Characterization of Two Human Small Cell Lung Carcinoma-reactive Monoclonal Antibodies Generated by a Novel Immunization Approach

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ABSTRACT

Two human small cell lung carcinoma cell lines, NCI-H69 and NCI-H128, were used as alternating sources of immunogen to generate monoclonal antibodies to small cell lung carcinoma-associated antigens. BALB/c mice were sensitized with seven injections of live tumor cells, four with NCI-H69 cells and three with NCI-H128 cells. Somatic cell hybridization was performed by fusion of the immune murine splenocytes using syngeneic myeloma cells from the SP2/0 Ag14 cell line. Hybridoma colonies were screened against small cell lung carcinoma cells and normal lung fibroblasts with an enzyme-linked immunosorbent assay. Compared to animals immunized with only NCI-H69 or NCI-H128 cells, alternate immunization resulted in the generation of a significantly higher number of hybridomas that reacted selectively with both tumor cell lines. Monoclonal antibodies from two reactive hybrid clones generated by alternate immunization, SCLC 2051 and SCLC 5023, were uniformly negative to normal human tissues including lung, kidney, liver, spleen, breast, thyroid, brain, small intestine, and colon. While both monoclonal antibodies were nonreactive to paraffin-embedded, formalin-fixed, nonmalignant lung biopsies, the monoclonal antibody SCLC 5023 reacted with tumor cell infiltrates in biopsies from small cell lung carcinoma patients (14 of 14 cases positive), using the immunoperoxidase technique. This monoclonal reagent also reacted with other lung tumor cell types, including atypical carcinoid (5 of 5 positive), epidermoid (4 of 6 positive), undifferentiated and bronchoalveolar (3 of 4 cases each positive) carcinomas. By contrast, monoclonal antibody SCLC 2051 apparently identified an antigen expressed preferentially on small cell lung carcinoma cells (12 of 14 positive) and only rarely reacted with other lung tumor cell types (2 of 34 positive). Both monoclonal antibodies were negative to colon carcinoma, epidermoid carcinoma of the floor of the mouth, breast adenocarcinoma, and B- and T-cell leukemia and lymphoma cells, as determined by the enzyme-linked immunosorbent assay, indirect immunofluorescence, and immunoperoxidase techniques. These observations suggest that SCLC 2051 and SCLC 5023 may be of value in identifying tumor-associated antigens expressed in small cell and other lung carcinomas. In addition, the generation of antibody-producing cells towards common tumor-associated antigens may be enhanced by immunization with multiple tumor cell lines of the same histological type.

INTRODUCTION

SCLC comprises approximately 20% of human lung cancers and is distinct from other lung carcinoma histological cell types with respect to cell of origin, natural history, and treatment. Believed to originate from transformation of the neuroendocrine granule-rich lung basal cells (K-cells), SCLC has a rapid growth rate and a high incidence of metastasis at an early stage (17). Although the majority of patients initially respond to conventional chemotherapy, long-term prognosis is poor (<5% 5-year survival). Furthermore, the administration of appropriate therapy requires accurate histological distinction of SCLCs from non-SCLCs; this distinction is often difficult (11, 17).

With the recent advent of the somatic hybridization technique (13), antigens preferentially expressed in various human tumors can be identified and characterized, using tumor-specific MoAbs. In various human tumor systems, such as melanoma (18) and lymphoreticular neoplasms (6), the identification of tumor antigen expression by specific MoAbs has contributed to better understanding of tumor biology and the malignant transformation event (7). MoAbs also have demonstrated potential usefulness in many clinical applications, including the immunohistochemical diagnosis and classification of primary cancers (8, 20), location of metastases by radioimaging (20), or use as therapeutic agents (14). However, few studies have focused on SCLC, due primarily to the limited availability of specific monoclonal reagents (1–3, 12, 15, 16, 19).

