Monoclonal Antibodies to Human Sarcoma and Connective Tissue Differentiation Antigens

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ABSTRACT

The use of monoclonal antibodies to distinguish human sarcoma from carcinoma cells has been explored. Spleen cells from a BALB/c mouse immunized with a human malignant fibrohistiocytoma were fused with cells of the mouse P3U1 plasmacytoma cell line. Antibodies were then screened for reactivity against human sarcoma and carcinoma cells growing in culture. This work has yielded 2 immunoglobulin G monoclonal antibodies VIE4 and VIF3 which, respectively, reacted with 85% (17 of 20) and 90% (18 of 20) of sarcoma lines tested but with none of eight carcinoma cell line prepartions. Reactivity against normal fibroblasts was also demonstrated. By immunofluorescence, the antigens detected by the two antibodies appear to have distinctive intracellular distributions. Immunoprecipitation with VIF3 has shown that it is detecting a protein with a molecular weight of 70,000. When tested against pathologized frozen tissue sections, VIF3 reacted with four of 11 and VIE4 with three of 11 human sarcomas but with none of ten carcinomas tested. VIF3 occasionally bound to normal adult connective tissues, whereas no such reactivity was seen with VIE4. These antibodies appear to be directed to fibroblastic markers associated with sarcomas and connective tissue differentiation antigens.

INTRODUCTION

While the first immunological investigations of human cancer were initiated more than 50 years ago, progress in defining tumor-distictive antigens in humans has been slow. In 1968, Morton and coworkers (2, 12, 20) described an antigen common to human sarcomas using patient sera as sources of antibody. This work led to our own extensive investigations of allogeneic and syngeneic responses of sarcoma patients to their tumors (5, 6, 13, 17, 19). Eventually, we identified 3 sarcoma-associated antigens of which 2, S1 and S3, are heterophile in nature and the other, S2, is an oncofetal marker (6, 13, 17). The nature and pathogenetic significance of the sarcoma-associated antigens remain to be further defined. However, there has been general disappointment with the lack of specificity of tumor markers detected with patient sera.

Recently, methods for generating mouse monoclonal antibodies have been developed (8). Some of these permit reagents of high titer and specificity to be produced in almost limitless quantities. The principal challenge faced in the use of this technology is not in securing a sufficient number of monospecific antibodies but in developing means for selecting those antibodies with "relevant" specificities which warrant further characterization. Given the impasse reached in immunological studies of human tumors using patient sera, there is increasing interest (7, 9, 16) in developing monoclonal antibodies to human tumor markers.

This paper reports the results of our initial studies directed to the detection of sarcoma-related antigens using monoclonal antibody methods.

MATERIALS AND METHODS

Human Sarcoma Tissues. A human malignant fibrohistiocytoma was obtained directly from the operating room at the MSKCC, New York, NY. The tumor was dissected out of surrounding normal tissues, immediately minced, passed through a sterile mesh, and then passed through needles of diminishing size to 25 gauge. Aliquots of the cell suspension were frozen and stored at -20° to be used for immunizations.

Immunization Protocol. A sarcoma cell suspension (0.5 ml) containing 1 x 10^6 cells mixed with 0.5 ml of incomplete Freund's adjuvant was injected s.c. into each of 5 BALB/c mice (The Jackson Laboratory, Bar Harbor, ME). Using the cell suspension only, mice received rejections of 0.5 ml of the sarcoma cells at biweekly intervals 4 additional times. They then were permitted to rest for 2 months before being given a final booster immunization prior to fusion.

