BIO DISTRIBUTION OF A MONOCLONAL ANTIBODY (RNL-1) AGAINST THE NEURAL CELL ADHESION MOLECULE (NCAM) IN ATHYMIC MICE BEARING HUMAN SMALL-CELL LUNG-CANCER XENOGRAFTS

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The purpose of this investigation was to determine the target potential of the murine monoclonal antibody (MAB) RNL-1 for human small-cell lung cancer (SCLC) in a nude mouse model. RNL-1 is preferentially reactive with SCLC and lung carcinoids, and was classified as a cluster-I MAB as defined by the International Workshop on Small-Cell Lung Cancer Antigens. From the intercellular location of the target antigen and its reactivity with 3T3 cells transfected with nucleic acid sequences encoding for the neural cell adhesion molecule (NCAM), it was concluded that RNL-1 is directed against NCAM. RNL-1 was radiolabelled with either 125Iodine or 111Indium and injected into nude mice bearing NCI-H82 SCLC xenografts. The biodistribution of the radiolabels was determined up to 120 hr post injection. Maximum tumour accretion for 111In-RNL-1 was 11.8%ID/g and 6.5%ID/g for 125I-RNL-1. The accumulation of 111In-RNL-1 could be visualized clearly by gamma scintigraphy without background subtraction techniques. Autoradiographs of whole-body sections from animals injected with 125I-RNL-1 showed that activity in the SCLC xenografts was mainly peripheral, suggesting that tumour uptake is dependent on the vascularization of the tumour tissue.

The subdivision of lung cancer into small-cell lung cancer (SCLC) and non-SCLC is of clinical significance, since initially SCLC, unlike the other forms of lung cancer, is highly sensitive to both chemotherapeutic agents and radiotherapy (Minna et al., 1985). However, in most cases recurring tumours develop, which in general are extremely therapy-resistant. Therefore, there is continuous interest in the application of immunological techniques for the management of the disease.

Monoclonal antibodies recognizing SCLC associated antigens have been developed in several laboratories (De Leij et al., 1985; Bernal and Speck, 1984; Wahid et al., 1987). In a recent International Workshop on Small Cell Lung Cancer Antigens (Sourhami et al., 1988; Beverley et al., 1988) a cluster of antibodies, the so-called cluster-1 antibodies, was distinguished which was characterized by the recognition of an antigen commonly present on the SCLC cell surface but absent in most non-SCLC. Later, this group of antibodies appeared to recognize NCAM epitopes (Patel et al., 1989). We have recently developed a monoclonal antibody (RNL-1) recognizing NCAM, which was shown to detect SCLC (Broers et al., 1991).

Since radiolabelled antibodies against SCLC cell surface antigens are potential tools for radio-immunodetection, and may have therapeutic potential if conjugated to drugs, toxins or radionuclides, we have investigated the possibilities of using the radiolabelled RNL-1 antibody to localize human SCLC xenografts in a nude mouse model.

MATERIAL AND METHODS

Cell line

The NCI-H82 cell line (Carmey et al., 1985) was grown in RPMI 1640-based culture medium (Gibco, Paisley, UK) supplemented with 13% newborn calf serum (Flow, Rickmanworth, UK).

1Monoclonal antibodies

The RNL-1 antibody (IgG, isotype) raised against the variant SCLC cell line NCI-H82 has been shown to be reactive with SCLC, lung carcinoids, renal cancers, and some sarcomas. The antibody was reactive in normal neuronal tissues, and endocrine glands, such as pancreatic islet cells, pituitary gland, and adrenal medulla. Expression was also observed in Leydig cells of the testis, in the thyroid and in smooth-muscle cells of the small intestine, colon and bladder (Broers et al., 1991). The anti-interferon MAb B140 (IgG, isotype), kindly provided by Dr. C. Pak (Canton, Malvern, PA), was used as a control antibody in the animal experiments.

