Demonstration of Common Sarcoma-associated Antigen(s) in an Established Human Neurogenic Sarcoma Cell Line

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SUMMARY
An established human neurogenic sarcoma cell line presented a tumor-associated antigen common to various sarcoma tumors. The antigen was detected by immunofluorescent techniques with the use of the sera of patients bearing such tumors and is located mainly on the cellular surface. The tumor-specific antibody of most sera showed only membrane immunofluorescence while five sera revealed both intracytoplasmic and membrane immunofluorescence. In one case, only intracytoplasmic fluorescence was detected. Sixteen of 22 sera of patients with sarcoma gave positive reactions with antibody titers ranging from 40 to 160. Only 1 of 16 sera from patients with other neoplastic diseases and 1 serum of 18 normal sera gave positive results. The tumor antigen(s) present in this established cell line may provide an easy, standard source in the development of a clinical laboratory screening and monitoring assay for sarcoma tumors.

INTRODUCTION
Recent data demonstrate convincingly that human tumor cells may carry specific tumor-associated antigens (7, 10, 19, 21, 23).

That malignant cells do synthesize tumor antigens that may evoke specific antibodies from the host has prompted investigations to generate practical diagnostic and monitoring methods (5, 12, 22). The present study was designed to demonstrate, by indirect immunofluorescent techniques, the presence of sarcoma-associated antigens in an established neurogenic sarcoma cell line. The presence of such antigens in the cells of standardized cultures could eventually be applicable as a clinical laboratory screening and monitoring test to detect sarcoma-specific antibodies.

MATERIALS AND METHODS

Cell Lines
T₂ cells, from an established human neurogenic sarcoma line (20), were utilized as the target cells. The cells were grown in Ham's F-10 medium supplemented with 20% fetal calf serum, vitamins, and glutamine; to control airborne bacteria and Mycoplasma contamination, penicillin (100 µg/ml) and tylosin (60 µg/ml, Grand Island Biological Co., Grand Island, N. Y.) (4) were added to the medium. Periodic checks for Mycoplasma contamination were carried out by morphological examination both at the light (3) and electron microscope level and by biochemical determinations for the presence of arginine-deaminase (1). The cultures were always free of Mycoplasma. Methods for serial subcultures and single-cell suspensions have been described elsewhere (2). Control cells were obtained from established human lines of melanoma, colon adenocarcinoma, and lymphoma growing in our own laboratory and from other tissue culture laboratories in our institution.

Sera
Serum was obtained from patients with established histological diagnosis at the time of their arrival at M. D. Anderson Hospital before chemotherapy was instituted. In 7 cases surgical procedures had been carried out within a 2- to 3-week interval before the collection of the sera but no other therapy had been given. Samples from patients with previous transfusions or pregnancy were rejected. Normal serum was obtained from presumably healthy blood bank donors or from laboratory personnel.

The blood samples were allowed to clot and were then centrifuged for 10 min. The supernatant was carefully aspirated, avoiding mixing with red blood cells and the buffy coat. The serum was decomplemented by incubation in a water bath at 56°C for 1 hr. For absorption of possible blood group-specific antibodies, the test sera were incubated at 37°C for 1 hr with washed AB human red blood cells and then centrifuged for 5 min. The cell plug was discarded, and the supernatant was incubated in a 37°C water bath for 1 hr with a R₁₁ r cell panel (Selectogen I, Ortho Corp., Raritan, N. J.) and a R₂₂ r cell panel (Selectogen II). After centrifugation for 5 min the cell plug was discarded. The serum was immediately frozen at -20°C.

Immunofluorescent Methods
Multispot Slide Technique for Intracytoplasmic Antigens. Slides were rinsed in absolute alcohol for 24 hr and air dried. Eight drops of glycerol were properly spaced on the slide and a fluorocarbon resin (Fluoro Glide, Chem. Plast, Inc., Wayne, N. J.) was sprayed over the preparation. The slides were exposed to UV for 30 min and were then placed in sterile
150-mm Petri dishes (4 slides/dish). Medium was poured over the slides and about 4 × 10^6 T2 cells were seeded. The dishes were incubated at 37° in a 5% CO₂ humid atmosphere for 1 week. The slides were removed from the dishes, rinsed in a 0.9% NaCl solution, and fixed in acetone for 10 min. The slides were stored at −20° if not used immediately. Twenty μl of test and control sera were added, to separate spots on the slides. The slides were incubated in a wet chamber at room temperature for 30 min. The cells were briefly rinsed in FTA buffer and washed in FTA buffer for 2 separate 15-min periods. The slides were then counterstained with 0.006% Evans blue in FTA buffer for 2 min and washed in buffer for 5 min. All the slides were then dried in a 37° incubator following which a drop of glycerol in Sorensen’s buffer at pH 8.2 was added to each test spot. The slides were mounted and examined with a UV Zeiss microscope with a UV Osram HBO-200 lamp and UG 38 and UG 1 exciting filters, a 41 chamber for 30 min at room temperature, rinsed in FTA human serum. The slides were then incubated in a wet chamber at room temperature, rinsed in FTA buffer and washed in FTA buffer for 2 separate 15-min periods. The slides were then counterstained with 0.006% Evans blue in FTA buffer for 2 min and washed in buffer for 5 min. All the slides were then dried in a 37° incubator following which a drop of glycerol in Sorensen’s buffer at pH 8.2 was added to each test spot. The slides were mounted and examined with a UV Zeiss microscope with a UV Osram HBO-200 lamp and UG 38 and UG 1 exciting filters, a 41 barrier filter, a dark field condenser and a 40X Planapochromat Neofluor oil immersion objective lens provided with an iris. Similar tests were carried out with the cells from the other neoplastic lines and all of the cells were examined as unknowns by 2 independent observers.

