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# Immunodetection of Small Cell Lung Cancer Metastases in Bone Marrow Using Three Monoclonal Antibodies

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**Abstract**—*Detection of bone marrow metastases by indirect immunofluorescence methods was investigated using three monoclonal antibodies (MoAbs) raised against small cell lung cancer (SCLC). These antibodies, designated anti-LCA1, -LCA2 and -LCA3, recognize three different antigens on the surface of SCLC cells. Eighty-four bone marrow samples from 74 different patients were studied. Whereas tumor cells were found in 32 (38%) by MoAb staining, only 10 (12%) were positively identified using conventional morphological methods. Nine out of the morphologically positive specimens showed reactivity with at least two monoclonal antibodies. Among the 32 samples proven positive by immunofluorescence, an important antigeneic variability was noted. Anti-LCA1 recognized tumor cells in 62%, anti-LCA2 and anti-LCA3 in 53%. Due to the recognition of bone marrow involvement by fluorescence methods in 26% of the 34 patients classified as limited disease, a new subgroup of limited disease patients was defined whose prognosis remains undetermined. Our results confirm the utility of immunodetection in the diagnosis of SCLC bone marrow metastases and emphasize the advantage of using a panel of MoAbs with different antigeneic specificities. Further study is needed to determine the prognostic significance of bone marrow involvement established by immunodetection.*

## INTRODUCTION

IT IS well known that small cell lung cancer (SCLC) has a high propensity to metastasize to the bone marrow and that such involvement is a prognostic factor [1]. Depending on the series, the incidence of positive bone marrow at the time of diagnosis is reported to be 17–29% [2–6]. Detection of lung cancer cells in the bone marrow is also of an obvious interest in the setting of autologous bone marrow transplantation, where the presence of clonogeneic tumor cells in the graft should be avoided. This can be achieved by using appropriate screening tests to determine whether the bone marrow is contaminated, in which case either the patient is considered ineligible for autografting or the marrow is submitted to *in vitro* purging.

To increase detection sensitivity, immunocytological staining with monoclonal antibodies (MoAbs) against SCLC has been reported [7]. Considerable antigeneic heterogeneity exists not only between tumors from different SCLC patients but also between cells originating from the same tumor [8]. Therefore, the use of a panel of MoAbs with different specificities appears to be of particular interest. The purpose of the present study was to compare the results obtained by conventional morphological methods with those obtained by means of an indirect immunofluorescence test using three monoclonal antibodies, each recognizing a different SCLC surface membrane antigen while not reacting with peripheral blood and bone marrow cells [9].

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## MATERIALS AND METHODS

*Patients*

Between November 1985 and April 1987, 84 bone marrow samples from 74 patients suffering from histologically or cytologically proven SCLC were included in this study. The staging procedure included a general physical examination, a chest X-ray with hilar tomography, fibroscropy with brushing, washing and biopsies, CT scan of the chest, abdomen and brain, isotopic bone scan and a bone marrow aspirate and biopsy. Limited disease was defined as disease confined to one hemithorax and to regional lymph nodes including mediastinum, contralateral hilar and ipsilateral supraclavicular lymph nodes. Any disease beyond these limits was defined as extensive.

A total of fifteen normal bone marrow samples were obtained from among six healthy donors and five patients (1 Ewing's sarcoma and 4 neuroblastoma).

*Collection of bone marrow*

Bone marrow aspirates were obtained from the posterior iliac crest and anticoagulated with 125 units/ml of calparine (Choay, Paris, France). The samples were then diluted with an equal volume of calcium- and magnesium-free Hank's medium. Mononuclear cells were concentrated by density centrifugation on Ficoll-Hypaque (1077 g/cm$^3$) (Pharmacia, Uppsala, Sweden) at 400 ***g*** on the interface during 30 min at 16°C. Next, the cell layer at the interface was collected, washed in Hank's medium and centrifuged at 400 ***g*** for 20 min. Finally, the pellet was counted using a Coulter Counter (Coulter Electronics 2 TD, U.K.) and resuspended in calcium- and magnesium-free PBS medium at a concentration of $2 \times 10^7$ cells/ml.