In order to develop monoclonal reagents suitable for the study of SCLC, we have utilized an alternating immunization approach in an attempt to select for tumor-reactive antibody-producing cells. Because immunization with tumor cells from a single cell line generally results in a poor yield of tumor-reactive hybridomas, cells from 2 distinct human SCLC cell lines were used as alternate sources of immunogen. Somatic hybridization of spleen cells from the alternatively immunized animals resulted in significantly higher numbers of clones reactive to SCLC cell lines and tissue biopsies. Two of the MoAbs described in this report, SCLC 2051 and SCLC 5023, were found to be unreactive with various normal human tissues and with other human malignant tumors. SCLC 5023 was reactive with SCLC and, to a lesser extent, with various non-SCLC types. By contrast, SCLC 2051 identified an antigen primarily expressed on SCLC cells but was largely negative with non-SCLC lung carcinomas. Both antibodies inhibited SCLC clonal growth in vitro. These properties suggest that...
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these MoAbs may be of value in further clinical and biological studies of human SCLC.

MATERIALS AND METHODS

Source of Cells and Tissues. The SCLC cell lines NCI-H69 and NCI-H128 were kindly provided by Dr. Adi F. Gazdar, National Cancer Institute; UM-1, floor of the mouth epidermoid carcinoma cell line, and UM-2, a lung epidermoid carcinoma cell line, were provided by Dr. Tom Carey, University of Michigan Medical Center. The mouse myeloma cell line, SP2/0 Ag14, and human myeloma cell line JLC-MON-HMy 2 were obtained from Dr. Denis Burger, VA Medical Center, Portland, OR. LUF cell lines Ag 4432 and Ag 2262 were obtained from the NIA Aging Cell Repository, Camden, NJ; the epidermoid lung carcinoma cell lines Calu-2 and Sk-MES-1, lung adenocarcinoma cell lines Calu-3 and Sk-Lu-1, anaplastic lung carcinoma Calu-6, and undifferentiated lung carcinoma cell line A-427 were obtained from the American Type Culture Collection, Rockville, MD. Peripheral blood lymphocytes from leukemia and lymphoma patients were derived from blood samples drawn by venipuncture with prior consent. Normal brain tissue was kindly provided by Dr. J. Luby, University of Texas Health Science Center at Dallas. Biopsies of other normal tissues and formalin-fixed, paraffin-embedded tissue blocks of confirmed cases of various lung tumors were obtained from the Department of Surgical Pathology, Baylor University Medical Center. All procedures involving the handling of human tissues were previously approved by the Institutional Review Board for Human Protection, Baylor University Medical Center.

Fusion Protocol. Two 2-month-old female BALB/c mice were immunized to 2 human SCLC cell lines (NCI-H69 and NCI-H128). Each mouse received 7 i.p. injections of 1 × 10⁶ cells, alternating H69 with H128 cells. The first 4 injections were made weekly, followed by boosts 3 days apart. Other mice were immunized with 7 injections of each SCLC cell line alone. Somatic hybridization was performed 3 days following the last injection. "Filler cells" were prepared using thymocytes from the same and 2 other mice of the same age. A single-cell suspension was prepared in IMDM (Grand Island Biological, Grand Island, NY) and suspended at 1 × 10⁶/ml in IMDM (Grand Island Biological) and 100 mm hypoxanthine: 0.4 mm aminopterin: 16 mm thymidine (Sigma Chemical Co., St. Louis, MO). One hundred μl of the thymocyte suspension were dispensed into each well of 5 96-well microtiter plates and stored at 37°.

Spleen cells from an immunized animal were extracted by gently teasing the spleen with a pair of forceps. The cells were washed twice in IMDM and then resuspended in 25 ml of IMDM (5 to 10 × 10⁶/ml). Myeloma cells from the nonsecretory SP2/0 Ag14 cell line were collected at logarithmic growth phase, washed twice in IMDM, and then pelleted in 100 ml of IMDM and then resuspended in 25 ml of IMDM (5 to 10 × 10⁶/ml). The culture was closely monitored daily to observe for the emergence of clones. Culture wells containing single clones were noted by Days 5 to 7 and tested at confluency. Hybrid colonies were cloned 2 to 3 times to ensure monoclonality.