Surface Antigen Immunofluorescent Technique. T2 cells and the other neoplastic cell lines were harvested, and an aliquot of 7 × 10^6 cells were dispensed to nonwettable 150-mm plastic Petri dishes containing 20 ml of medium. The cells were incubated at 37° in a 5% CO₂ humid atmosphere. The cells grew as round, single cells or in easily dispersable clumps. Initially, samples were taken at 24-, 48-, 72-, and 96-hr intervals for the membrane immunofluorescent assay. Subsequently, a 72-hr incubation was the standard procedure. The cells were processed following the technique of Møller (13). The cell suspension was aspirated from the Petri dishes and occasional clumps were disrupted mechanically. The cells were washed twice with prewarmed 0.9% NaCl solution and the suspension was adjusted to 5 × 10^6 cells/ml. Aliquots of 0.1 ml were then pipetted into 0.5-ml polystyrene centrifuge tubes and incubated with 0.1 ml of the test and control sera for 30 min at 37°. The cells were centrifuged at 2800 rpm for 5 min. The supernatant was discarded and the cells were washed twice with FTA buffer. The cells were then mixed with 0.1 ml of a 1:5 dilution of FITC-labeled goat anti-human globulin serum and incubated for 30 min at 37°. This was followed by a double wash with FTA buffer. The cells were then resuspended in buffered glycerol at pH 8.2, and a drop of each suspension was placed on individual spots of the multispot slides described in the previous section. A similar procedure was carried out with lymphoma, melanoma, and adenocarcinoma cells. After mounting, the slides were examined as

The abbreviations used are: FTA, fluorescent antibody buffer; FITC, fluorescein isothiocyanate.

RESULTS

Patients with established histological diagnoses and no previous transfusions or pregnancies provided the test sera. The serum was obtained before chemo- or radiotherapy was instituted. The sarcoma group totaled 22 cases consisting of 2 rhabdomyosarcomas, 3 fibrosarcomas, 4 osteogenic sarcomas, 2 liposarcomas, 2 leiomyosarcomas, 2 undifferentiated sarcomas, 1 pleomorphic sarcoma, 1 synovial sarcoma, and 5 neurogenic sarcomas. Other neoplasias included 3 cases of chronic granulocytic leukemia, 2 cases of acute leukemias, 3 melanomas, 2 squamous cell carcinomas, 1 oat cell carcinoma, 2 lymphomas, 1 parotid gland carcinoma, 1 bronchogenic carcinoma, and 1 adenocarcinoma of the colon, giving a total of 16 cases. Serum from 18 healthy blood bank donors or laboratory personnel provided control sera.

The criteria for positive fluorescence in fixed cells were multiple bright apple-green intracytoplasmic dots or masses of perinuclear fluorescence (Fig. 1). The percentage of fluorescent cells in the positive test sera ranged from 25 to 75%, while the fluorescent and pooled normal sera controls both gave less than 10% positive cells. It was not necessary, therefore, to resort to a fluorescent index as proposed by Klein et al. (9). Sera producing between 10 and 24% positive cells were considered nonspecific and were not recorded as positive.

The criteria for positive fluorescence in live cells were: bright, apple-green, multiple membrane dots, crescent forms, and sectorial fluorescence (Fig. 2). Full fluorescent circles were rarely seen. Diffuse intracytoplasmic fluorescence was considered a sign of cellular death. Controls usually presented no fluorescent cells at all or at most 5% positive cells. Sera giving less than 20% fluorescent cells were considered equivocal and were not recorded as positive for the presence of antibodies. T2 cells derived from 24-hr cultures after the trypsinization procedures performed to obtain a single-cell suspension displayed no fluorescence when tested with specific sera. After 48 hr a small proportion of the cells presented membrane fluorescence. After 72 hr the maximum percentage of fluorescent cells were elicited since no further increase was observed after 96-hr culture. The 72-hr cultured cells were the standard elements for subsequent testings. Similar time incubations were used for the lymphoma, melanoma, and adenocarcinoma cell lines.

