Monoclonal Antibodies to Surface Antigens of Small Cell Carcinoma of the Lung

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ABSTRACT

Monoclonal antibodies to membrane antigens of human small cell carcinoma of the lung were produced by fusion of P3X63/Ag8U1 mouse myeloma cells with spleen cells from BALB/c mice immunized against the intact cells of the small cell carcinomas grown in BALB/c nude mice. The hybrids were screened for antibody production using intact cells in a solid-phase radioimmunoassay or in a membrane fluorescence with a fluorescence-activated cell sorter. Four monoclonal antibodies were chosen that demonstrated reactivities with human small cell carcinoma of the lung and not with apparently normal diploid fibroblasts or lymphoblastoid cells. The antibodies designated as TFS-1 and TFS-2 rather demonstrated “pancarcinoma” reactivity, showing binding to the other types of lung cancer (adenocarcinoma, squamous cell carcinoma, and large cell carcinoma) and carcinomas derived from other organs, such as colon, pancreas, or stomach. The monoclonal antibodies TFS-3 and TFS-4 preferentially bound to small cell carcinoma cells and neuroblastoma cells, but not to non-small cell carcinomas (adenocarcinoma, squamous cell, or large cell). Especially, TFS-4 did not bind to a variety of normal or malignant cells. Immunoprecipitation of the antigens by monoclonal antibodies and sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that they had different molecular weights.

INTRODUCTION

There has been a tendency among clinicians to divide lung cancer into 2 major clinicotherapeutic categories: SCCL and NSCCL. While there are differences in presentation, response to therapy, and survival among NSCCL, they are relatively modest. SCCL has a much greater metastatic capacity and responsiveness to treatment than does NSCCL. SCCL stands out as a distinct entity (11). Antibodies with sufficient specificity for SCCL or NSCCL may be potentially important diagnostic and therapeutic tools. Several groups have prepared polyclonal antibodies with potential specificity for lung cancer by using standard immunological methods (5, 6, 8, 9, 18, 34, 37, 40). However, since the development of somatic cell hybrid technology for the production of monoclonal antibodies has been reported, only a few monoclonal antibodies have been developed with various degrees of specificity to human lung tumors (12, 28). In this study, we wanted to make monoclonal antibodies that react with SCCL but not with NSCCL. Thus, we immunized mice with tumors transplanted into nude mice and screened the resultant hybrid cell antibody for reactivity against the immunizing cells and other SCCL tumor cells.

MATERIALS AND METHODS

Cells. The sources of references for the various cell lines used are shown in Tables 1 to 4, and they were grown as reported previously (30-33) in F-10, RPMI-1640, or Dulbecco’s modified Eagle’s medium supplemented with 5% FBS (Flow Laboratories Inc., Rockville, MD). All human tumor cell lines, established in our laboratory, were from patients with documented histology of the stated tumor type, and all induced human mouse heterotransplants with a histology similar to that seen in the patients’ tumors. Small cell carcinomas, TNSC-1 and TNSC-2, have been maintained in BALB/c nude mice. Heterotransplants of nude mouse tumors were histologically and functionally similar to the patients’ tumors (23, 43). TNSC-1 cells synthesized antidiuretic hormone and neurophysin, and nude mice bearing the tumor developed “syndrome of inappropriate secretion of antidiuretic hormone.” TNSC-2 produced adrenocorticotropic hormone.

Immunization of Mice. Small cell carcinomas, TNSC-1 and TNSC-2, which had been maintained in BALB/c nude mice, were used for boosted immunization. The fresh tumor cells were obtained by mincing the tumor which had been maintained in BALB/c nude mice, were used for boosted immunization. The fresh tumor cells were obtained by mincing the tumor that had been maintained in BALB/c nude mice, were used for boosted immunization. The fresh tumor cells were obtained by mincing the tumor and cultured in culture medium. The mouse myeloma cell line P3X63Ag8U1 (P3U1), which was kindly provided by Dr. T. Watanabe, Saga Medical School, Saga, Japan, according to the method of Köhler and Milstein (22). The fusion reagent was 45% polyethylene glycol 4000 (Sigma Chemical Co., St. Louis, MO). Fused cells were distributed into 24-well Nunc plates in Dulbecco’s modified Eagle’s medium (Flow) supplemented with 15% FBS (Centaurus Biological Corp., Anaheim, CA), 2 mM L-glutamine, 2 x 10^-5 M 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 µg/ml), and gentamicin (80 µg/ml). The cells were fed with selective hypoxanthine:aminopterin/thymidine medium on alternative days and incubated until growth could be detected microscopically. Plates with growth in 20 to 80% of the cells were selected for antibody-screening assays. Hybrid cells were subsequently cloned several times by the limiting dilution method in 96-well culture plates, using a mouse thymocyte cell feeder layer.

