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Monoclonal Antibodies for the *In Vitro* Detection of Small Cell Lung Cancer Metastases in Human Bone Marrow

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Abstract—*Three rat monoclonal antibodies were selected for the immunodetection of small cell lung cancer metastases in bone marrow and other hematologic samples. By membrane immunofluorescence, they define three distinct surface antigens here termed lung cancer-associated antigens or LCAs. The latter are widely expressed on small cell lung cancer and non-small cell lung cancer cells/cell lines, but not detectable on a variety of normal and transformed bone marrow, blood and lymphoid cells. Anti-LCA1 (IgM) is similar to the many anti-lacto-N-fucopentaose III IgM antibodies raised against human tumors. In contrast, anti-LCA2 (IgG2b) and anti-LCA3 (IgG2a) define surface proteins of* 29, 32, 41 *and* 98 *kilodaltons, respectively, that have not been reported earlier. These three reagents have immunodiagnostic potential, since in combination they label all* 49 *lung cancer cell lines tested. Their ability to detect lung cancer metastases in patient's bone marrow samples is documented in an accompanying paper.*

INTRODUCTION

SMALL CELL LUNG CANCERS (SCLC) have unique clinical, morphological and biological properties that distinguish them from the other types of lung cancers [1]. Clinically relevant properties are their early dissemination to distant sites and their sensitivity to a variety of cytostatic drugs, two features that justify their treatment by combination chemotherapy [2] and, more recently, by intensive chemotherapy supported by autologous bone marrow transplantation [3, 4]. In the latter trials, it is essential to diagnose SCLC metastases, including micrometastases, in bone marrow samples prior to transplantation. In this paper, we describe three monoclonal antibodies that allow detection of SCLC cells as well as a variety of other cancer cells in bone marrow aspirates and in other hematological samples. Two of these reagents seem to recognize novel lung cancer-associated surface antigens that have not been defined by anti-SCLC monoclonal antibodies reported previously [5–14].

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MATERIALS AND METHODS

Normal hematologic cells

Heparinized blood was from healthy young volunteers (University of Louvain). Heparinized bone marrow samples were from two patients with acute leukemia in complete remission, from nine patients with dyshematopoiesis and from four patients with neuroblastoma without morphologically detectable bone marrow involvement. Thoracic duct lymph was surgically drained from a kidney transplant recipient. Thymus and tonsils were from children undergoing cardio-vascular surgery and tonsillectomy, respectively. Blood buffy coats were prepared by sedimentation at 37°C. Blood and bone marrow mononuclear cells were purified by density centrifugation on Ficoll-Hypaque (1.077 g/cm^3). Purified red blood cells and platelets were prepared by

appropriate centrifugation on Percoll gradients and lymphocytes by centrifugation on Ficoll-Hypaque after the removal of iron-loaded monocytes with a magnet. Cells were either kept at 4°C in Eagle's minimum essential medium (MEM) with 20 mM hepes pH 7.2 and 10% fetal calf serum, or frozen (−1°C/min) in MEM with 20% fetal calf serum and 7.5% dimethyl sulfoxide before storage in liquid nitrogen. Viability, assessed by trypan blue exclusion, was above 85% in all fresh and cryopreserved samples. Differential nucleated cell counts were done on May–Grunwald and Giemsa-stained cytocentrifuge cell smears. Differential T and B lymphocyte counts were done by indirect immunofluorescence, using monoclonal antibodies OKT11 and B1 (Coulter Immunology) and a commercial goat anti-mouse IgG fluoresceinated conjugate (Tago).