ELISA Assay. An ELISA technique utilizing intact target cells was used for screening antibody activity in hybridoma supernatant fluids (22). Target cells were washed twice and suspended in PBS at 1 × 10⁶/ml. Fifty μl of the cell suspension (5 × 10⁴ cells) were added to each well of a 96-well microtiter plate (96 U-bottomed wells; Falcon Plasticware, Becton Dickinson Labware, Oxnard, CA). The plates were dried at 37° overnight and then sealed and placed at 4° for storage. On the day of evaluation, the antigen-coated plates were flooded with 200 μl of 1% bovine serum albumin (Sigma) in PBS for 30 min to block the remaining protein-binding sites. Fifty μl of supernatant fluid were added to each well, incubated for 1 hr at 37°, and then washed 3 times with PBS. Fifty μl of a peroxidase-conjugated secondary antibody (peroxidase-conjugated goat anti-mouse IgG + IgM, 1:3000; Tago, Burlingame, CA) were added, and the plate was further incubated for 1 hr at 37°. After a washing, 50 μl of freshly prepared substrate solution (4 mg o-phenylenediamine in 10 ml 80 mm citrate-phosphate buffer, with 4 μl of 30% H₂O₂ were added and incubated for 30 min at 37°, followed by 25 μl of 4 M H₂SO₄ immediately before reading. Antibody binding was determined as a function of absorbance at 492 nm (Titertek Multiskan MC; Flow Laboratories, McLean, VA). Antibody binding in each well was graded against negative controls (treated with an irrelevant primary antibody) as + (≥0.1 absorbance unit higher than control, triplicate determinations), ++ (≥0.2 absorbance unit over control), or +++ (≥0.3 absorbance unit over control).

MoAb Isotyping. An ELISA procedure similar to that described above was used. However, subclass-specific rabbit anti-mouse immunoglobulins were used as secondary reagents (Mouse Immunoglobulins Subtype Identification Kit; Boehringer Mannheim Biochemicals, Indianapolis, IN). A peroxidase-conjugated goat anti-rabbit antibody was added as tertiary antibody.

Immunofluorescence Assay. Target cells from cell line cultures or Hytypaque:Ficol (Sigma)-separated peripheral blood lymphocytes were washed twice with PBS and then resuspended at 2 × 10⁶/ml in PBS containing 0.02% sodium azide. Fifty μl of tissue culture supernatant fluid were added to a 12- x 75-mm plastic disposable tube (Falcon) containing 1 × 10⁶ cells. The reaction mixtures were incubated in an ice bath (2-8°) for 1 hr and then washed twice with PBS:azide at 4°. Fifty μl of a fluorescein isothiocyanate-conjugated secondary antibody [F(ab')₂] goat anti-mouse immunoglobulin (1:10; Cappel Laboratories, Westchester, PA) were added (4°, 60 min). The reaction mixtures were washed twice and fixed with 1% formaldehyde in PBS. The frequency of antibody-reactive cells in the target cell population was determined by flow cytometry (Becton-Dickinson FACS Systems, Mountain View, CA), using the parameters of cell size (forward angle scatter) versus green fluorescence.

Immunoperoxidase Protocol. The MoAb reactivity to normal tissues was evaluated by the immunoperoxidase technique (29) with a panel of fresh tissues cryopreserved within 1 hr of extraction. Sections (5 μm) from each sample were prepared on the day of evaluation, fixed with acetone (10 min, 23°), and washed twice by submerging in PBS. These sections were treated with 1% H₂O₂ in absolute methanol (30 min, 23°), washed in distilled water and PBS, and then treated with normal horse serum and subsequent reagents as described below. The MoAb reactivity to various lung carcinomas was determined using formalin-fixed, paraffin-
embedded tissue blocks from previously confirmed cases diagnosed by the Department of Surgical Pathology, Baylor University Medical Center. The sections were deparaffinized, rehydrated in xylene and graded alcohols, and then incubated with 1% H2O2 in methanol for 30 min. After a thorough rinsing in distilled water and PBS, the sections were treated first with diluted normal horse serum for 20 min and then with the primary antibody (culture supernatant) for 30 min, washed, and then treated with the secondary antibody (biotinylated horse anti-mouse IgG, 30 min, 23°C). The samples were again washed in PBS and treated with avidin-biotin-conjugated horseradish peroxidase (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) for 60 min. All incubations were performed at room temperature in a humidity chamber. Freshly prepared substrate (0.02% 3-amino-9-ethylcarbazole; 0.03% H2O2; 5% A/W-dimethylformamide in 0.1 M acetate buffer, pH 5.2) was added for 30 min, followed by the counterstain (0.125% methylene blue) and mounting solution (glycerol gelatin; Sigma).