*Cell line*

NCI-H69, a SCLC cell line, was grown in HITES-RPMI medium at 37°C with a partial pressure of 5% $O_2$ and 5% $CO_2$ [10]. Cells were centrifuged at 300 ***g*** during 20 min and then resuspended in Hank's medium. The viability was determined by Trypan blue exclusion. Only samples having a viability of more than 70% were used and brought to a final concentration of $10 \times 10^6$ cells/ml.

*Anti-rat monoclonal antibodies*

Rat–rat hybridomas were generated and the resulting MoAbs were purified as previously described [11]. Anti-LCA1, -LCA2 and -LCA3 were selected because of the strong expression of their corresponding antigen on SCLC cell lines and their lack of reaction with normal hematologic cells. They are fully described in the accompanying paper [9]. The antibodies were brought to a concentration of 1 mg/ml using 0.2 molar carbonate–bicarbonate (pH 8.8) and 0.15 molar NaCl solution. While stirring, 20 µl of a solution containing 2 mg biotin *N*-hydroxysuccinimide ester diluted in 500 µl anhydrous *N*-dimethylformamide was added. This solution was incubated for 15 min at 25°C and then dialyzed for 24 h at 4°C against 0.15 molar PBS (pH 7.2).

*Indirect immunofluorescence assay*

This assay was performed using each of the three MoAbs separately. Two hundred and fifty thousand cells from the SCLC cell line or 500,000 isolated marrow cells were incubated at 4°C during 30 min with 25 µl of biotinylated MoAbs diluted to 1 : 100. After two washings with PBS (2%) FCS (5%) azide, 50 µl of fluorescent Avidine (Becton Dickinson, California) diluted to 1 : 100 was added to the pellet. Following an incubation period of 60 min at 4°C, the cells were washed again twice. After having mounted cytocentrifuged cell smears in Tris-buffered glycerol (pH 9), the slides were examined with a reflected light fluorescent microscope equipped with a 40 × objective (Olympus Optical Co., Japan). The entire smear was scanned and scored positive if at least one cell with bright surface fluorescence was detected. Positive controls, using the NCI-H69 line, were made with each test. Two separate assays, with each MoAb, were performed on each bone marrow sample to avoid technical errors.

*Cytological and histological examination*

Bone marrow aspirates were stained with May–Grunwald–Giemsa stain and bone marrow biopsies with hemalin–eosin stain. All cytological and histological material was reclassified by a single pathologist.

## RESULTS

Fifteen bone marrow samples originating from individuals free of small cell lung cancer were tested using three anti-LCA MoAbs. Although some granular cells displayed discrete intra-cytoplasmic fluorescent dots, no fluorescent patterns were observed on the surface membrane.

On the contrary, bone marrow aspirates from patients with SCLC may exhibit cells with bright surface fluorescence. Examination of the fluorescent cells under phase contrast revealed living cells consistent with small cell lung cancer. Thus, 84 bone marrow samples from 74 different patients with SCLC, confirmed by cytology and/or biopsy, were studied by morphological and immunological methods: 63 at the time of diagnosis and 21 after at least three cycles of chemotherapy. Whereas bone marrow metastases were diagnosed by classical mor-

*Table 1. Immunodetection of SCLC metastases in bone marrow using three different monoclonal antibodies*

| | No. of marrows tested (%) | No. of positive marrows using MoAbs (%) |
|---|---|---|
| Morphologically positive | 10 (12) | 10 (12) |
| Morphologically negative | 74 (88) | 22 (26) |
| Total | 84 (100) | 32 (38) |

*Table 2. LCA expression in 32 bone marrows from SCLC patients*

| No. of bone marrows | | LCA1 | LCA2 | LCA3 |
|---|---|---|---|---|
| | 7 | + | + | + |
| | 4 | + | + | − |
| | 1 | + | − | + |
| | 3 | − | + | + |
| | 8 | + | − | − |
| | 3 | − | + | − |
| | 6 | − | − | + |
| Total | 32 | 20 (62%) | 17 (53%) | 17 (53%) |

phology in 10 different specimens (12%) (Table 1), tumor cells were recognized in 32 cases (38%) by means of indirect immunofluorescence using the three anti-LCAs separately.

