Immunotargeting of human small cell lung cancer xenografts in athymic mice using a monoclonal antibody (RNL-1) against a neuroendocrine-related antigen

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Summary A mouse monoclonal antibody (RNL-1) was raised against the variant small cell lung cancer (SCLC) cell line NCI-H82. Immunohistochemical studies on frozen sections showed that the antibody was reactive with most SCLC (15 out of 16) and lung carcinoids (six out of seven), while in general adenocarcinomas and squamous cell carcinomas of the lung were negative. Immunocytochemical studies on 29 different cell lines derived from human lung tumours confirmed the neuroendocrine-related expression of the RNL-1 defined antigenic determinant. Immunoelectron microscopy showed that RNL-1 recognises an extracellular membrane domain, concentrated at adhesion sites between adjacent cells. The tissue distribution of the RNL-1 defined antigen was mainly restricted to neural and neuroendocrine tissues. These immunohistochemical data suggest that RNL-1 is directed against a neuroendocrine-related antigen. Being reactive with an epitope expressed on the surface of most neuroendocrine malignant cells, RNL-1 (IgG isotype) is a potential vehicle for targeting SCLC in vivo. We evaluated the ability of radiolabelled RNL-1 to localise human SCLC xenografts in nude mice as a first step in determining the in vivo value for radioimmunodetection. RNL-1 was radiolabelled using the Bolton-Hunter labelling technique. Nude mice bearing NCI-H82 xenografts were injected intravenously with the radiolabelled RNL-1 preparations, and animals were dissected 4, 24, 48, 72 and 120 h post injection (p.i.) to determine the biodistribution of the radiolabel. The iodine-125 label accumulated in the tumour up to 48 h p.i. (6.5% injected dose per gram of tissue [ID g−1]), while the label content of the normal tissues decreased with time. Tumour/non-tumour ratios 72 h p.i. ranged from 47 (tumour/brain) to 3.1 (tumour/lung). These data suggest that RNL-1 is a promising candidate for in vivo applications.

Lung cancer is still one of the main causes for cancer deaths, especially amongst men in Western countries. Small cell lung cancer (SCLC) accounts for 20–25% of all new cases of primary lung cancer. Unlike the other major histological types of lung cancer (collectively referred to as non-SCLC), SCLC is highly sensitive to both chemotherapeutic agents and radiation therapy, so that 90% of patients with SCLC initially will achieve a clinical remission after cytotoxic therapy. However, the options for the treatment of recurrent disease occurring in most cases, are extremely limited.

Given the difficulties in treating SCLC by means of conventional therapeutic protocols, there is a continued interest in the application of immunological techniques for the management of this disease. A series of monoclonal antibodies (MAbs) against antigens associated with SCLC has been developed in various laboratories (see for review: Southam et al., 1988). Labelled with appropriate radionuclides these antibodies may be used for radioimmunodetection of (un)suspected tumour lesions. Linked to drugs, toxins or radionuclides anti-SCLC MAbs may also have therapeutic potential.

In this report we describe the development and characterisation of a new MAb, RNL-1, reactive with SCLCs and lung carcinoids. As a first step to evaluate the potential application of the antibody for in vivo diagnostic and therapeutic use, we investigated its ability to localise human SCLC in a nude mouse model.

Materials and methods

Tissues, cell lines and immunocytochemical procedures

Human tissue samples were obtained freshly at surgery or autopsy, snap-frozen in liquid nitrogen and stored either in liquid nitrogen or at −80°C. Cryostat sections (4–7 μm thick) were air dried and fixed in acetone for 5 min. Indirect immunofluorescence and immunoperoxidase assays were performed as described previously (Broers et al., 1986).

Twenty-one SCLC cell lines and eight non-SCLC cell lines were used in this study (Carney et al., 1985a,b; Gazdar et al., 1985; de Leij et al., 1985r; Bepler et al., 1987a,b, 1988). All cell lines were grown in RPMI 1640-based culture medium (Gibco, Paisley, UK) supplemented with 15% foetal calf serum (Flow, Herts, UK).

Production of the monoclonal antibody RNL-1

Balb/c mice were immunised with cell suspensions of NCI-H82 cells. For this purpose cells were harvested and washed once with 50 mM phosphate-buffered saline, pH 7.4 (PBS). On day 0 and day 14, mice received i.p. injections of 10⁶ cells mixed with equal volumes of Freund's complete and incomplete adjuvant, respectively. Thereafter, mice were boosted four times at weekly intervals, without addition of adjuvant to the cell suspension. Three days after the last booster mice were sacrificed and spleen cells were fused with Sp2/0-Ag14 myeloma cells at a ratio of 2:1, essentially as described by Köhler and Milstein (1975), using PEG 4000 (E. Merck, Darmstadt, FRG). Hybridoma clones producing relevant monoclonal antibodies (MAbs) were selected using an enzyme-linked immunosorbent assay (ELISA) as described (Suster et al., 1980). In the ELISA, NCI-H82 cells coated onto the wells of 96-well plates were used as the solid phase. In addition, positive wells were immediately screened using the indirect immunofluorescence technique on cytospin preparations of NCI-H82 and NCI-H69, as well as on frozen sections of a SCLC, an adenocarcinoma, and a squamous cell carcinoma of the lung. Clones reacting only with SCLC were selected, subcloned three times and used for further studies.

