Expression of antigens defined by antibodies 3B12 and 6.10 in frozen sections of tumor, as detected by immunohistology

A modification of the Sternberger PAP technique (6) was used to demonstrate antigen expression in frozen sections of various tissues, as described under "Materials and Methods." The degree of staining detected was graded from - (negative) through + (weakly positive) to ++++ (very strongly positive). Essentially all (±90%) carcinoma cells were stained within positive tumors, although their degree of staining varied from ++ to ++++.

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>3B12</th>
<th>6.10</th>
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<tbody>
<tr>
<td>Bladder carcinoma</td>
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<tr>
<td>1656-Primary (MCA)</td>
<td>++</td>
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<tr>
<td>1656-16 passage (MCA)</td>
<td>+++</td>
<td>+++</td>
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<td>1657-14 passage (MCA)</td>
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<td>++++</td>
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<tr>
<td>1660-Primary (MCA)</td>
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<td>++++</td>
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<tr>
<td>1660-15 passage (MCA)</td>
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<tr>
<td>1673-14 passage (MCA)</td>
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<td>++++</td>
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<tr>
<td>1682-16 passage (FANFT)</td>
<td>++++</td>
<td>++++</td>
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<tr>
<td>Mammary carcinoma</td>
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<tr>
<td>1651</td>
<td>++</td>
<td>-</td>
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<tr>
<td>Melanoma</td>
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<tr>
<td>B16</td>
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<tr>
<td>Sarcoma</td>
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<td>Lymphosarcoma</td>
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<tr>
<td>1647</td>
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MOUSE BLADDER TUMOR ANTIGENS

on tumor (and normal) tissue samples obtained directly from mice. However, we also used cells grown in vitro as described previously (4); studies on living cells were done for preliminary screening of hybridomas and to establish that the antibodies are to cell surface antigens.

Spleen cells were hybridized with NS-1 cells, an azaguanine-resistant BALB/c myeloma line (7).

Growth Selection and Cloning of Hybridomas. Two hybridomas, 3B12 and 6.10, were selected from screening a total of approximately 7000 rat hybridomas.

Hybridoma 3B12 was obtained from the same fusion as a previously reported hybridoma, 2HS, by immunizing with a Wistar/Furth rat (4).

Hybridoma 6.10 was obtained by immunizing a Sprague-Dawley rat with irradiated 1657-14 bladder carcinoma cells, 106 cells being given intraperitoneally on 3 occasions with weekly intervals. Spleen cells from the immunized rat were fused, using thymocytes as feeder cells, followed by overnight incubation in hypoxanthine-aminopterin-thymine medium as described previously (4). The next day, the cells were spun down gently at 200 x g and resuspended in new medium; they were fed on every second day, starting on Day 7. On Day 11, the first screening of hybridoma supernatant was done on 1657 bladder carcinoma cell extracts, as described under "Binding Assays" below (4). Supernatants binding to the bladder tumor extracts were screened on intact cells from cultures of Carcinoma 1657, as well as on normal kidney and liver extracts; those supernatants only binding to 1657 cells were selected for tests on frozen sections of bladder carcinoma 1657, normal bladder, and normal kidney using the Sternberger PAP technique (5) as described below.

Of 23 hybrids screened by the PAP technique, 3 appeared to be bladder carcinoma specific and were chosen for cloning, expansion, and further testing. The most specific one of these, 6.10, is described under "Results."

Supernatants from hybridoma cultures were used as source of antibody, one pool being established for each hybridoma. Hybridoma 6.10 can also be grown as an ascites tumor following injection of cultured cells into pristane-primed, nude BALB/c mice so that ascites from this hybridoma can be purified and used as additional source of antibody. Hybridoma 3B12 does not grow in nude mice but has been adapted to grow as an ascites tumor in nude rats. Microzone electrophoresis (8) shows that the antibodies are monoclonal, and immunodiffusion studies performed on antibodies formed by hybridomas 3B12 and 6.10 determined that both are rat monoclonal IgG2a.

Binding Assays. As part of the screening of hybridomas, binding assays were performed on intact cells and cell extracts, as described previously (4). Briefly, cultured cells were suspended in a solution of 0.5 mM EDTA:0.14 mM NaCl:2.3 mM KCl, plated at 35,000 cells/well into Costar 96-well tissue culture cluster (No. 3596; Costar, Cambridge, MA), and incubated at 37°C. After washing, 50 μl of spent hybridoma medium or control medium were added to the adherent monolayer. The plates were incubated at 20°C for 1 h, after which the medium was aspirated and the plates were washed. Subsequently, 50 μl of affinity-purified 125I-labeled rabbit anti-rat IgG (Zymed Laboratory, Burlingame, CA), in 1% bovine serum albumin in PBS was added and diluted so as to give 1 to 2.5 x 106 cpm/well. The plates were incubated for 1 h at 20°C and washed extensively. The attached cells were removed with 2 mM NaOH and counted in a gamma counter. To establish the specificity of antibody binding, we also tested whether the binding of directly 125I-labeled anti-tumor-antibody could be competitively inhibited by preincubation with an excess of unlabeled antibody (9).

Binding assays were also performed on cell extracts which were prepared and tested as described previously (4).