Flow cytometric analysis

The reactivity of RNL-1 with human blood cells was tested by flow cytometry. Blood mononuclear cells were isolated fromuffy coats by density centrifugation on 1.077 g/ml Ficoll (Pharmacia, Uppsala, Sweden). Red blood cells remaining in theuffy-coat fraction were lysed with 20 volumes of lysing solution (155 mM NH4Cl, 10 mM KHCO3, 0.11 mM EDTA). The white blood cells were washed with PBS. Following an immunofluorescence procedure in suspension (Broers et al., 1986), the cells were analyzed in an Ortho 50H flow cytometer (Ortho, Westwood, MA).

Immuno-electron microscopy

A suspension of 2.5 x 10^6 NCI-H82 cells was subjected to a standard indirect immunoperoxidase procedure (Broers et al., 1986). The cells were then fixed in 3% glutaraldehyde (1 hr), washed in PBS, and postfixed in 1% osmium tetroxide (1 hr). Cells were dehydrated to 70% ethanol, stained with 0.5% uranyl acetate in 70% ethanol, further dehydrated towards 100% ethanol, and embedded in Epon 812. Ultra-thin sections were viewed with a Philips EM 300 electron microscope (Eindhoven, The Netherlands).

Transfection studies

3T3 cells were transfected with a cDNA clone coding for the 125-KDa isoform of muscle NCAM (Gower et al., 1988). Reactivity of RNL-1 with transfected 3T3 cells was tested with an indirect immunofluorescence assay, using untransfected 3T3 cells as a control.

Radiolabelling and quality control

Ascitic fluid was generated by intraperitoneal (i.p.) inoculation of 5 x 10^6 RNL-1-secreting hybridoma cells into pristane-primed BALB/c mice. RNL-1 was purified from ascitic

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fluid using a protein A-Sepharose 4B column (Pharmacia) (Ey et al., 1978). Antibody concentrations were quantitated by absorbance at 280 nm, assuming that 1 mg/ml reads an extinction of 1.38. The preparations were stored at −20°C until use.

The purified RNL-1 was radioiodinated by either the iodogen method (Fraker and Speck, 1978) or the Bolton and Hunter method (1973). For the iodogen labelling procedure 100 μg of RNL-1 in 50 mM phosphate buffer (pH 7.2) and 0.3

\[ \text{mcI}^{125}\text{I} \] (Amersham, Little Chalfont, UK) were added to glass tubes that had been pre-coated with 1,3,4,6-tetra-chloro-3a,6a-diphenylacyclohex (Pierce, Rockford, IL) (15 μg/100 μl), and the reaction was allowed to proceed for 5 min. For the Bolton-Hunter labelling method, 400 μg of RNL-1 in 0.1 M borate buffer, pH 8.5, was added to 1 mcI of dried

\[ \text{Bolton-Hunter reagent (iodinated hydroxyxysuccinimide ester from 3-(4-hydroxyphenyl)propionic acid; Amersham) and incubated for 15 min at 0°C, according to the manufacturer's instructions. Following both labelling reactions, the reaction mixtures were applied to a Sephadex G-25 column (Pharmacia) and eluted with PBS. The fractions containing the labelled antibody were pooled and used for in vivo studies within 24 hr.}

Purified anti-interferon MAb B140 was radiolabelled with 125I (Amersham), using the iodogen method as described above.

RNL-1 was labelled with 125I-indium using the bicyclic anhydride of diethylentriamine penta acetic acid (dTPA) as a bifunctional chelating agent (Hnatowich et al., 1983). Briefly, 3 mg of RNL-1 in 0.1 M NaHCO3 (3 mg/ml) was incubated for 1 hr with a 5-fold molar excess of dTPA. After removing unconjugated DTPA by Sephadex G-25 column chromatography, 100 μg of DTPA-conjugated RNL-1 in 0.1 M citrate buffer (pH 5.0) was incubated with 400 μcI 111InCl3 (Amersham) for 20 min. The reaction mixture was eluted on Sephadex G-25 to remove unincorporated 111In, and the fraction of unbound 111In was determined by ITLC using 0.1 M citrate buffer, pH 5.0, as a solvent.