Clonal Growth Assay. The assay was a modification of the soft-agar growth assay described by Minna et al. (16). Cells from the NCI-H69 cell line were harvested from culture, washed twice, and resuspended in serum-free IMDM at 1 x 10^6/ml. One hundred μl of the approximately diluted supernatant fluids from SCLC 2051, SCLC 5023, and an H69-nonreactive clone were added to 17 x 134-mm sterile disposable centrefuge tubes (Falcon) containing 1 x 10^5 cells and incubated at room temperature for 1 hr. Two μl of 0.3% agar in IMDM:15% FCS were added, and equal volumes of the mixture were layered in duplicate onto a 6-well culture plate (Falcon) with a previously prepared base layer (0.5% agar in IMDM:15% FCS). The plates were incubated at 37°C for 1 week. The number of colonies (with ≥50 cells) in each well was enumerated under low magnification (x10). Growth inhibition of the culture supernatant was determined as

% of colony growth inhibition
\[ = \left( 1 - \frac{\text{Mean no. of colonies with antibody treatment}}{\text{Mean no. of colonies without antibody treatment}} \right) \times 100 \]

Statistical Analysis. The expected yield of hybridomas per well, or the frequency of SCLC-reactive hybridomas per well was calculated according to Poisson distribution analysis by the formula

\[ P_e = e^{-x} \]

where \( P_e \) is the observed frequency of zero events (number of negative wells/288), and \( x \) is the expected mean number of events per well (28).

The \( \chi^2 \) test (27) for comparing yields of SCLC-reactive hybridomas per well was calculated by the formula

\[ \chi^2 = \sum \frac{(\text{observed} - \text{expected})^2}{\text{expected}}, \text{ d.f.} = 1 \]

RESULTS

Generation and Screening of MoAbs. In an effort to enrich for antibody-producing cells to common SCLC antigens, we immunized mice with alternate injections of 2 different human SCLC cell lines (NCI-H69 and H128). To determine the efficacy of this alternate immunization protocol, parallel somatic hybridization experiments were performed with animals that had received the same number of injections of either H69 or H128 cells alone. When cultures were examined on Day 12, 47 to 53% of the culture wells from H128- or H69-immunized animals generated one or more hybrid colonies. By contrast, alternate immunization with both cell lines yielded hybrid colonies in over 90% of the wells (Table 1). The number of colonies remained unchanged up to Day 21. An ELISA assay was used for screening against the SCLC cell lines, as well as LUFB of fetal (Ag 4432) and nonfetal (Ag 2262) origin, in order to identify hybridomas with selective reactivity towards common SCLC-associated antigens. Alternate immunization resulted in 65 colonies that reacted with one of the 2 SCLC cell lines (43 reactive to H69, 22 reactive to H128) but not with either of the fibroblast cell lines (Table 1). Eleven colonies reacted with both the SCLC cell lines (H69 positive, H128 positive, LUFB negative). By comparison, sensitization only with H69 cells generated 30 SCLC-reactive colonies (22 reactive to H69, 8 reactive to H128), 3 of which were reactive with both SCLC cell lines. Sixteen SCLC-reactive colonies were isolated after sensitization with H128 cells (8 reactive to H69, 8 reactive to H128), one of which reacted with both H69 and H128. \( \chi^2 \) analysis indicated that the yield of H69-positive, H128-positive, LUFB-negative hybridomas generated by alternate immunization was significantly higher than that with H69 (\( \chi^2 = 4.57, p < 0.05 \)) or H128 (\( \chi^2 = 8.33, p < 0.005 \)) cells alone.

Poisson distribution analysis was performed to estimate the theoretical precursor frequencies of hybridomas per well (28). Based on the number of wells with no colony growth, 2.77 hybridoma colonies/well can be expected by alternate immunization, as compared to 0.75 and 0.64 in H69 and H128 immunized animals, respectively. The detection of H69-positive, H128-positive, LUFB-negative hybridomas in 11 of 288 wells following alternate immunization represents a mean precursor frequency of 0.04 per well, as compared with 0.01 (3 of 288) and 0.004 (1 of 288) per well in H69- and H128-immunized animals, respectively.