Production of Hybridoma. The mouse used for the studies reported in this paper received a booster immunization, i.e., 4 days before the fusion attempt. On the day of fusion, 15 x 10^7 spleen cells were mixed with 3 x 10^7 cells of the mouse P3U1 plasmacytoma cell line in the presence of 0.2 ml of 40% Polyethylene Glycol 4000 (Sigma Chemical Co., St. Louis, MO) prepared in PBS containing in g/liter: CaCl2 (0.1); KCl (0.2); KH2PO4 (0.2); MgCl2·6H2O (0.1); NaCl (9.0); Na2HPO4·7H2O (2.16) with 15% dimethyl sulfoxide (Fisher Scientific, Pittsburgh, PA). After 2 min at room temperature, the polyethylene glycol was slowly diluted with 10 ml of RPMI-1640 and 15% fetal calf serum (Media Lab., MSKCC). The cells were then spun down at 500 x g for 10 min. The pellet was resuspended in RPMI-1640 containing 15% fetal calf serum and 2% hypoxanthine:aminopterin:thymidine (Media Lab., MSKCC) and plated out in 96-well plates (Flow Laboratories, MacLean, VA). Twenty-one days postfusion, supernatants from wells containing hybridomas were obtained for screening. Cloning of selected hybridomas was performed by the limiting dilution method (11).

Determination of Immunoglobulin Type. The immunoglobulin type of specific monoclonal antibodies was determined by using heavy chain-specific fluorescently labeled goat anti-mouse antisera (Meloy Laboratories, Springfield, MA) in an immunofluorescence assay and similar unlabeled goat antisera for testing by the Ouchterloney method (Cappel Laboratories, Downingtown, PA).

Ascites Production. BALB/c mice were initially primed with 0.5 ml of 2,6,10,14-tetramethylpentadecane (Pristane; Aldrich Chemical Co., Milwaukee, WI) injected i.p. One week later, 1 to 5 x 10^6 hybridoma cells were injected into the abdomen. When ascites developed, the fluid was tapped and then divided into aliquots and frozen at -20°.

Slide Preparation for Immunofluorescence. Twenty-five μl of a tumor cell suspension (5 x 10^6 cells/ml) were placed in each of the wells on plastic-coated multiwell slides (Hendley-Essex, Sussex, England). These slides were then permitted to rest for 2 months before being given a final booster immunization prior to fusion.
slides were incubated at 37° in an atmosphere of 5% CO₂ for 48 hr. This process permits the antigen-bearing cell to adhere to the glass and spread out. In this way, antigen distribution can subsequently be more readily determined. The slides were then washed twice with PBS, air dried, fixed in acetone for 10 min, and stored at -20°C.

Cell Lines. Methods used for establishing tumor cell lines have been described previously (4, 18). The variety of cell lines used in this study is shown in Table 2. A total of 20 human sarcoma cell lines, 8 carcinoma lines, and 6 fibroblastic cell lines originating from the normal skin of cancer patients was used in the study. Two of the cell lines, one from a patient with leiomyosarcoma (ALMS) and the other (1) from a patient with breast carcinoma (ALAB), were used for initial screening to select hybridomas producing antibodies to sarcomas as distinguished from carcinomas. The other cell lines were used to establish more definitively the specificity of selected antibodies. All cell lines were checked routinely for Mycoplasma using Mycotrim-TC (Hana Media, Inc., Berkeley, CA).

Hybridomas producing antibodies to sarcomas, were established. The horseradish peroxidase-labeled myeloma supernatants were tested in the indirect immunofluorescence method, using a sarcoma cell line (ALMS) and a carcinoma cell line (ALAB). Twenty-one wells contained monoclonal antibodies interacting only with ALMS, and 18 clones were found to secrete antibodies reacting only with the ALAB cell line. Another 9 monoclonal antibodies were able to recognize cells of both lines.

With repeated cloning, 7 of the 21 hybridoma clones secreting sarcoma-associated antibodies were successfully isolated and expanded. Table 1 shows the morphology of the fluorescence reaction seen when these 7 antibodies reacted with ALMS. Also given is their immunoglobulin class. Five antibodies gave fibrillar reaction (Fig. 1A). One produced a punctate cytoplasmic pattern (Fig. 1B), and one yielded a diffuse cytoplasmic reaction. All antibodies were of the IgG class except IIIF6 and IIE5, which were IgM. Supernatant titers ranged between 1:10 and 1:640.

In ascites fluid, titers as high as 1:10,000 were observed.