Immunoreactivity of the labelled antibody preparations was checked in a competitive binding assay carried out as described (Boerman et al., 1989), using NCI-H82-coated 96-well plates as the solid phase. Briefly, after the plates were coated with 0.01% poly-L-lysine in PBS for 30 min, NCI-H82 cells (150,000 cells/well) were spun onto the bottom of the wells, fixed in 0.25% glutaraldehyde in PBS (15 min, 20°C) and saturated with 0.5% BSA in PBS (1 hr, 20°C). After washing, the cells were incubated with 50 μl of a fixed amount of antibody (0.5 μg/ml) in the presence of various dilutions (10−1 to 10−8 μg/ml) of unlabelled RNL-1 for 4 hr at 20°C. After washing, the retained radioactivity was determined in a gamma counter. The affinity constant of the antibody was calculated as the reciprocal concentration of unlabelled MAb required for 50% displacement of the 125I-RNL-1 (Giacomin et al., 1985).

Biodistribution studies

Male nude BALB/c mice bearing subcutaneous (s.c.) human SCLC xenografts (NCI-H82) were used. Tumours were initiated by injecting 3 × 104 NCI-H82 cells. When tumours had grown to approximately 1 cm in diameter, they were resected aseptically, minced into small pieces of 2 to 3 mm diameter and serially transplanted in anaesthetized animals. Biodistribution studies were initiated 4 weeks after transplantation, when tumours measured approximately 1.0 cm in diameter (0.5–2.0 g).

NCI-H82 xenograft-bearing mice received a mixture of 10 μg of 125I-RNL-1 and 10 μg of 111In-B140 in the tail vein. Another set of tumour-bearing mice received 10 μg of

\[ \text{11In-RNL-1}. \] At 5 different time intervals after injection (4, 24, 48, 72 and 120 hr), groups of 3 mice were bled and killed under ether anaesthesia. Tumours and selected tissues were removed, washed, blotted dry, weighed and analyzed in a gamma counter for 125I, 111I or 11In activity. The results were expressed as the percentage of the injected dose per gram of tissue (%D/g), and the radioactivity in tumour as compared to that in normal tissues (tissue/normal-tissue ratios). Radio localization indices (RI) were calculated for each tissue for each time point according to the formula:

\[ \text{RI} = \frac{\% \text{D/g of RNL-1 in tissue}}{\% \text{D/g of B140 in tissue}} \times \frac{\% \text{D/g of B140 in blood}}{\% \text{D/g of RNL-1 in blood}} \]

Biodistribution experiments were done on 2 occasions to check the reproducibility of the determinations. Mice injected with 111In-RNL-1 were scanned 24, 48, 72 and 120 hr after injection with a single-head gamma camera with a parallel-hole, medium energy collimator (Siemens, Type Orbiter, Des Plains, IL). Digital acquisition was recorded on a Siemens Schintview data processing system. Both 173-keV and 247-keV gamma-ray peaks of 111In with symmetric 20% windows were used. Between 100,000 (24 hr) and 25,000 (120 hr) counts were acquired for the images. Animals were anaesthetized during imaging by i.p. injection of Nembutal.

Whole-body section autoradiography

The distribution of the radiolabel was visualized by whole-body-section autoradiography in tumour-bearing animals. Briefly, 2 and 3 days after injection of 125I-RNL-1 animals were anaesthetized and snap-frozen as a whole in dry-ice-cooled isopentane. Frozen animals were embedded in 8% carboxymethyl cellulose and 30-μm-thick sections were cut at −20°C, using a LKB-2250 PMV cryomicrotome (LKB, Stockholm, Sweden) as described by Rijnstrij et al. (1979). Freeze-drying of the sections was carried out for 3 to 4 days at −20°C and registration of radioactivity was made by exposure of desiccated sections on Kodak X-Omat AR films.