Seven of 11 H69-positive, H128-positive, LUFB-negative hybridomas derived from alternate immunization were subsequently cloned by limiting dilution. Two of these hybrid clones, SCLC 2051 and SCLC 5023, were found to have minimal reactivity to normal human tissues and were further characterized as described below.

Reactivity with Human Cell Lines. The reactivity of MoAbs 2051 and 5023 against various human cell lines was determined by the ELISA assay (Table 2). Supernatant fluids from the hybrid clone 2051, which secretes a IgG1 λ antibody, and the IgG2a λ-secreting 5023 were collected from confluent cultures. Both MoAbs reacted with cells from the SCLC cell lines, NCI-H69 and H128, but were negative against other human cell lines, including other lung carcinomas (epidermoid, adenocarcinoma, undifferentiated and anaplastic carcinoma), head and neck epidermoid carcinoma, and LUFB of fetal and nonfetal origin. Moreover, no
reactivity was observed with peripheral blood lymphocytes derived from normal donors, or from patients with B- or T-cell leukemia, or diffuse poorly differentiated lymphocytic lymphoma. These observations were corroborated by cell-binding studies, using the indirect immunofluorescence technique (Table 2). Significant, although relatively low, levels of SCLC binding (6 to 26%) were detected with both MoAbs. No significant binding by SCLC 2051 to other cell lines was observed. SCLC 5023 reacted with 10% of cells from the epidermoid lung carcinoma cell line, UM2, but this MoAb did not react with other target cells.

Immunohistochemical Characterization. The immunoperoxidase technique was used to examine the reactivity of SCLC 2051 and SCLC 5023 to normal human tissues (Table 3). Both MoAbs were unreactive to cryopreserved, acetone-fixed, non-malignant tissues from lungs and other organs, including kidney, spleen, small bowel, breast, liver, colon, thyroid, and brain. SCLC 2051 and SCLC 5023 were positive to similarly fixed cytospin preparations of H69 and H128 cells but exhibited no reactivity with breast or colon carcinomas.

The reactivity of SCLC 2051 and SCLC 5023 against SCLC, non-SCLC lung tumors, and nonmalignant lung was determined by the immunoperoxidase technique, using paraffin-embedded, formalin-fixed samples. Positive biopsy sections from independently diagnosed cases were treated in a blind manner and then evaluated by a second observer. The reactivity of each reagent was graded by comparing against a section treated with a negative control antibody (MM 5001) that reacted with human plasmacytes, but not with normal or malignant lung tissues.

The MoAb SCLC 2051 reacted with all SCLC biopsies from primary sites (8 of 8 cases (Table 4)). This antibody also demonstrated strong reactivity with SCLC biopsies from metastatic sites (4 of 6 cases; lymph node biopsies). Ten to 50% of the tumor cell infiltrates were SCLC 2051 positive. There was little or no cellular reactivity to adjacent normal tissues. Staining patterns were consistent with binding to tumor cell membrane as well as to cytoplasmic determinants and were not affected by trypsinization of the tissue sections. This antibody did not react with nonmalignant lung biopsies (0 of 6 cases positive). Similarly, biopsies from non-SCLC lung tumor cases also were unreactive, although some weak staining was observed in 2 of the 34 specimens examined (Table 5).

The MoAb SCLC 5023 reacted strongly with all of 14 SCLC biopsies (Table 4). In general, it demonstrated a higher staining intensity than did SCLC 2051 and identified 10 to 75% of the tumor cell infiltrates. This antibody was also positive with other non-SCLC lung tumors (Table 5), including atypical carcinoid (5 of 5 cases), epidermoid carcinoma (4 of 6 cases), bronchoalveolar and undifferentiated carcinoma (3 of 4 cases each), and adenocarcinoma (1 of 7 cases). SCLC 5023 reacted predominantly with cell surface determinants, although some cytoplasmic reactivity was noted. Little or no cellular reactivity to adjacent normal tissue constituents was observed. This MoAb was uniformly unreactive to nonmalignant lung biopsies [0 of 6 cases (Table 5)].

