported for 3F8 (11), whereas its B_max on SKN-AS cells (40,000–50,000, Fig. 3) is about 100-fold less than the B_max of 3F8 on its target cells (11). Among the many factors contributing to the tumor targeting ability of a Mab, a high number of binding sites at the surfaces of target cells is thought to be important. Our results with chCE7 indicate that other properties of this Mab, such as high affinity as well as possible in vivo properties of the xenograft system used (tumor vascularization for instance), can lead to good tumor uptake despite not very high antigen expression. Another factor involved in tumor uptake is modulation of antibody binding at the tumor cell surface by shedding or internalization of the antigen. In contrast to the G02 antigen, we could find no evidence for shedding of the antigen recognized by chCE7. However a number of binding experiments with SKN-AS neuroblastoma cells (Figs. 1 and 2) indicate that the antigen turns over rapidly at the cell surface. Binding sites at the cell surface were measured as well as acid-stable binding [presumed to be due to the intracellular antibody pool (13)]. It was found that at 37°C about half of the binding sites are lost from the cell surface (Fig. 2A) after 2 hr.
independent of the presence or absence of unlabeled antibody and that concomitantly acid stable binding increased to up to 40% of the total binding (Fig. 2B). These results suggest that about 40% of the total immunocomplexes present at the cell surface are internalized within 2 hr at 37°C and that this process is not mediated by the antibody itself.

Binding of 125I-chCE7 to SKN-AS cells is highly temperature-dependent (Fig. 1), with binding at 37°C increasing rapidly and falling off after 4 hr of incubation, whereas binding at 0°C increases more slowly and stays at maximal levels up to 24 hr. The decrease in binding over time at 37°C is difficult to dissect experimentally, since it could consist of effects of degradation and release of radioactivity from internalized antibody as well as of other effects due to decreased cell vitality.

Tumor uptake of iodinated chCE7 was found to be high (Tables 1–3). However, it is known, that when iodinated antibodies are internalized and degraded intracellularly, radioactivity is released. It could be expected that chCE7, which is in part internalized, will show even higher tumor uptake and stability when labeled with metallic radio nuclides which are retained within cells.

The parent murine CE7 Mab shows good tumor uptake comparable to the chimeric CE7 in neuroblastoma-bearing nude mice Table 2 (14). CE7 recognizes a 190,000 Mr cell surface glycoprotein and was found to be highly specific for neuroblastomas, independent of their histological grades, when tumors of 15 patients of various stages were tested (8). In this respect, it should be superior to the neuroblastoma-seeking drug metaiodobenzylguanidine (MIBG), which is not always taken up into metastases (15). CE7 showed no binding to blood cells and normal non-neuroectodermal tissues and very weak binding to some normal sympatbo-adreno-medullary cells (8). Although the murine parent CE7 Mab does not activate human complement, the chimeric chCE7 is able to activate complement to the same degree as purified human IgG, as will be reported elsewhere. It remains to be seen how this effector function influences biodistributions in patients. The two neuroblastoma Mabs that have been undergoing clinical trials (3F8, UJ13A) also show no binding to non-neuroectodermal tissues, but their antigen is localized on some peripheral sympathetic nerves, notably on peripheral pain fibers (4). The reported transient side effects of 3F8, including severe pain (11) may either be due to this property or may be induced by the cytotoxic IgG3-isotype of this Mab. Such side effects, however, do not seem to be apparent in 131I-labeled preparations of this Mab (7).

Neuroblastoma tends to metastasize early during the progress of the disease and radiosensitive, disseminated tumors have been suggested as promising cases for the application of RIT. This underlines the potential of RIT for the treatment of neuroblastoma and warrants the clinical testing of new neuroblastoma-specific antibodies. Our results on the tumor targeting ability of the chimeric Mab chCE7 provide the rationale for starting its clinical evaluation.

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