Radioimmunossays. Affinity-purified goat anti-mouse immunoglobulin which were directed against the mouse immunoglobulin F(ab')2, was labeled with 125I (New England Nuclear, Boston, MA) by the chloramine-T method to specific activity of 5 to 10 µCi/µg protein (16). Test cells (1 x 10^6) were washed and suspended in PBS containing 1% bovine serum albumin and 10 mM NaCl. The cells were added to each tube precoated with bovine serum albumin solution; 100 µl of culture fluid supernatants were added to each well, incubated for 90 min at 4°. After washing the cells, 125I-labeled anti-mouse F(ab')2 (1 x 10^6 to 20,000 cpm/tube) was added, and the cells were incubated for another 15 hr. Then, the cells were washed 3 times with PBS, and radioactivities were measured in a γ-counter. All the procedures were performed at 4°. In all experiments,
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P3X63Ag8 culture fluid supernatant was used as a negative control, and the proportion of each count to the count of negative control was expressed as the specific binding.

Fluorescence-activated Cell Sorter Analysis. The cells were washed and suspended in Dulbecco's modified Eagle's medium containing 2% FBS and 0.1% NaN3. The cells (2 x 10^6) were stained by incubating for 45 min at 4°C with 100 μl of culture fluid supernatants. The cells were then washed and treated for 45 min at 4°C with fluorescein isothiocyanate-conjugated antimouse IgG (Meloy Laboratories, Springfield, VA). After washing twice, the stained cells were processed on a FACS IV (Becton Dickinson Electronics Laboratories, CA).

Assay for Complement-mediated Lysis. Cells (5 x 10^6) were labeled with 0.1 ml of 51Cr sodium chromate (1 mCi/ml, New England Nuclear) for 1 hr at 37°C. The cells were washed twice and brought to a final concentration of 6 x 10^6 cells/ml in RPMI 1640 containing 5% FBS; 3 x 10^6 radiolabeled target cells (50 μl) were incubated with various dilutions of the monoclonal antibody (50 μl) on ice for 30 min followed by addition of rabbit complement (50 μl, 1:3 dilution; Rockland, Inc., Gilbertsville, PA) and further incubation at 37°C in a humidified atmosphere of 5% CO2 in air for 2 hr. To elude nonspecific lysis, rabbit complement absorbed 3 times against many cell lines was used. Cell lysis was detected by release of 51Cr into supernatant fluids. Maximal 51Cr release was determined after freeze-thawing target cells. Percentage of cytotoxicity was determined by

\[
\% \text{ of cytotoxicity} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total releasable counts} - \text{spontaneous release}} \times 100
\]

Table 1

Lack of binding of lymphoblastoid cells to anti-SCCL monoclonal antibodies

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<th>Target cells</th>
<th>Ref. or source</th>
<th>TFS-1</th>
<th>TFS-2</th>
<th>TFS-3</th>
<th>TFS-4</th>
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<tr>
<td>Namalva</td>
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<td>Daudi</td>
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<td>1.2</td>
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<td>1.0</td>
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<tr>
<td>CCRF-CEM</td>
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<td>1.1</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>CCRF-HSB-2</td>
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Table 2

Binding of monoclonal antibodies to human lung cancer cells

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<th>Ref. or source</th>
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<th>TFS-2</th>
<th>TFS-3</th>
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RESULTS

Production and Screening of Monoclonal Antibody. Fusion experiments were performed 4 times by immunizing mice with TNSC-1 cells, and once by the use of TNSC-2 cells. Approximately 90% of the wells had growing hybrids, and about 10 to 30% of the hybrid cultures reacted with TNSC-1 cells; 20 to 30% of the hybrid cultures showing selective binding to TNSC-1 cells but not lymphoblastoid cell lines (Table 1) were used for cloning. About 70% of the hybrids showing selectivity in the initial screening could be established as stable antibody-producing clonal lines. Four monoclonal antibodies were chosen that demonstrated reactivities with small cell carcinoma of the lung but not with normal lung fibroblasts for further studies and were designated as TFS-1, TFS-2, TFS-3, and TFS-4.