### Table 2

MoAb reactivity to human cell lines and peripheral blood lymphocytes

<table>
<thead>
<tr>
<th>Target cell</th>
<th>SCLC 2051 reactivity</th>
<th>SCLC 5023 reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA&lt;br&gt;Immunofluorescence&lt;br&gt;ELISA</td>
<td>ELISA&lt;br&gt;Immunofluorescence&lt;br&gt;ELISA</td>
</tr>
<tr>
<td>SCLC</td>
<td>++&lt;sup&gt;a&lt;/sup&gt; 15</td>
<td>+ 7 &lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>H69</td>
<td>+ 6</td>
<td>26</td>
</tr>
<tr>
<td>H128</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Non-SCLCs

<table>
<thead>
<tr>
<th>Epidermoid</th>
<th>SCLC 2051</th>
<th>SCLC 5023</th>
</tr>
</thead>
<tbody>
<tr>
<td>UM2</td>
<td>-&lt;1</td>
<td>10</td>
</tr>
<tr>
<td>Calu-1</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>Sk-MES-1</td>
<td>- 1</td>
<td>NT</td>
</tr>
</tbody>
</table>

Adenocarcinoma

<table>
<thead>
<tr>
<th>Cell-3</th>
<th>SCLC 2051</th>
<th>SCLC 5023</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
| SCLC 2051 and SCLC 5023 reactivity with normal human tissues (Table 3). Both MoAbs were unreactive to cryopreserved, acetone-fixed, non-malignant tissues from lungs and other organs, including kidney, spleen, small bowel, breast, liver, colon, thyroid, and brain. SCLC 2051 and SCLC 5023 were positive to similarly fixed cytospin preparations of H69 and H128 cells but exhibited no reactivity with breast or colon carcinomas.

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### Table 3

Immunoperoxidase reactivity of SCLC 2051 and SCLC 5023 with normal human tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Immunoperoxidase reactivity&lt;sup&gt;b&lt;/sup&gt; (no. positive/no. treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>SCLC 2051: 0/3, SCLC 5023: 0/1</td>
</tr>
<tr>
<td>Spleen</td>
<td>SCLC 2051: 0/2, SCLC 5023: 0/2</td>
</tr>
<tr>
<td>Kidney</td>
<td>SCLC 2051: 0/2, SCLC 5023: 0/2</td>
</tr>
<tr>
<td>Liver</td>
<td>SCLC 2051: 0/1, SCLC 5023: 0/1</td>
</tr>
<tr>
<td>Small bowel</td>
<td>SCLC 2051: 0/1, SCLC 5023: 0/1</td>
</tr>
<tr>
<td>Breast</td>
<td>SCLC 2051: 0/1, SCLC 5023: 0/1</td>
</tr>
<tr>
<td>Colon</td>
<td>SCLC 2051: 0/1, SCLC 5023: 0/1</td>
</tr>
<tr>
<td>Thyroid</td>
<td>SCLC 2051: 0/1, SCLC 5023: 0/1</td>
</tr>
<tr>
<td>Brain</td>
<td>SCLC 2051: 0/1, SCLC 5023: 0/1</td>
</tr>
</tbody>
</table>

<sup>a</sup> By the avidin:biotin complex immunoperoxidase technique (see text).

<sup>b</sup> Determined by absorbance.

<sup>c</sup> Percentage of cell binding as determined by flow cytometry per 10<sup>6</sup> target cells.

<sup>d</sup> NT, not determined.

### Table 4

Immunoperoxidase reactivity of SCLC 2051 and SCLC 5023 MoAb with SCLC

<table>
<thead>
<tr>
<th>Biopsy site&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SCLC 2051</th>
<th>SCLC 5023</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td>Metastatic</td>
<td>4/6</td>
<td>6/6</td>
</tr>
<tr>
<td>Total</td>
<td>12/14</td>
<td>14/14</td>
</tr>
</tbody>
</table>

<sup>a</sup> From histologically confirmed cases of SCLC.