RESULTS

Reactivity pattern of RNL-1

The reactivity of RNL-1 with the variant SCLC cell line NCI-H82 was studied immunocytochemically. Indirect immunofluorescence staining indicated that RNL-1 is reactive principally with the tumour cell membrane (Fig. 1a). Immunoelectron microscopy corroborated that the RNL-1-defined antigen was localized on the cell surface, and especially at adherent junctions between adjacent cells, while cell-surface regions not in contact with each other showed a less intense staining reaction (Fig. 1b). Flow cytometric analysis indicated that RNL-1 was unreactive with various types of human blood cells (erythrocytes, monocytes, granulocytes, lymphocytes).

RNL-1 reacted positively in the immunofluorescence assay with the NCAM-transfected 3T3 cells, while the antibody did not react with untransfected 3T3 cells, confirming the suggestion that RNL-1 is directed against an NCAM epitope (experiments kindly performed by Dr. F. Walsh, London).

Radiolabelling and quality control

The method of radio-iodination of the purified RNL-1 antibody appeared to be critical and influenced its immunoreactivity markedly. According to the competitive binding assay on 0.025% glutaraldehyde-fixed NCI-H82 cells, the antibody completely lost its immunoreactivity upon oxidative radioiodination (iodogen method). However, RNL-1 labelled by the Bolton-Hunter method was immunoreactive, suggesting that the linker of the 125I-labelled Bolton-Hunter reagent (80% incorporation) preserved the antigen-binding properties of the antibody. Fifty percent displacement of the 125I-RNL-1 was obtained at an unlabelled antibody concentration of 6 μg/ml, indicating an affinity constant of 2.7 × 10⁷ m⁻¹

Biodistribution studies

Maximum tumour accretion of 125I-RNL-1 was achieved at
approximately 48 hr, reaching a level of 6.5 %ID/g (Table 1, Fig. 2a). The activity in all of the normal mouse tissues decreased over time. The levels of activity in the normal tissues correlated to their blood contents. The lungs showed highest label uptake (7.5 %ID/g, 48 hr p.i.), and brain tissue showed the least uptake (0.2 %ID/g, 48 hr p.i.) (Table 1, Fig. 2a). Blood clearance of the radiolabel was slow, and therefore tumour/blood ratio did not exceed 1.0 during the first 3 days after injection.

Comparing the biodistribution of the radio-iodinated RNL-1 with the biodistribution of the anti-interferon MAb (111I-B140) indicates that RNL-1 specifically accumulates at the tumour xenografts. The radiolocalization indices (RI) for the tumour increased during the experiment from 1.8 at 4 hr to 4.3 at 120 hr (Fig. 3), while the RI of the normal mouse tissues ranged from 0.5 to 1.8 and were stable over time, except for the RI of the intestines, which decreased between 4 and 120 hr p.i. from 4.0 to 1.7.

Tumour accretion was higher with the 111In-labelled RNL-1 in comparison with radio-iodinated RNL-1. Tumour uptake reached 11.8% at 72 hr p.i. and remained at that level at least until 5 days after injection (Table 1B, Fig. 2b). However, 111In uptake in most non-tumour tissues, especially the spleen, liver, and kidney, was also higher as compared with the 1231 uptake, and persisted in these organs over time. Consequently, most tumour/non-tumour ratios obtained with 1231-RNL-1 were much higher than those obtained with 111In-RNL-1. Blood activity for both radiolabels was in the same range.

The selective tumour accretion of 111In-RNL-1 was confirmed by external scintigraphy, as shown in Figure 4. Tumours were already clearly visible at day 1, and apparently background activity was further reduced at days 2, 3 and 4. Imaging of 3 animals bearing different-sized tumours (0.27 g, 1.2 g, and 2.2 g) (Fig. 4a) with different tumour uptake levels, as determined 5 days after injection (14.5, 13.4 and 7.9 %ID/g respectively), showed that the smallest tumour is less photodense (Fig. 4b). The liver and probably the kidneys also showed enhanced 111In uptake.