Table 1 shows the results obtained by testing sera of control and sarcoma patients on live and fixed T2 cells. Sixteen of 22 sera from sarcoma patients gave positive membrane immunofluorescent results while only 6 of 17 sera presented intracytoplasmic fluorescence. Five of the cases with intracytoplasmic fluorescence also had membrane fluorescence. The single exception was a case of osteogenic
Fig. 1. T₄ cells grown on slides, fixed in acetone, and exposed to the serum of a patient with rhabdomyosarcoma. Intracytoplasmic antigen demonstrated by FITC-labeled goat anti-human γ-globulin serum.

Fig. 2. T₄ cells in suspension incubated with serum of a patient with neurofibrosarcoma. Membrane-bound antigen demonstrated by FITC-labeled goat anti-human γ-globulin serum.

sarcoma; this serum indicated only intracytoplasmic antigen but not a membrane-bound one. Only 1 serum of 18 normal ones presented intracytoplasmic fluorescence while 1 case of chronic granulocytic leukemia (out of 16 non-sarcoma neoplasias) revealed membrane fluorescence. Testing live cells with serial dilutions of sera demonstrated that the percentage of fluorescent cells drastically dropped with a 1:40 to 1:160 dilution in 13 sera from sarcoma patients while the normal controls showed absolutely no positive fluorescent cells beyond the 1:5 dilution (Table 2). The sera from patients with sarcoma were also tested with other in vitro neoplastic lines (lymphoma, melanoma, and adenocarcinoma). These cells failed to show any fluorescent cells at all.

The positive sera of patients with sarcoma were absorbed with lymphoma, melanoma, and adenocarcinoma cells and retested on T₂ cells. The proportion of fluorescent cells was similar to that of the unadsorbed serum.

DISCUSSION

Tumor-specific circulating antibodies may be used as a valuable screening and monitoring procedure in the diagnosis of sarcoma.
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Table 1
Demonstration of a sarcoma-associated antigen in T2 cells by
the immunofluorescent techniques

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Live cells (membrane fluorescence) no. of positive tests/total no. of cases</th>
<th>Fixed cells (intracytoplasmic fluorescence) no. of positive tests/total no. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum controls</td>
<td>0/18</td>
<td>1/18</td>
</tr>
<tr>
<td>Non-sarcoma neoplasias</td>
<td>1/16</td>
<td>0/16</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>2/2</td>
<td>1/2</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>3/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Osteogenic sarcoma</td>
<td>1/4</td>
<td>2/4</td>
</tr>
<tr>
<td>Lipoosarcoma</td>
<td>1/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>2/2</td>
<td>1/2</td>
</tr>
<tr>
<td>Neurogenic sarcoma</td>
<td>4/5</td>
<td>0/3</td>
</tr>
<tr>
<td>Undifferentiated sarcoma</td>
<td>1/2</td>
<td>0/1</td>
</tr>
<tr>
<td>Pleomorphic sarcoma</td>
<td>1/1</td>
<td>Not done</td>
</tr>
<tr>
<td>Sinovial sarcoma</td>
<td>1/1</td>
<td>Not done</td>
</tr>
<tr>
<td>Total</td>
<td>16/22</td>
<td>6/17</td>
</tr>
</tbody>
</table>

Table 2
Titration of tumor-specific antibodies on T2 cells

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of sera</th>
<th>Reciprocal of highest titer showing fluorescent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>2</td>
<td>40–80</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>2</td>
<td>80–160</td>
</tr>
<tr>
<td>Osteogenic sarcoma</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>Lipoosarcoma</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>2</td>
<td>40–160</td>
</tr>
<tr>
<td>Neurogenic sarcoma</td>
<td>4</td>
<td>40–160</td>
</tr>
<tr>
<td>Undifferentiated sarcoma</td>
<td>1</td>
<td>20</td>
</tr>
</tbody>
</table>

and treatment of cancers (7). Furthermore, it has been shown that many neoplastic cells derived from similar tissues carry a common tissue-type antigen which could be detected by cross-reaction with the serum of different patients bearing this type of neoplasia (18). Evidence suggests that there are tissue-type-specific common tumor antigens in lymphomas (21), melanomas (17, 23), gastrointestinal tumors (6), hepatic tumors (22), and certain sarcomas (14, 16).