Immunoelectron microscopy

A suspension of 2.5 × 10⁶ NCI-H82 cells was subjected to a standard indirect immunoperoxidase procedure. After washing
in PBS the cells were fixed in 1.5% glutaraldehyde in PBS (10 min, 20°C). After subsequent washing the peroxidase activity was detected with 3,3'-diaminobenzidine-HCl (Sigma Chem. Co., St. Louis, MO) in the presence of 0.01% H2O2. Thereafter, cells were fixed in 3% glutaraldehyde (1 h), washed in PBS and postfixed in 1% osmium tetroxide (1 h). Cells were dehydrated in an ascending series of ethanol, embedded in Epon 812, and viewed with a Philips EM 300 electron microscope (Philips, Eindhoven, The Netherlands).

Radioiodination of antibody RNL-1

The antibody RNL-1 (IgGl subclass) was purified from ascitic fluid generated by inoculation of hybridoma cells into pristane-primed Balb/c mice, using a Protein A-Sepharose 4B column (Pharmacia, Uppsala, Sweden) (Ey et al., 1978). Antibody concentrations were quantitated by absorbance at 280 nm, assuming that 1 mg ml−1 corresponds with an extinction of 1.38. The preparations were stored at −20°C until use.

The purified RNL-1 was radioiodinated according to the iodogen method (Fraker & Speck, 1975) as well as according to the Bolton-Hunter method (Bolton & Hunter, 1973). For the iodogen labelling procedure 100 μg of RNL-1 in 50 ml phosphate buffer (pH 7.2) and 0.3 mci 125I (Amersham Int., Amersham, UK) were added to 10 ml plastic tubes that had been precleaned with 1,3,4,6-tetracloro-3a,6-diphenylglycoluril (Pierce Chemical Co., Rockford, IL) (15 μg 100 μl−1), 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, were added to 1 ml cdi of dried Bolton and Hunter reagent (iodinated hydroxyxuccinimide ester of 3-(4-hydroxyphenyl)propionic acid; Amersham Int., Amersham, UK) and incubated for 15 min at 0°C. Following both labelling reactions, the reaction mixtures were applied to a Sephadex G-25 column (Pharmacia, Sweden) and eluted with PBS. The fractions containing the labelled antibody were pooled and used for in vivo studies within 24 h.

Immunoreactivity of the labelled antibody preparations was checked in a competitive binding assay as described earlier (Boerman et al., 1990), using NCI-H82 coated 96-well plates as the solid phase.

Biolocalisation studies

Male nude Balb/c mice bearing a subcutaneously s.c. growing human SCLC xenograft (NCI-H82) were used. The tumour model was established by sc injection of 3 × 106 NCI-H82 cells. Tumours were resected aseptically, and small pieces of 2–3 mm diameter were serially transplanted s.c. under ether anaesthesia. Four weeks after transplantation tumours measured 0.7 to 1.5 cm in diameter (0.3–2.0 g). At that time mice were used for biodistribution studies.

NCI-H82 xenograft-bearing mice received 10 μg of 125I-labelled RNL-1 in the tail vein. At five different time points after injection (4 h, 24 h, 48 h, 72 h, 120 h) groups of three mice were bled and sacrificed under ether anaesthesia. Tumours and selected tissues were removed, washed in PBS, blotted dry, weighed and analysed in a gamma counter. The results were expressed as the percentage of the injected dose per gram of tissue (% ID g−1), and as the radioactivity in tumours compared to that in normal tissues (tumour/non-tumour ratios). All experiments were done on two different occasions to check the reproducibility of the method.

Results

Selection and cross-reactivity of RNL-1

The RNL-1 antibody was selected on basis of its reactivity with two human SCLC cell lines and three lung cancer specimens of different histology. From tests on this limited panel of lung cancer cell lines and tumour specimens we obtained the impression that amongst lung tumours RNL-1 specifically reacted with SCLC. Therefore RNL-1 was further tested in an extended series of lung cancer cell lines and solid lung tumours, but also in a panel of human non-lung cancers and normal human tissues. These results have been extensively described by Broers et al., 1991. In brief, all SCLC cell lines were clearly stained at the cell surface. More specifically, strong reactivity was seen at adherent junctions between adjacent cells, while cell surface regions not in contact with each other showed a less intense staining reaction (Figure 1a).

Immunoelectron microscopy showed that the RNL-1 defined antigen was indeed localised on the cell surface and especially at the cell adhesion sites (Figure 1b).