Reaction of Monoclonal Antibodies to Various Cell Types. The 4 monoclonal antibodies were tested for binding to malignant and nonmalignant human cell lines, erythrocytes, lymphocytes, and granulocytes. TFS-1 bound to all 6 of the SCCL cells and to other NSCCL cells (Table 2). The antibody also bound to other human malignant cells including stomach cancer, uterus cancer, or neuroblastoma cells (Table 3). The antibody did not give significant reactions with lymphoblastoid cells (Table 1), fibroblasts, lymphocytes, or RBC (Table 4). TFS-2 bound to 5 of 6 SCCL cells and to other NSCCL cells (Table 2). The antibody also reacted with other human malignant cells such as stomach...
cancer, uterus cancer, or choriocarcinoma. The antibody failed to react with lymphoblastoid cell, or other malignant cells (Tables 1 and 4). TFS-3 reacted with 3 of 6 SCCL cells, but the antibody did not bind to NSCCL cells (Table 2). It showed slight reactions with neuroblastoma and HeLa S3 cells, but not with any other malignant or nonmalignant cells tested (Tables 1, 3, and 4). TFS-4 was reactive with 4 of 6 SCCL cells but not with NSCCL cells. With the exception of neuroblastoma cells, this antibody failed to react sufficiently with any other malignant or nonmalignant cells examined (Tables 1 to 4).

Immunocytochemical and Fluorometric Analysis. Chart 1 shows immunofluorescence profiles of SCCL cells (NCI-H69) with each monoclonal antibody. As shown, virtually all NCI-H69 cells were reactive with TFS-1, TFS-2, and TFS-4. The cells showed considerably less reactivity with TFS-3 than the other monoclonal antibodies. Immunofluorescent staining of the intact cells with the antibodies is shown in Fig. 1. A ring pattern of cellular fluorescence is observed, which is consistent with surface membrane reactivity.

Characterization of Monoclonal Antibodies. To determine the immunoglobulin subclass of the monoclonal antibodies, Ouchterlony immunodiffusion was performed in 1.3% agar in 70 mm Veronal buffer (pH 8.6) by the use of anti-mouse immunoglobulin class-specific antisera. TFS-1 was IgG2a; TFS-2, IgG2b; TFS-3, IgG1; and TFS-4; IgG1. TFS-1 and TFS-2 showed complement-dependent cytotoxicity against target cells (Chart 2). TFS-3 or TFS-4 was not cytotoxic to the target cells.

Characterization of Antigens. TNSC-1 cells were surface iodinated with 125I by lactoperoxidase method. Radiolabeled cells were dissolved in Nonidet P-40, and the antigens were isolated by Sepharose 4B coupled with the monoclonal antibodies. So

molecular weight of 42,000. The antigen recognized by TFS-2 had an apparent molecular weight of 39,000. M, 110,000 antigen was precipitated by TFS-3. TFS-4 recognized the antigen with a molecular weight of 124,000 under the reducing condition.

DISCUSSION
The present study described 4 monoclonal antibodies with different specificity to small cell carcinoma of the lung. While 2 of them demonstrated "pancarcinoma" reactivity, the others were strongly reactive with SCCL cells but not with NSCCL cells. TFS-1 detected determinant present on SCCL and NSCCL tumors. The antibody reacted with stomach adenocarcinoma, neuroblastoma, and other human malignant cells. It did not react with lung fibroblasts, erythrocytes, or lymphocytes, but slightly bound to granulocytes. TFS-2 reacted with SCCL and NSCCL tumors but failed to react with neuroblastomas. The antibody showed significant reactions with other malignant cells such as choriocarcinoma, or adenocarcinomas of stomach and colon. It did not react with lung fibroblasts, lymphocytes, granulocytes, or erythrocytes. These 2 antibodies showed complement-dependent cytotoxicity against the target cells. While both of the monoclonal antibodies demonstrated rather "pancarcinoma" reactivity, the other 2 antibodies, TFS-3 and TFS-4, appear to have specificity for SCCL. They reacted with SCCL but not with NSCCL. Interestingly, TFS-4 detected the determinant expressed on SCCL and neuroblastoma cells but not on NSCCL or other human cell lines so far tested. These 2 monoclonal antibodies in conjunction with rabbit complement did not mediate lysis of the target cells.