Cancer cells and cell lines

Various specimens were obtained from patients (Cliniques Universitaires Saint Luc, Brussels) presenting with acute and chronic leukemia, with lymphoma, neuroblastoma, small cell lung carcinoma and lung adenocarcinoma. Heparinized blood, bone marrow aspirates and pleural effusions were fractionated on Ficoll-Hypaque. Surgically removed lymph nodes were teased into single cell suspensions in MEM with 10% fetal calf serum. Viability was above 85% in all freshly prepared cell suspensions as well as in cryopreserved leukemia and lymphoma samples; it was lower in cryopreserved neuroblastoma and lung carcinoma samples. Only samples that contained more than 70% of malignant cells, as established by cytology, and by membrane marker analysis for leukemia and lymphoma cells, were used in this study. The origins of most cancer cell lines used in this work were described earlier [5, 15]. Lung cancer cell lines comprise SCLC and non-SCLC lines, a classification based upon the unique biological properties of SCLC [1]. The latter have been further divided into classic lines, with typical morphology and neuroendocrine markers, and variant lines, with atypical morphology, selective loss of markers and more rapid growth [15]. SCLC lines LB-11-OC, LB-12-OC and LB-13-OC were established at the Ludwig Institute for Cancer Research, Brussels Branch.* Neuroblastoma lines CHP-100 and CHP-212 were kindly given by J. Kemshead [16], and KCNR, SAN, A6 and N1000 by P. Reynolds [17]. Colon cancer cell lines were established by H. Oie at the NCI-Navy Medical Oncology Branch. The 19 leukemia, lymphoma and myeloma lines were a generous gift of M.F. Greaves and G. Janossy [18]. All cell lines were maintained as recommended by the authors.

*Humblet, Y., in preparation.

Table 1. Anti-LCA monoclonal antibodies

Name	Class	SCLC cells used for immunization
Anti-LCA1	IgM	Metastatic lymph node cells
Anti-LCA2	IgG2b	Metastatic lymph node cells
Anti-LCA3	IgG2a	Cell line NCI-H69

Monoclonal antibodies

Rat–rat hybridomas were generated essentially as previously described [19]. Briefly, 2-month old female Louvain rats [20] were injected i.p. at weeks 0, 3–4 and 7–22 with 2×10^6 viable SCLC cells (Table 1). Three days after the last boost, immune spleen cells were fused in the presence of polyethylene glycol with the IR983F rat myeloma cell line [21]. Hybrids were selected in HAT medium and their supernatants were screened by indirect membrane immunofluorescence on SCLC cell lines NCI-H69, LB-11-OC and LB-12-OC. The latter line was derived from the clinical tumor sample used for immunization (Table 1). Hybrids associated with a bright surface labelling of at least one of these cell lines were cloned several times by limiting dilution and produced as ascitic tumors in Louvain rats. Ascites were stored at −70°C and diluted in MEM-1% fetal calf serum just before use. The heavy chain class of antibodies of interest was determined by immunodiffusion, using class-specific anti-rat immunoglobulin antisera [22].

Cell radiolabelling and immune precipitation

Cell-surface radiolabelling and immune precipitations were done as previously described [19]. Briefly, NCI-H69 cells were iodinated by the lactoperoxidase/glucose oxidase method [23]. Membrane proteins were solubilized by 0.5% Nonidet P-40 in 0.1 M Tris buffer pH 8.2 with 0.15 M NaCl and 2.5 mM phenylmethylsulfonate. After a preliminary precipitation with *Staphylococcus aureus*, immune precipitation was achieved with 1 or 2 μg of rat monoclonal antibody and 100 μl of a 10% suspension of *Staphylococcus aureus* precoated with rabbit anti-rat IgG antibodies. After washing, antigens were eluted in 4% sodium dodecyl sulfate (SDS), and submitted to polyacrylamide gel electrophoresis in reducing conditions [24] and eventually to autoradiography.

Membrane immunofluorescence

For indirect membrane immunofluorescence assays, 250,000 cells from cell lines or 500,000 cells from patients were incubated with saturating amounts of rat ascites in the first layer, and with F(ab')2 fragments of affinity-purified rabbit anti-rat IgG antibodies conjugated to fluorescein isothiocyanate (FITC) [19]. Cytocentrifuge cell smears were