Further analysis carried out with additional sarcoma and carcinoma cell lines showed that 2 of the 7 antibodies originally found to react only with ALMS reacted specifically with sarcoma and not with carcinoma lines. When tested, these antibodies, VIE4 and VIF3, reacted, respectively, with 85% (17 of 20) and 90% (18 of 20) of sarcoma lines tested but with none of 8 carcinoma cell line substrates. The remaining monoclonal antibodies were found to interact with 1 to 4 of 6 carcinoma cell lines. All the antibodies reacted with 3 or more of the fibroblastic cell lines tested (Table 2).

Table 2 describes the results of using the ELISA viable cell assay to determine the specificity of the same ALMS-positive monoclonal antibody cell lines. VIE4 reacted with 4 sarcoma and one fibroblast cell lines, but not with 3 carcinoma cell lines. Monoclonal antibodies IIIE5, IXG11, XVE6, and XIID8 interacted with cells of both sarcoma and carcinoma origin. IIIF6 was found to recognize an antigen on ANS and ALAB breast only. VIF3 was negative against all cell lines, with this assay.

Fig. 2 shows the SDS-PAGE pattern obtained by immunoprecipitating the antigen detected by 2 of the sarcoma-associated monoclonal antibodies. A molecular weight band of 70,000 was identified by monoclonal antibodies VIF3 and XVE6. The remaining antibodies (Table 1) yielded no detectable precipitation by the methods used, thus far.

When tested directly against pathological sections of 11 sarcomas (Table 4), VIF3 reacted with a chondrosarcoma, osteogenic sarcoma, fibrosarcoma, and a leiomyosarcoma. VIE4 reacted with the same chondrosarcoma and osteogenic sarcoma.

<table>
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<tr>
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<th>Morphology</th>
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<td>IgM</td>
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<td>IXG11</td>
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<td>Cytoplasmic</td>
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<td>XVE6</td>
<td>IgG</td>
<td>Fibillar</td>
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</table>

RESULTS

Of 1440 wells into which cells were placed after fusion, 1184 yielded one or more hybridoma clones (82.2%). Three hundred eighty wells which appeared to contain only single clones were selected for further study. The supernatants of these wells were screened by the indirect immunofluorescence method, using a sarcoma cell line (ALMS) and a carcinoma cell line (ALAB). Twenty-one wells contained monoclonal antibodies interacting only with ALMS, and 18 clones were found to secrete antibodies reacting only with the ALAB cell line. Another 9 monoclonal antibodies were able to recognize cells of both lines.
HUMAN SARCOMA AND CONNECTIVE TISSUE ANTIGENS

Table 2
Characterization of monoclonal antibodies by immunofluorescence using sarcoma, fibroblast, and carcinoma cell lines

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<tr>
<th>Cell line</th>
<th>Type</th>
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<th>VIE4</th>
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* ND, not determined.

Table 3
ELISA performed using cell lines to determine the expression of connective tissue antigens linked with sarcomas

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>Cell line</th>
<th>VIE4</th>
<th>VIF3</th>
<th>IIF6</th>
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but not against the other 2 tumors. In addition, it reacted with a second chondrosarcoma. No reactions were seen with 10 carcinomas tested (Table 4).

Tests were also completed against normal adult tissues. Findings are shown in Table 5. Epithelial cells of skin, lung, breast, colon, and prostate did not react with VIF3 or VIE4. However, VIF3 did bind to connective tissue cells in adult skin, lung, breast, and prostate. No reactivity was seen with VIE4.

DISCUSSION

Efforts to identify human tumor-specific antigens in humans have been hampered by a lack of well-defined reagents. It is now possible to develop large quantities of relatively purified, high-titer, specific antibodies to well-defined antigens. Unfortunately, human tumor antigens are so far poorly defined, and their only sources are crude tumor extracts. Tumor specificity, unique to tumors alone, therefore, remains difficult to establish even with monoclonal antibodies available. Our approach in this initial stage of developing such antibodies to sarcomas has been not to insist on absolute specificity. Instead, antibodies have been selected

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which are specific only in the relative sense of their ability to
distinguish sarcoma from other cells, particularly those derived
from carcinomas. No assumption is made that these differences are
characteristic only of tumors. It is more likely that differentia-
tion markers are being detected.