**Autoradiography**

The distribution of the activity 48 hr after the injection of 1231-RNL-1 is shown in the autoradiography of the whole-body section shown in Figure 5. Highest activity is seen in the thyroid, bladder, and the tumour. Thyroid uptake is most likely a

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**TABLE 1 - BIODISTRIBUTION OF RADIOACTIVITY 4, 24, 48, 72 AND 120 HR AFTER INJECTION OF MICE BEARING NCI-H82 XENOGRAFTS WITH 1231-RNL-1**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Tumour</th>
<th>Muscle</th>
<th>Liver</th>
<th>Intestine</th>
<th>Kidney</th>
<th>Heart</th>
<th>Lung</th>
<th>Brain</th>
<th>Blood</th>
</tr>
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<tr>
<td>4</td>
<td>2.2 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>3.6 ± 0.1</td>
<td>4.0 ± 1.9</td>
<td>5.0 ± 0.3</td>
<td>3.5 ± 0.3</td>
<td>4.0 ± 0.5</td>
<td>0.6 ± 0.1</td>
<td>14.3 ± 0.3</td>
</tr>
<tr>
<td>24</td>
<td>4.2 ± 0.7</td>
<td>0.6 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>1.3 ± 0.6</td>
<td>2.4 ± 0.3</td>
<td>1.8 ± 0.3</td>
<td>2.5 ± 0.2</td>
<td>0.2 ± 0.02</td>
<td>7.4 ± 0.7</td>
</tr>
<tr>
<td>48</td>
<td>6.5 ± 2.0</td>
<td>0.6 ± 0.3</td>
<td>1.8 ± 0.1</td>
<td>1.1 ± 0.5</td>
<td>2.1 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>2.5 ± 0.4</td>
<td>0.2 ± 0.03</td>
<td>6.6 ± 0.3</td>
</tr>
<tr>
<td>72</td>
<td>5.6 ± 1.7</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.4</td>
<td>0.6 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>0.1 ± 0.01</td>
<td>5.3 ± 0.9</td>
</tr>
<tr>
<td>120</td>
<td>4.1 ± 1.1</td>
<td>0.5 ± 0.1</td>
<td>1.0 ± 0.4</td>
<td>0.4 ± 0.1</td>
<td>1.1 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>1.4 ± 0.6</td>
<td>0.1 ± 0.01</td>
<td>3.4 ± 1.2</td>
</tr>
</tbody>
</table>

A: Tumour Bearing NCI-H82 Xenografts with 1231-RNL-1

B: Tumour Bearing NCI-H82 Xenografts with 111In-RNL-1

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1Section A. 2Section B. %ID/g ± so, n = 3.
vessels within the NCI-H82 xenograft. The distribution of activity within the tumour (Fig. 5, c-f) was similar to the distribution of the blood vessels. The autoradiography of sections from animals frozen 72 hr p.i. were similar to those taken 48 hr p.i.

**DISCUSSION**

We have presented the tumour-targeting properties of RNL-1, a newly developed murine MAb raised against the variant SCLC cell line NCI-H82 (Broers et al., 1991). Because of its reactivity with normal and neoplastic tissues, RNL-1 was grouped among the cluster-1 antibodies, as defined by the International Workshop on SCLC antigens (Souhami et al., 1988). Since the cluster-1 antibodies have optimal binding to small-cell carcinomas, neuroblastomas, sarcomas and some melanomas, they may be suitable vehicles for targeting drugs, toxins or radionuclides to these tumour types.

Immunocytochemical studies on NCAM-transfected 3T3 cells confirmed the suggestion that RNL-1, like 15 other cluster-1 antibodies (Patel et al., 1989), recognizes an epitope on the 125 kDa isoform of the neural cell adhesion molecule. Flow cytometric analysis showed no reactivity of RNL-1 with most human blood cells, a prerequisite for in vivo use of MAb. NCAM has been reported to be expressed on NK-cells (Abo et al., 1981); however, the interaction of RNL-1 with this (or minor) sub-population of white blood cells was not detected primarily, because the flow cytometer was not set to detect NK-cells.