It has been advanced that in sarcomas of osteogenic origin, the common antigen might be viral dependent (14). A similar situation is encountered in Burkitt's lymphoma, and, possibly in nasopharyngeal carcinoma, where at least one of the tumor-associated antigens is Epstein-Barr virus dependent (11). In experimental animal systems, common tumor antigens specific for a given virus are commonly demonstrated regardless of the tissue undergoing malignant transformation (18).

In the present studies, an established culture of human neurogenic sarcoma cells was utilized to search for the presence of tumor-associated antigens common to sarcoma neoplasias. If such an antigen could be demonstrated in this long-term line (over 5 years in culture) the cells could provide an easy source of specific antigen for diagnostic purposes. The sera of patients with sarcoma were used as the source of tumor-specific antibodies which were demonstrated by immunofluorescent techniques. Controls were sera from patients with tumors other than sarcoma and sera from healthy blood bank donors and laboratory personnel. One problem with immunofluorescent techniques is the definition and interpretation of positive fluorescent cells. In this study, criteria for positive fluorescent cells were clearly outlined and rigidly adhered to in the microscopic examination of the cells. Probably because of the high-quality optics used for the examination, positive cells were rather easily detected even when very few fluorescent spots were present on the cells.

Sixteen of 22 sera from patients with sarcoma reacted with T2 cells producing positive immunofluorescence. Four of the 6 patients with negative sera had undergone surgery for the removal of the tumor mass 2 to 3 weeks before the serum was obtained. It is possible that the tumor-specific antibody of these patients had a short biological half-life and that removal of the antigenic tumor mass resulted in the disappearance of detectable tumor-specific antibody titers. Alternatively, these patients may have never produced antibody either because their immunocompetence was depressed or because the tumor cells were not sufficiently immunogenic. Unfortunately, we were unable to test these tumor cells with positive sera. In only one instance did the normal serum controls give origin to a significant number of positive fluorescent cells. When the sera from patients with neoplastic diseases other than sarcoma were tested on T2 cells, no significant number of fluorescent cells were elicited except in one case of chronic granulocytic leukemia. In both cases fluorescent cells were absent after a dilution of 1:5. The positive sera from sarcoma patients were negative when tested on other human neoplastic cells grown in vitro (lymphoma, melanoma, and adenocarcinoma). After absorption with these cells, the positive sera still originated positive fluorescent cells when retested on the sarcoma line with a proportion of fluorescent cells similar to that of the unabsorbed sera.

At least one of the tumor-associated antigens was demonstrated to be located principally in the cellular membrane of T2 cells. Most of the sera gave only membrane fluorescence while only 5 demonstrated both intracytoplasmic and membrane fluorescence. One serum, of a patient with osteogenic sarcoma, yielded only intracytoplasmic fluorescence. This suggests that there may be 2 different types of tumor-associated antigens present in T2 cells both with different immunogenic capabilities evoking different specific antibodies from the host. On the other hand, the fact that freshly trypsinized cells failed to demonstrate membrane antigens and that these were recovered after 72-hr incubation suggest an active synthesis, and possibly a constant turnover, of the tumor-associated antigen. The intracytoplasmic antigen could represent the "native moiety" detected at the site of its synthesis while the membrane-bound antigen would represent the terminal element fully integrated into the membrane. Failure to detect intracytoplasmic antigens with many of the sera could then be due to technical problems or due to various kinds of antibodies produced by different patients. These antibodies would have different shapes or molecular weights. Some would be able to penetrate into the cytoplasm of fixed cells while others are excluded. Switching off of the synthesis...
of antigen by the cells (antigenic modulation) cannot be considered since all of the tests were carried out simultaneously on replicate cell aliquots.

It has been shown that in Burkitt's lymphoma, intracytoplasmic antigens depend on the demonstration of Epstein-Barr virus particles (8), whereas membrane antigens depend on the Epstein-Barr virus genome, probably integrated into the cellular DNA (11). T2 cells never presented viral particles under the electron microscope (20). Yet it is possible that the tumor-associated antigens detected in the present studies depend on a latherto underdemonstrated viral genome present in T2 cells. However, if so, this virus may be different from the one postulated by Morton et al. (15) for osteogenic sarcomas since only 1 of 4 patients with osteogenic sarcoma demonstrated membrane antigens while the serum of patients with other types of sarcoma gave almost invariable positive reactions.

This study has shown the probable existence of tumor antigen(s) common to various neoplasms of mesenchymal origin in an established human neurogenic sarcoma cell line. The antigens remained demonstrable after absorbing the test sera with blood group-specific antigens and other neoplastic cells. The sera showed no cross-reactivity with a variety of antigens common to various neoplasms of mesenchymal origin in an established human neurogenic sarcoma cell line. The authors wish to thank Mrs. Eunice Clemens for her secretarial assistance in the preparation of this manuscript and Dr. Benjamin Lichtiger for participating in some parts of the investigation.

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