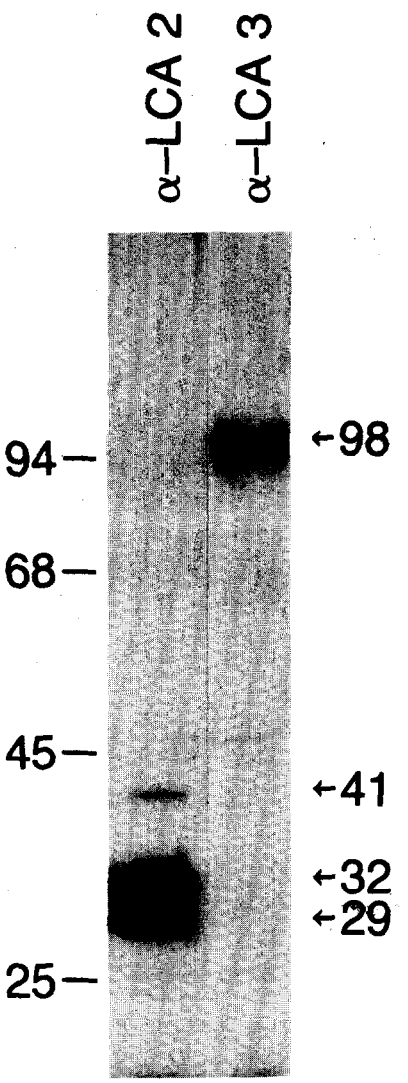

Fig. 1. Surface-^{125}I-labelled extracts of SCLC line NCI-H69 immunoprecipitated with anti-LCA2 and anti-LCA3; autoradiography after electrophoresis in reducing conditions on SDS-polyacrylamide gel; molecular weight markers in kilodaltons are indicated on the left; the molecular weight of the precipitated proteins is shown on the right.