The 2 monoclonal antibodies described, VIF3 and VIE4, distin-
guish fixed sarcoma from carcinoma cell lines. One of these
antibodies, VIE4, is also able to distinguish between viable
sarcoma and carcinoma cell lines. Consistent with the assump-
tion that the antigens being detected are related to cell differen-
tiation is the finding that the same antibodies react with cultured
normal fibroblasts.

Studies with more than 20 sarcoma cell lines indicate that
monoclonal antibodies VIF3 and VIE4 recognize most forms of
sarcoma in culture. The isolation of antibodies with such broad
reactivity may in part be due to the use of an allogeneic sarcoma
cell lines (ALMS) and not the original immunogen to screen the
first hybridoma supernatant for reactivity.

The antigens detected by VIF3 and VIE4 within sarcoma cells
differ in their intracellular distribution. VIF3 reacts with an antigen
located in cellular structures whose size and configuration respec-
tively resemble lysosomes or mitochondria. VIE4 produces a "filamentous"
fluorescence reaction pattern which may be related to the pres-
ence of the associated antigen on the cell surface. An advantage
of developing several monoclonal antibodies with similar cell
specificities is, therefore, that this provides additional insight into
the variety of determinants that are unique to given cell types.
Unfortunately, of these 2 monoclonal antibodies:antigen com-
plexes, only that related to VIF3 could be precipitated, so that
the 2 antigens cannot as yet be compared on immunochemo-
ical grounds.

While monoclonal antibody specificity determined using cul-
tured cells as substrates is of interest, the relevance of such
specificity to clinical problems must remain in doubt until an
antibody is subjected to actual testing against cells present in
the complex setting of whole tissues. It is, therefore, reassuring
that both VIF3 and VIE4 remain specific for connective tissues
when tested against pathological sections. The reactivity of VIF3
with a subset of fibroblasts within normal adult connective tis-
sues confirms that this antibody detects a connective tissue
differentiation antigen. VIE4 is more selective in its reactivity,
since it reacts only with normal connective tissues after they
have been placed in culture, a setting in which some other defferen-
tiation may occur. This greater selectivity of VIE4 may ultimately
make it a more useful reagent for the detection of sarcomas.
VIF3 and VIE4 react only with 4 of 11 and 3 of 11 human sarcoma
tissue sections, respectively, and with none of 10 carcinoma
tissues tested. Antibodies to vimentin have also been demonstrated
to be capable of distinguishing carcinomas from sarcomas (14).

The currently reported monoclonal antibodies are expected to serve as probes in the immunopathological diagnosis
of human sarcomas. As the number of such antibodies increases,
more definitive classification of human sarcomas should become
possible. This in turn can be expected to lead to more effective
therapies tailored to specific subclassifications of sarcomas.

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Table 5

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<tr>
<th>Tissue</th>
<th>VIF3</th>
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<td>Breast</td>
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<tr>
<td>Duct cells</td>
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<tr>
<td>Connective tissue</td>
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Tissue sections were from postmortem specimens obtained less than 9 hr after death.
Fig. 1. Photomicrographs of indirect immunofluorescence produced by monoclonal antibodies on a human osteogenic sarcoma cell line (MOS-229). A, monoclonal antibody VIE4, followed by fluorescein isothiocyanate goat anti-mouse IgG, counterstained in Evan's blue, x 250. B, monoclonal antibody VIF3, same procedure, x 250.

Fig. 2. SDS-PAGE pattern of 125I-labeled antigens precipitated by monoclonal antibodies from MOS-229 cells. Immunoprecipitation was done as described in "Materials and Methods." Goat anti-human β2-microglobulin heterologous antibodies precipitated two bands: one of 12,000 (β2-microglobulin) and another one of 43,000, presumably HLA antigens. Two antibodies, XVE6 and VIF3, but not VIE4, precipitated M, 70,000 material.
Monoclonal Antibodies to Human Sarcoma and Connective Tissue Differentiation Antigens

Carl Feit, Arie H. Bartal, Barbara Fass, et al.


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