### Table 5

MoAb reactivity to biopsies from nonmalignant lung and non-SCLCs

<table>
<thead>
<tr>
<th>Biopsy diagnosis</th>
<th>SCLC 2051</th>
<th>SCLC 5023</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonmalignant lung</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Lung tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>0/7</td>
<td>1/7</td>
</tr>
<tr>
<td>Atypical carcinoid</td>
<td>0/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Bronchoalveolar</td>
<td>1/4</td>
<td>3/4</td>
</tr>
<tr>
<td>Epidermoid</td>
<td>0/6</td>
<td>4/6</td>
</tr>
<tr>
<td>Large cell</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>1/4</td>
<td>3/4</td>
</tr>
</tbody>
</table>
Inhibition of Colony Growth. The data described above indicated the presence of SCLC 2051- and SCLC 5021-reactive antigens among SCLCs. In order to examine the biological effect of these antibodies on SCLC cells, we determined the colony growth of cells from the NCI-H69 cell line after MoAb treatment, using an in vitro semisolid agar growth assay (16).

Compared to samples that were not exposed to antibody, NCI-H69 cells treated with SCLC 2051 or SCLC 5023 demonstrated significant inhibition of clonal growth over a wide range of dilutions (Table 6). SCLC 2051 inhibited 46% of colony formation at a final dilution of 1:10,000. Similarly, SCLC 5023 was effective in inhibiting clonal growth (>60%) at various dilutions. Growth inhibition appeared related to specific antibody binding, since neither antibody inhibited clonal growth of an irrelevant human myeloma cell line (LICR-MON-HMy 2) within the same dilution range.

**DISCUSSION**

Immunization with intact tumor cells has the theoretical advantage of presenting tumor-associated antigens in their native form but is inefficient in generating tumor-reactive antibody-producing cells. Antibodies specific for tumor-associated antigens constitute only a portion of the humoral response, with the majority of antibodies directed to other normally occurring surface antigens, such as histocompatibility antigens or blood group substances (24). Repeated boosts tend to result in suppression of the overall response, rather than having the desired effect of enhancing antibody production to "weak" tumor antigens (7, 26).

In order to select for antibody-producing cells that were tumor antigen specific, we immunized animals to alternate sources of SCLC antigens expressed in 2 separate cell lines. Each of these cell lines previously has been found to elicit SCLC-specific responses (3). Our thesis is similar to the concept of "original antigenic sin" of Fazekas de St. Groth, who described the synergistic effect of a previous virus infection on the humoral response to a subsequent infection by another cross-reacting strain (4, 5, 25). Antibodies that reacted equally with both viruses were directed towards common determinants and had the high avidity of a secondary humoral response. Our observations suggest that a similar mechanism may be operative in the antibody response to SCLC. In initial attempts to generate SCLC-reactive hybridomas, we noticed that higher numbers of hybrid colonies resulted from boosting H128-immunized animals with cells from the H69 cell line. Subsequently, animals were sensitized alternately with H69 and H128 cells to maximize the generation of SCLC-reactive hybridomas. Parallel fusion experiments using each cell line alone indicated that alternate immunization yielded significantly more hybridomas, with a proportionate increase in SCLC-reactive clones. Although the binding avidities of these MoAbs remain to be determined, the majority were of the IgG isotype, a finding consistent with an anamnestic response. This is in contrast to most other SCLC-reactive MoAbs generated by hyperimmunization with a single SCLC cell line, which have been of the IgM isotype (1, 3, 23). Thus, alternate immunization may be an effective approach in enhancing sensitization to common antigenic determinants expressed by SCLC or other tumor cell lines.