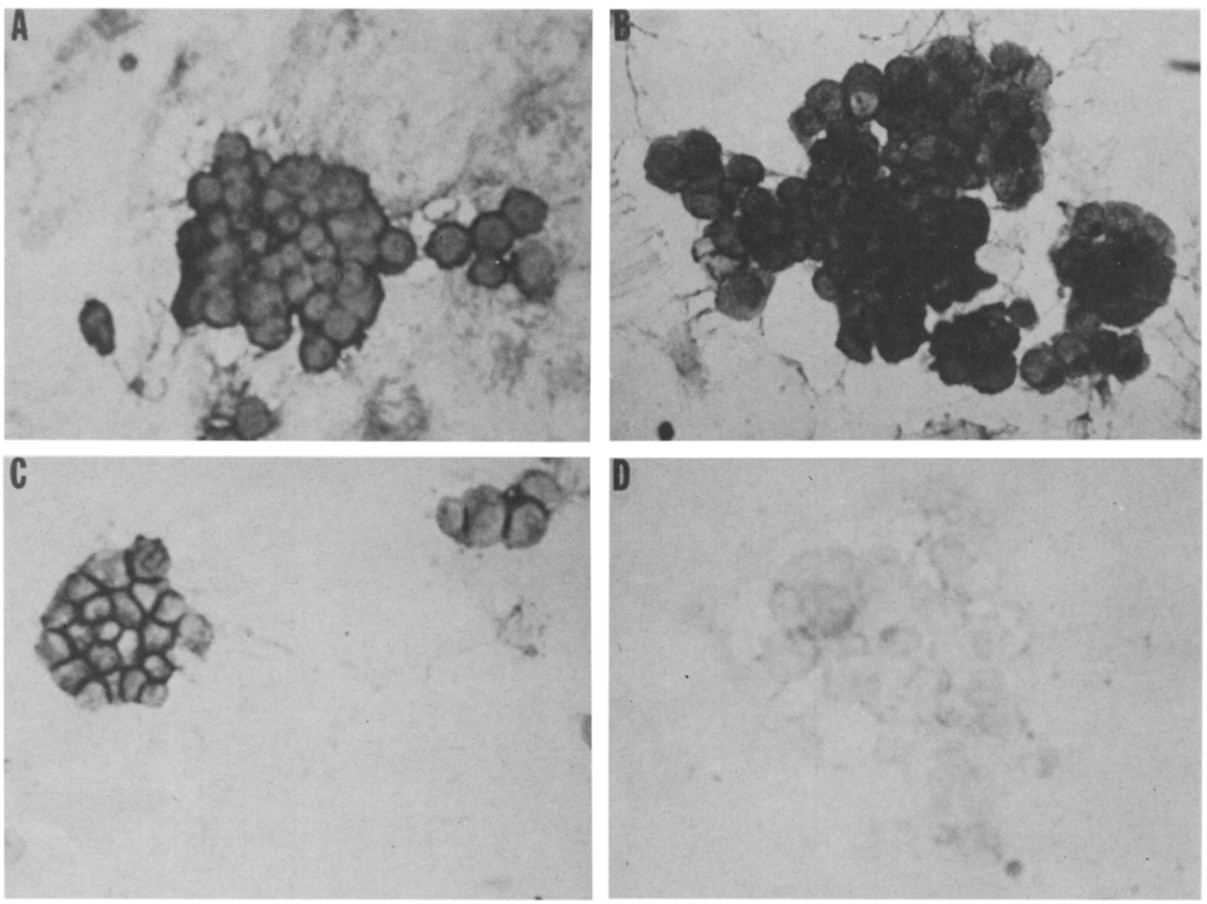

Fig. 2. Immunoperoxidase stain with 50 ng *anti-LCA*1 (*A*), *anti-LCA*2 (*B*) *and anti-LCA*3 (*C*) *rat monoclonal antibody on acetone-fixed smears of SCLC line NCI-H*69; *in* (*D*), 50 ng *of rat IgG2b myeloma proteins are used as a negative control.*

mounted in Tris-buffered glycerol (pH 9) and examined under a Leitz Dialux fluorescence microscope equipped with an epiilluminator and phase contrast optics. Rat myeloma ascites with IgG2b or IgM paraproteins [20] and ascites induced by the non-secreting IR983F rat myeloma line [21] were used as negative controls. Positive controls were performed simultaneously on reference cell lines. All tests were duplicated at least once.

Immunocytochemistry

Cytocentrifuge cell smears were fixed for 15 min in cold acetone, air-dried and assayed sequentially with appropriately diluted rat ascites, biotinylated rabbit anti-rat IgG antibody, and a mixture of avidin and biotinylated horseradish peroxidase (Vector Laboratories; [25]). Smears were fixed in 2% osmium vapors and counter-stained with 0.03% light green. Rat IgG2b myeloma ascites was used as a negative control and simultaneous positive controls were performed on reference cell lines.

RESULTS

Selection of three monoclonal antibodies defining lung cancer-associated antigens

Forty-two distinct anti-SCLC monoclonal ascites were screened by indirect membrane immunofluorescence on four SCLC cell lines, on 19 leukemia, lymphoma and myeloma lines as well as on peripheral blood buffy coat cells and mononuclear cells. Only 6/42 monoclonal antibodies labelled brightly at least 2/4 SCLC cell lines while exhibiting insignificant immunofluorescence on the 21 hematological samples tested. These six antibodies appeared to define only three distinct surface antigens, hereafter termed lung cancer-associated antigens or LCAs. The origin and class of anti-LCA1, anti-LCA2 and anti-LCA3 monoclonal antibodies are summarized in Table 1.

Biochemical nature of lung cancer-associated antigens

Surface-^{125}I-labelled extracts of SCLC line NCI-H69 were analyzed by immunoprecipitation with the three anti-LCA monoclonal antibodies (Fig. 1). Anti-LCA2 clearly precipitated three polypeptides with apparent molecular weights of 29, 32 and 41 kilodaltons (kd) and anti-LCA3 precipitated a single molecule of 98 kd. Identical immunoprecipitates were obtained with anti-LCA2 and anti-LCA3 after [^{35}S]methionine labelling of NCI-H69 cells (not shown). No detectable antigens were immunoprecipitated with anti-LCA1, either after iodination of accessible tyrosyl residues or after [^{35}S]methionine incorporation into cell proteins of SCLC lines NCI-H69 and LB-11-OC.

Surface lung cancer-associated antigens are expressed on solid tumor cells/cell lines but not on hematologic cells/cell lines

The cellular specificity of the three anti-LCA monoclonal antibodies was extensively investigated by membrane immunofluorescence (Tables 2, 3). They reacted variably with SCLC metastatic cells and SCLC cell lines, but none of them detected 100% of tumor cells in the five samples tested. They also reacted with lung adenocarcinoma cells and with a neuroblastoma cell line, thus defining lung cancer-associated antigens rather than SCLC-restricted or lung cancer-restricted antigens. In contrast, they failed to react with 40 samples of normal hematologic cells derived from blood, bone marrow and lymphoid organs. They were also tested on acute leukemia and lymphoma cells, that are thought to represent clonal expansions of rare hematopoietic precursors. They were totally unreactive with 33 clinical samples and with 19 cell lines, thus confirming the earlier screening results. However, when tested against chronic myeloid and myelomonocytic leukemia cells, anti-LCA1, but neither anti-LCA2 nor anti-LCA3, labelled 5–10% of monocytoid-like cells found exclusively, so far, in blood and bone marrow mononuclear cell suspensions from these patients.