Initially, our biodistribution studies with RNL-1 were hampered because of the loss of immunoreactivity upon oxidative radio-iodination by the iodogen method. In contrast, radiolabelling by the Bolton-Hunter method or with 111In using the cyclic anhydride, did not affect the immunoreactivity of the antibody. RNL-1 radio-iodinated by the Bolton-Hunter method showed specific tumour accretion following i.v. injection into nude mice with NCI-H82 heterotransplants. The RNL-1 antibody labelled according to the iodogen method injected in the nude mouse/NCI-H82 human SCLC model revealed similar tumour/blood ratios as obtained with the anti-interferon B140 antibody (data not shown). These results suggest that tyrosine residues in the antibody molecule may be essential for antigen binding.

Tumour accretion was higher with 111In-RNL-1 in comparison with 125I-labelled RNL-1. This is consistent with previous studies (Brown et al., 1987; Andrew et al., 1988). Apparently, radio-iodinated antibodies are metabolized in the tumour and the free 125I label is rapidly removed via the blood (Halpern et al., 1987). In contrast, with 111In-labelled antibodies virtually all of the activity is retained in the tumour. The comparison of the activity of both radionuclides in the normal tissues at consecutive time points after injection suggests that this phenomenon also occurs in non-tumour tissues.

It has been suggested that the sub-cellular localization of the antigen is a critically important factor determining the accessibility of the tumour antigenic sites in tumour targeting (Matzku et al., 1988; Perverez et al., 1988, 1989), and thus its suitability for tumour targeting. Our immunocytochemical studies showed that on the NCI-H82 cells the NCAM antigen is most abundantly expressed at adherent junctions between adjacent cells, and therefore NCAM may not be the most optimal target for antibodies. The limited accessibility of the RNL-1-defined antigenic determinant at the NCI-H82 cells may account for the moderate tumour uptake and tumour/ non-tumour ratios obtained in our study as compared with other targeting studies in nude mouse/human tumour models (Smith et al., 1989; Jones et al., 1986; Sharkey et al., 1988). But nevertheless, in vivo the NCAM epitopes appear to acces-

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**Figure 2** - Tissue distribution of 125I-labelled (a) and 111In-labelled (b) RNL-1 (IgG3) in NCI-H82-xenograft-bearing nude mice 4, 24, 48, 72 and 120 hr p.i. The mean of 3 animals is presented.

**Figure 3** - Radiolocalization indices of 125I-RNL-1 compared with 131I-B140 anti-interferon antibody in nude mice bearing NCI-H82 xenografts.
sible to a degree allowing them to be used as a target in immunodetection procedures. In addition, the relatively low affinity of the antibody (2.7 x 10^7 M^-1) might also limit the tumour accretion of the antibody. Further studies are needed to determine whether other SCLC antigens may be more suitable targets for antibodies in vivo.

RNL-1 is reactive with a wide range of neuronal and endocrine tissues, including brain and peripheral nerve. No accumulation was seen in murine brain during our in vivo experiments. However, RNL-1 probably does not cross-react with murine NCAM, and the blood-brain barrier will also prevent the radiolabelled antibody from penetrating the brain tissue. Wilson et al. (1990) have shown in a rabbit model that the anti-NCAM antibody, LS2D617, specifically accumulates in antigen-positive normal tissues except brain tissue.

The autoradiographs of the nude mice injected with radiiodinated antibodies not only visualized distribution of the radiolabel within the animal, but also the limited penetration of the radiolabelled antibody into the tumour tissue, in agreement with other reports (Perevez et al., 1988; Fand et al., 1987). Apparently, after the antibody leaves the circulation and enters the interstitial fluid of the tumour, it principally binds to tumour cells in the immediate vicinity of the blood vessels and capillaries. Whether this is due to physiological barriers, such as increased interstitial pressure, or to avid binding of antigen remains to be determined.

Our study shows the specific tumour accretion of radiolabelled RNL-1 in the human SCLC NCI-H82 nude mouse host model, suggesting the possible in vivo applicability of this antibody. However, it remains to be determined whether the expression of its target antigen (NCAM) in certain normal human tissues will limit its clinical usefulness.

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