The MoAbs 5023 and 2051 described in this report identify tumor antigens expressed both in SCLC cell lines, as well as histological tissue sections. Determinations by ELISA, immunofluorescence, and immunoperoxidase techniques indicated that the SCLC 2051-reactive antigen was poorly expressed in normal tissues, most non-SCLC lung carcinomas, or non-lung carcinomas (Tables 3 and 5). By contrast, SCLC 5023 recognized an antigen expressed by most lung carcinoma subtypes, including small cell. Since SCLC 5023 was unreactive with nonmalignant lung tissues and several non-SCLC tumor cell lines, its reactivity is probably directed to a common lung carcinoma-related antigen. Other investigators have produced SCLC-reactive MoAbs that also react with other lung carcinoma cell types but not with normal tissue constituents. MoAbs described by Cuttitta et al. (3) and Rosen et al. (23) reacted with SCLC, as well as adenocarcinoma and squamous cell carcinoma of the lung. Alternatively, immunization with a large cell carcinoma cell line resulted in a MoAb that reacted with adenocarcinoma, epidermoid, and large cell lung carcinoma (19). Our SCLC 5023 MoAb reacted with a lung tumor antigen that is expressed on small cell, as well as bronchoalveolar and epidermoid carcinoma, but not on large cell and or most adenocarcinomas of the lung. Although each of these MoAbs reacts with multiple lung carcinoma subtypes, their pattern of reactivity suggests that they probably recognize different tumor antigenic determinants.

MoAbs that are relatively subtype specific also have been reported. These include 9.2.2, an epidermoid-carcinoma specific antibody (2), and the SCLC-specific antibody SM-1 described by Bernal et al. (1), as well as SCLC 2051 described in this study. SM-1, an IgM antibody, and SCLC 2051, an IgG1 antibody, have similar reactivity largely restricted to SCLC. SM-1 reacted with antigenic determinants with molecular weights of 50,000 and 25,000, whereas SCLC 2051 immunoprecipitated antigenic determinants with molecular weights of approximately 30,000 and 25,000. Further biochemical characterization will be required to determine whether these 2 MoAbs identify the same SCLC-related antigen.

It is of biological interest that both SCLC 2051 and SCLC 5023 inhibited the clonal growth of H69 cells in vitro. This effect appeared to be related to specific antigen binding, since no growth inhibition of an unrelated myeloma cell line was observed. Several considerations may explain the significantly higher levels of inhibition compared to the frequency of cell binding observed by indirect immunofluorescence. Immunofluorescence, a rela-

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**Table 6**

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Final concentration</th>
<th>H69</th>
<th>LICR-MON-HMy 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC 2051</td>
<td>1:10</td>
<td>93</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>91</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>91</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>1:10000</td>
<td>91</td>
<td>0.67</td>
</tr>
<tr>
<td>SCLC 5023</td>
<td>1:10</td>
<td>71</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>71</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>71</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>1:10000</td>
<td>71</td>
<td>0.65</td>
</tr>
</tbody>
</table>

* By the soft-agar growth assay (see text).
* NT, not tested.

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8 Preliminary observations.
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tively insensitive technique, may fail to detect antibody binding to cells with low antigenic densities on their surface. Alternatively, the expression of tumor antigens may be cell cycle related, accounting in part for the antigenic heterogeneity of most tumor cell populations (10). It is also possible that the antigens reactive to SCLC 2051 and 5023 may be selectively expressed on subpopulations required for clonal growth.

The pattern of reactivity of SCLC 2051 and SCLC 5023 suggests that each identified distinct antigenic determinants. However, certain evidence indicated that both antigens may be expressed on the same cell subpopulation in SCLC. The 2 antibodies when combined elicited a partially additive response expressed on the same cell subpopulation in SCLC. The 2 subpopulations required for donor growth.


Clinically, the ability to identify subtype-specific lung tumor antigens may be of value for histopathological identification of primary tumors or metastases (8, 11, 20). SCLC 2051 and SCLC 5023 may be useful for this purpose, since their reactive antigens were preserved in paraffin-embedded, formalin-fixed biopsy samples and were unaffected by trypsinization. The high incidence of reactive SCLC cases observed with both MoAbs indicate that these antigens were expressed commonly. SCLC 2051 may be of value in differentiating SCLCs from non-SCLCs, including atypical carcinoids which have similar histological and biochemical features but which carry a distinct prognosis (17). Although each MoAb reacted with only a fraction of the total tumor cell infiltrate, sensitivity of detection can be enhanced by using a panel of several SCLC-reactive monoclonal reagents. The efficacy of such a panel in the in vitro diagnosis of small cell versus non-SCLCs will be the subject of a separate report.4

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Characterization of Two Human Small Cell Lung Carcinoma-reactive Monoclonal Antibodies Generated by a Novel Immunization Approach

Alex W. Tong, Jennifer Lee and Marvin J. Stone


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