LCA expression by 62 cancer cell lines

The diagnostic potential of the three anti-LCA monoclonal antibodies was established using immunocytochemistry on a panel of 62 cancer cell lines that included 32 SCLC lines, 17 non-SCLC lines and 13 non-lung cancer lines (Table 4). The labelling of NCI-H69 SCLC cells by the three anti-LCAs is shown in Fig. 2. The morphology of the labelling suggests that LCA1 and LCA2 are expressed not only on the cell surface, as demonstrated by membrane immunofluorescence, but also in the cytoplasm, whereas LCA3 seems to be more restricted to the plasma membrane. The three anti-LCAs are overwhelmingly expressed in 20 classic cell lines and somewhat more sporadically in 12 variant SCLC lines [15]. However, none of these 32 SCLC lines escaped labelling by at least one of the three anti-LCAs, suggesting that, in combination, these three reagents have good immunodiagnostic potential. All 17 non-SCLC lines and over half of the 12 non-lung cancer lines were also labelled by at least one anti-LCA. The staining of the HL60 cell line by all three antibodies is noteworthy, since no surface LCAs could be detected on these cells by membrane immunofluorescence (Table 3). Hence, HL60, and presumably other hematologic cells, express LCAs in their cytoplasm although not on their surface.

Table 2. Tumor cells/cell lines with detectable surface LCAs*

	% fluorescent cells		
	LCA1	LCA2	LCA3
Small cell lung cancer			
Metastatic lymph node cells‡	79	92	68
NCI-H69	>90	>90	>90
LB-11-OC	>90	—†	>90
LB-12-OC	>90	>90	>90
LB-13-OC	—	—	75
Lung adenocarcinoma			
Metastatic pleural effusions§			
1	10	79	71
2	—	—	—
Neuroblastoma			
Metastatic bone marrow cells‖ 1,2,3	—	—	—
CHP-100	75	—	50
CHP-212	—	—	—
Chronic myeloid and myelomonocytic leukemia			
Bone marrow/blood mononuclear cells (n = 5)	5–10	—	—

*Indirect membrane immunofluorescence with 250 ng of rat monoclonal antibody and affinity-purified rabbit anti-rat IgG F(ab')2-FITC conjugate [19].
†— = less than 0.5% stained cells.

‡, §, ‖Over 90, 80 and 70% tumor cells in samples, respectively.

Table 3. Hematologic cells/cell lines without detectable surface LCAs*

Normal cells

Blood:
- buffy coat cells (4)†
- mononuclear cells (6)
- lymphocytes (2)
- platelets (2)
- ABO red blood cells (3)
- monocytes (2)
- granulocytes (2)

Bone marrow: mononuclear cells (15)
Thoracic duct lymphocytes (1)
Thymus lymphocytes (2)
Tonsil lymphocytes (3)

Leukemia/lymphoma cells‡	*Cell lines*‡
T-ALL (2)	T-ALL: HPB-ALL, JM, Jurkat, Molt-4
c-ALL (11)	c-ALL: KM3, Nalm-1, Nalm-6
B-NHL (4)	B-NHL: Riva, Raji, Daudi
B-CLL (3)	B-LBL: IM9. UC-729-6
AML (M2,M3) (7)	ANLL: KG1, ML3, HL60, U937, K562
AMMoL, AMoL (M4,M5) (6)	Myeloma: ARH77, U266

*Less than 0.5% fluorescent cells in membrane immunofluorescence assay (see footnote to Table 2).
†( ) = number of samples tested.
‡T-ALL: T acute lymphoblastic leukemia, >90% OKT11+ cells; c-ALL: common acute lymphoblastic leukemia, >90% AL2+ cells (19); B-NHL: B non-Hodgkin's lymphoma, > 70% kappa chain+ cells; B-CLL: B chronic lymphocytic leukemia, > 90% B1+ cells; AML, AMMoL and AMoL: acute myeloblastic, myelomonoblastic and monoblastic leukemia, M2–M5 FAB classification; ANLL: acute non lymphoblastic leukemia; B-LBL: B lymphoblastoid cell lines.