The antigens precipitated by each monoclonal antibody revealed single peaks for each antigen in polyacrylamide gel electrophoresis. Under reducing conditions, molecular weights of the antigens recognized were: by TFS-1, M, 42,000; by TFS-2, M, 39,000; by TFS-3, M, 110,000; and by TFS-4, M, 124,000. Since the molecular weights of the antigens precipitated by TFS-1 and TFS-2 were similar to HLA-DR (la-like) antigens or HLA-A, B, C antigen, binding inhibition experiments were performed to exclude the possibility that the monoclonal antibodies recognized HLA antigens. To examine whether the antibodies would react with HLA-A, B, C antigen, NCI-H69 cells which were shown to possess HLA-A, B, C antigen but not la-like antigens, were
Chart 1. Immunofluorescence profiles of NCI-H69 cells with monoclonal antibodies, TFS-1 (A), TFS-2 (B), TFS-3 (C), and TFS-4 (D). Background fluorescence staining (-----) was obtained by incubating NCI-H69 cells with supernatant fluid of P3X63Ag cells.

Fig. 1. Immunofluorescence pattern of TFS-4 antibody on intact NCI-H69 cells, showing cellular ring pattern.

incubated with anti-HLA-A, B, C xenoantiserum and reacted with the monoclonal antibodies (Table 5) (17). Coating of NCI-H69 cells with the anti-HLA-A, B C xenoantiserum did not significantly affect the reactivity with the monoclonal antibodies, TFS-1, TFS-2, TFS-3, or TFS-4, although it strongly inhibited the binding of monoclonal antibody to human β2 microglobulin. Several lines of evidence excluded the possibility that the monoclonal antibodies described here recognized determinants expressed on la-like antigenic structures; the antibodies did not react with a panel of cultured B-lymphoblastoid cells that expressed la-like antigens in large amounts but displayed strong binding to SCCL cells (NCI-H69) that did not express la-like antigens. These observations strongly suggested that the determinants recognized by our monoclonal antibodies were not HLA antigens.
Although reactivity patterns of these 4 monoclonal antibodies suggested that they recognized different epitopes, immunoprecipitations of the antigens showed that the molecular weights of TFS-1 and -2, or those of TFS-3 and -4 were similar. To see whether TFS-3 and -4 would detect different epitopes, competition studies were performed. TFS-3 did not compete for the epitope recognized by TFS-4 (Table 6). TFS-1 and TFS-2 have been shown to recognize different epitopes (data not shown).

Among 4 antibodies, TFS-3 and TFS-4 differ from those described by Cuttitta et al. (12), which were found to be reactive with 3 of the major types of human lung cancer (small cell, adenocarcinoma, and squamous cell carcinoma). They differ from those described by Mazauric et al. (27) and Mulshine et al. (28), which were reactive primarily with NSCCL tumors. The most interesting possibility may be that TFS-3 and TFS-4 can distinguish SCCL from NSCCL tumors. Our recent observations showed that 2 SCCL tumors freshly isolated from patients were strongly reactive with the antibodies. To determine the usefulness of these antibodies in diagnosis and treatment of lung cancer, a large survey of additional tumors and normal tissue specimens is now ongoing in our laboratory.

Biochemical properties of SCCL have been extensively investigated (1, 7, 14, 36). Pearse (35) identified a widely distributed system of cells having APUD properties (35). SCCL tumors and cell lines have been shown to express these APUD cell properties (7). Compared to the well-investigated biochemical characteristics, our knowledge of surface antigens expressed on SCCL cells is pitifully meager (8, 9, 12). TFS-1 and TFS-2 cross-reacted with some of NSCCL tumors, but TFS-3 and TSF-4 reacted selectively with SCCL. Thus, different types of lung cancer may share certain antigens as well as express specific antigens. Although SCCL cells express many of APUD cell properties, it

![Chart 2. Complement-dependent cytotoxicity of monoclonal antibody.](image)

![Chart 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of surface antigens.](image)

![Table 6. Competition study of monoclonal antibodies.](table)

![Table 5. Binding experiments with anti-HLA-A, B, C xenoantiserum.](table)
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has not been known if SCCL cells arise from neural crest or endodermal structures as do other bronchogenic carcinomas. Interestingly, TFS-4 cross-reacted with neuroblastoma cells. Recent immunohistochemical studies showed that a carcinoid tumor directly obtained from a patient was strongly stained with TFS-4. These antibodies should aid in the study of the embryonic origin and interrelationship between neural crest and endodermal tumors.

ACKNOWLEDGMENTS

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