Nearly all SCLC tumour samples examined (13 out of 14, Figure 1c), and lung carcinoids (six out of seven) were positive. Some squamous cell carcinomas of the lung (11 out of 38) did also react with RNL-1, while all adenocarcinomas of the lung (n = 15) were negative. With respect to expression of the RNL-1 epitope in normal human tissues it can be stated that the antibody appears to be reactive in all neuronal tissues. Strong staining was seen in all nerves and nerve fibers. In addition, RNL-1 was reactive with neuroendocrine tissues, such as pancreatic islet cells, some cells in the pituitary gland and in the 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. All other normal human tissues examined (brain Purkinje cells, epidermis, tongue, oesophagus, stomach, spleen, liver, mammary gland, cervix, ovary, prostate, kidney, lymph nodes, blood cells and bone marrow) were negative.

Biolocalisation studies

Radioiodination of the purified RNL-1 antibody appeared to be critical and influenced its immunoreactivity. According to the competitive binding assay on 0.025% glutaraldehyde fixed NCI-H82 cells, the antibody lost its immunoreactivity upon oxidative radiiodination (iodogen method), while ester linkage of the 125I-labelled Bolton-Hunter reagent preserved the immunoreactivity of RNL-1. With the latter preparation 50% displacement of the 125I-RNL-1 was obtained at an unlabelled antibody concentration of 6 μg ml−1, indicating an affinity constant of 2.7 × 106 M−1. This preparation was used in the biolocalisation studies.

Following i.v. injection of the 125I-RNL-1 preparation into xenograft bearing mice, the 125I-label accumulated in the tumour, reaching a maximum level of 6.5% ID g−1 h−1 (Figure 2). The label content of the normal mouse tissues decreased during the experiment. Throughout the experiment the 125I content of the different solid tissues varied considerably, being the highest for kidney and the lowest for brain tissue (Figure 3). Blood clearance of the radiolabel was slow (7.4% ID g−1 at 24 h, 3.4% ID g−1 at 120 h), and
and was grouped in cluster 1 at the Second Workshop. The expression of the RNL-1 antigen in normal human tissues, as well as our immunoelectron microscopical observations are in agreement with the findings of Kibbelaar et al. (1989), suggesting a close relationship between cluster 1 antigens and the neural cell adhesion molecule (NCAM). In addition, cluster 1 antibodies have been shown to react with NCAM using transfectants (Patel et al., 1989).

Initially our biolocalisation studies with RNL-1 were hampered because of the loss of immunoreactivity upon oxidative radiodination. This problem could be overcome by using the Bolton-Hunter labelling technique. We suspect that the iodogen labelling technique may cause the modification of particular tyrosine residues of the MAb that are essential for antigen binding. In contrast, the Bolton-Hunter reagent is linked to free amino-groups of the antibody molecule. The immunoreactive fraction of the radiiodinated antibody could not be deduced from the binding assay because the assay was not performed at antigen excess conditions.

Our immunotargeting studies showed that radioactivity levels in all normal tissues of the mice decreased during the biolocalisation experiments, while the uptake into the xenografted human SCLC increased up to 6.5% ID g⁻¹ during the first 48 h after injection. This indicates a specific tumour accumulation of the RNL-1 antibody. Although RNL-1 is reactive with a wide range of neuroendocrine tissues, including human brain, no accumulation is seen in murine brain during our biolocalisation experiments. Whether this is due to lack of cross-reactivity of the MAb with murine neuroendocrine tissues or the inability of the immunon conjugate to penetrate the blood-brain barrier, is unclear. Furthermore, it remains to be defined whether the expression of the cluster 1 antigen on nerve fibers and other neural tissues will give rise to severe side-effects when these antibodies are applied in man.

After 48 h the ¹²⁵I content of the xenografts decreased gradually with time. Probably this decrease is due to dehalogenation of the radiiodinated antibody at the tumour site, a generally observed phenomenon when radiiodinated antibodies are applied in in vivo studies (Halpern & Dillman, 1987). Indeed, preliminary results indicate that label uptake into the xenografts is higher and more persistent when In-111 labelled RNL-1 is used in this in vivo model.

It has been suggested that the mode of presentation of a target antigen in the tumour may be essential for tumour uptake of antibody (Matzku et al., 1988). Theoretically, the RNL-1 antigen, predominantly expressed at adherent junctions between adjacent cells, could be considered a suboptimal target for tumour targeting. Our results, however, indicate that even such ‘hidden’ antigens can be reached by antibodies. It would therefore be worthwhile to compare the biolocalisation characteristics of different cluster 1 MAbs in a nude mouse model. Such a comparative study could demonstrate whether the relatively limited accessibility of the cluster 1 antigen actually limits its potential as a target for radioimmuni imaging and possibly radio-immuno therapy of SCLC.

We wish to thank Drs G. Bepler, L. de Leij, D.N. Carney and S. Wagenaar for providing cell lines and tissues, and G. Schaart, O. Moesk, W. van den Broek, J. Koedam and G. Grutters for their skilled technical assistance.

References


PATEL, K., MOORE, S.E., DICKSON, G. & 4 others (1989). Neural cell adhesion molecule (NCAM) is the antigen recognized by monoclonal antibodies of similar specificity in small-cell lung carcinoma and neuroblastoma. Int. J. Cancer, 44, 573.