Table 4. LCA expression in 62 cancer cell lines*

	Number positive†/number tested			
	LCA1	LCA2	LCA3	LCA1/2/3
Small cell lung cancer lines‡				
Classic	16/20	20/20	18/20	20/20
Variant	7/12	6/12	11/12	12/12
Non small cell lung cancer lines§				
Adenocarcinoma	5/8	5/8	6/8	8/8
Adenosquamous carcinoma	2/4	3/4	4/4	4/4
Squamous carcinoma	0/1	1/1	1/1	1/1
Large cell carcinoma	3/4	3/4	3/4	4/4
Non lung cancer lines§				
Mesothelioma	1/2	1/2	1/2	1/2
Neuroblastoma	0/4	0/4	2/4	2/4
Melanoma	0/1	0/1	0/1	0/1
Colon carcinoma	4/4	4/4	3/4	4/4
Colon carcinoid	0/1	0/1	0/1	0/1
Acute promyelocytic leukemia	1/1	1/1	1/1	1/1

*Immunocytochemistry, with 50 ng of rat monoclonal antibody and the ABC immunoperoxidase technique.

†Positive samples contain at least 5% and usually above 25% of stained cells.

‡Classic lines: NCI-H64, -H69, -H128, -H146, -H182, -H187, -H209, -H220, -H249, -H250, -H345, -H369, -H378, -H390, -H510, -H615, -H660, -H678, -H735, -H792; variant lines: NCI-H60, -H177, -H182, -H196, -H211, -H289, -H360, -H417, H446, -H524, -H526, -H719.

§Adenocarcinoma: NCI-H23, -H125, -H358, -H676, -H726, -H717, -H650, A549; adenosquamous carcinoma: NCI-H522, -H596, -H647, -H292; squamous carcinoma: NCI-H520; large cell carcinoma: NCI-H157, -H460, -H661, -H666; mesothelioma: NCI-H28, -H226; neuroblastoma: N1000, San, KCNR, A6; melanoma: NCI-H234; colon carcinoma: NCI-H548, -H630, -H747, -H768; colon carcinoid: NCI-H716; acute promyelocytic leukemia: HL60.

DISCUSSION

In this paper, we describe three monoclonal antibodies that were selected for the *in vitro* immunodetection of SCLC metastases in bone marrow samples, using cell surface labelling techniques. A number of monoclonal antibodies aimed at SCLC surface antigens have been described earlier [7–14]. The anti-LCA1 IgM monoclonal antibody is similar to the numerous IgM monoclonal antibodies raised against SCLC cells [5, 6, 26], other tumor cells [27] and blood cells [28]. However, none of the previously reported antibodies seem to recognize either LCA2 (p29, 32, 41) or LCA3 (p98). Thus, monoclonal antibodies SM1 (p25, 50) [7], TsF3 (p110) and TsF4 (p124) [8], SCC 41, 114, 124 and 175 [9], SCLC 2051 and 2053 [10], B10/12 (p100) [13] and LAM8 (p90,135) [14] react quite selectively with SCLC cells. In contrast to anti-LCA2 and anti-LCA3, they usually fail to react with non-SCLC and non-pulmonary tumor cells. The same selectivity for SCLC cells is displayed by antibodies defining the 100 kd Leu-7 antigen [29–31]. Monoclonal antibodies MOC-1 (p45, 50, 60) [11], E10/5 (p80) [13] and LAM2 (p45/125) [12] have a cellular immunoreactivity more compatible with that displayed by anti-LCA2 and anti-LCA3 but define polypeptides of differing molecular weights. Only monoclonal antibodies TsF1 (p42) and TsF2 (p39) [8] have an immunoreactivity comparable to that of anti-LCA2 and might conceivably bind to the heaviest of the three polypeptides (p29, 32, 41) immunoprecipitated by anti-LCA2. The key feature of our three monoclonal antibodies is their total absence of reactivity with surface antigens expressed on a wide variety of hematologic cells of all lineages and most developmental stages, combined with their broad reactivity with malignant cells and cell lines. Thus, when taken individually, they labelled 80–100% of the classic SCLC lines and 50–90% of the variant SCLC lines, and none of these 32 SCLC lines escaped detection by at least one of them. All 17 non-SCLC lines also reacted with at least one of the anti-LCAs. In conclusion, this set of reagents seems adequate for the *in vitro* immunodetection of lung cancer metastases in bone marrow, blood and lymphoid samples, when cell surface labelling techniques such as membrane immunofluorescence are being used. Their reactivity with normal tissues other than hematopoietic cells is irrelevant to the diagnostic application for which they have been tailored with success, as reported in the accompanying paper [32].

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