Immunofluorescence identified positive tumor cells in each of the 10 samples. Nine out of these then positive specimens showed reactivity with at least two monoclonal antibodies. Twenty-two samples, previously found to be negative using cytological and histological methods, were proven positive through immunodetection.

A remarkable heterogeneity in tumor antigen recognition was noted (Table 2). Anti-LCA1 recognized tumor cells in 20 of the 32 immunopositive marrows and anti-LCA2 and -LCA3 each recognized 17 out of these 32. Moreover, involvement was detected exclusively by anti-LCA1 in eight cases (25%), by anti-LCA2 in three cases (9%) and by anti-LCA3 in six cases (19%). Overall, out of 32 samples, anti-LCA1 recognized positive cells in 62%, -LCA2 and -LCA3 in 53%.

Sixty-three patients were at the time of initial staging when entered into the study (Table 3). Of these, 34 (54%) were classified as limited disease before immunodetection. However, MoAb positive cells were detected in nine of the marrow samples thereby leaving only 25 (40%) 'sensu stricto' limited disease patients. Among the 29 extensive disease patients, 10 manifested bone marrow involvement detected by morphology and six additional cases were revealed by immunodetection.

*Sensitivity = (true positives)/(true positives + false negatives).

## DISCUSSION

The interest provided by immunological staining for the purpose of detecting bone marrow metastases in solid tumors has been established in cases of breast cancer, neuroblastoma and SCLC [7, 12, 13]. Our study using three monoclonal antibodies confirms and extends previous results of Stahel *et al.* [7] showing the higher sensitivity of bone marrow metastasis detection in SCLC when staining with a single antibody. None of the 15 non-SCLC marrow samples reacted with anti-LCA MoAbs confirming the specificity of the antibodies. In our series, 38% of the samples studied revealed the presence of metastatic cells whereas only 12% were diagnosed by conventional morphological examination (Table 1). The correlation between morphological evidence of marrow involvement by SCLC and immunodetection is excellent. All patients with morphological evidence of metastasis to the bone marrow had SCLC identified by at least one of the three antibodies, the sensitivity* of the immunofluorescence being therefore 1.

The application of bone marrow immunodetection can improve the quality of the initial staging. A positive pretreatment bone marrow is an unfavorable prognostic indicator both for survival and duration of remission [1]. In point of fact, nine patients classified as having limited disease showed marrow metastases using immunofluorescence (Table 3). Nevertheless, it should be pointed out that the prognostic significance of bone marrow micrometastases in otherwise limited disease patients is not yet known. Time will tell if this technique identifies patients with outcomes significantly different from those of other limited disease patients with negative bone marrow.

Previous studies have shown that anti-LCA1, -LCA2 and -LCA3 recognize three different lung cancer associated antigens. Expression of LCA on 32 SCLC cell lines (classic and variant) was studied by radiobinding assay and immunocytochemistry. Even though considerable antigeneic heterogeneity was shown between the cell lines themselves, all lines were detected by at least one or more of these three MoAbs [9]. Antigeneic variability was also noted when performing MoAb staining on bone marrow from SCLC patients. Anti-LCA2 and

*Table 3. Correlation between clinical stage and bone marrow involvement*

| Clinical stage | No. of patients | No. of patients (%) with bone marrow involvement detected | |
|---|---|---|---|
| | | By morphology | By immunofluorescence |
| Limited disease | 34 | 0 (0) | 9 (26) |
| Extensive disease | 29 | 10 (34) | 16 (55) |
| Total | 63 | 10 (13) | 25 (40) |

-LCA3 detected tumoral cells in 53% of the immunopositive samples while anti-LCA1 recognized involvement in 62%. In 17 out of 32 immunopositive samples, tumor cells were detected exclusively by one of the three MoAbs used. This highlights the advantage of using a panel of MoAbs with different antigeneic specificities for micrometastasis immunodetection. In future studies, this methodology should be prospectively applied to specify the prognostic significance of micrometastasis immunodetection at time of diagnosis, to monitor the residual disease following chemotherapy, to select candidates for autologous bone marrow transplantation and to evaluate the efficacy of purging procedures.

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