Immunoprecipitation. Mouse bladder carcinoma cells were surface-labeled by incubating a confluent monolayer of cells in a 75-cm2 flask with 2 ml of PBS containing 5 mM glucose, 200 μg glucose oxidase (Calbiochem-Behring Corp., La Jolla, CA), 40 μg lactorperoxidase (Calbiochem-Behring), and 3 mCi Na125I. After 15 min at 20°C, the cells were washed 5 times with 5 ml of PBS and lysed with 1 ml of TNEN buffer for 10 min at 0°C. The lysate was centrifuged for 5 min at 2000 x g to remove nuclei. The supernatant was used immediately for immunoprecipitation.

Radioiodinated membrane proteins (2 x 105 cpm) were incubated with 100 μl of hybridoma culture medium or control medium for 1 h at 0°C. Then, 5 μl of a 1:2 dilution of rabbit antiserum to rat IgG were added for a 30-min incubation. Immune complexes were adsorbed to 3 mg of Staphylococcus aureus in 1 ml of TNEN buffer and washed twice with 1 ml of TNEN buffer and twice with TNEN diluted 1:10 with water. The bacteria were then resuspended in 50 μl of SDS-sample buffer containing 5% 2-mercaptoethanol (10), heated at 100°C for 5 min, and pelleted. The supernatant was analyzed by discontinuous electrophoresis on a 6% SDS polyacrylamide slab gel (10). The gel was dried and autoradiographed for 4 h at ~70°C with preflashed Kodak AR-2 film and a Rarex B mid speed-intensifying screen. Relative molecular weights were determined by comparison of migration of protein standards on the same gel.

Immunohistological Procedures. Tumor and normal tissue samples were frozen immediately upon removal from mice, using liquid nitrogen (11), after which they were stored at ~70°C until used.

Frozen sections, approximately 5 to 6 μm thick, were prepared and air dried for a minimum of 2 h. After treatment with acetone at ~20°C for 10 min, they were dried quickly with an air jet. Sections used for histological evaluation were stained with hematoxylin, while those for immunohistological staining were preincubated for 30 min with normal mouse serum diluted 1:5 in PBS.

Immunohistological staining was performed using the PAP technique of Sternberger (5), as modified previously (6). The test antibodies, rabbit anti-rat IgG and the rat PAP (Sternberger-Meyer Cytoimmunochemicals, Inc., Jarrettsville, MD), were diluted in a solution of 10% normal mouse serum and 3% rabbit serum in PBS. The staining procedure consisted of the following steps: (a) treatment for 1 h of serial sections with either specific or control antibody supernatant diluted 1:2 in the serum mixture above; (b) application of rabbit anti-rat IgG diluted 1:30; and (c) exposure to rat PAP complex diluted 1:80. All antisera were incubated with the sections for 30 min at room temperature. Following each antibody treatment, the slides were rinsed lightly with a stream of PBS and then washed twice in PBS.

The immunohistological reaction was developed with freshly prepared 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 M Tris buffer, pH 7.6, for 8 min (12). Further exposure to a 1% OsO4 solution for 15 min intensified the reaction product. The sections were rinsed briefly with water, passed through increasing con-
concentrations of alcohol followed by xylene, and mounted with coverslips. The same slides were observed independently in a light microscope by two investigators who did not have knowledge of whether specific or control antibody had been used. Attempts were made to quantitate degree of staining of tumor (or normal) cells from + (weak) to ++++ (very strongly positive). Typical slides were photographed.

RESULTS

The specificity profile of the antibodies produced by hybridomas 3B12 and 6.10 was established by immunohistological studies on biopsy material. The immunohistological data are summarized in Tables 1 and 2 and illustrated in Figures 1 to 4. The binding of antibody 3B12 to cultured bladder carcinoma cells could not be competitively inhibited by antibody 6.10 (Table 3), which indicates that the 2 antibodies are to different antigenic determinants. We shall comment on our findings for the 2 hybridomas separately.

Hybridoma 3B12. Hybridoma 3B12 makes an antibody which, in preliminary tests, bound strongly to cell extracts and to the surface of intact cultured cells from bladder carcinomas, as detected by immunofluorescence assays with living cells and binding assays with radiolabeled antibodies (Table 3).

When antibody 3B12 was tested in immunohistological assays on frozen sections (Tables 1 and 2), strong staining was detected with all bladder carcinomas tested (Figs. 1 and 2), primary as well as transplanted, FANFT-induced as well as MCA-induced, and an intermediary degree of staining was detected with a breast carcinoma, while cells from other tumors were negative. The staining was limited to the cell surface, as far as can be judged by immunohistological assays. The findings presented in Figure 2 for antibody 3B12 (and also for antibody 6.10) illustrate antibody specificity for carcinoma cells; in a transplanted bladder tumor that consisted of both carcinomatous and sarcomatous elements, essentially all of the carcinoma cells were stained strongly, and none of the cells in the sarcomatous part were stained at all.

A weak but definitive staining was seen when antibody 3B12 was tested with epithelial cells from several organs, including bladder epithelium (Fig. 3), and epineurial cells. Epidermal cells from mouse embryos were uniformly stained (Fig. 4), while such cells of adult mice were negative.

In radioimmunoprecipitation assays followed by gel electrophoresis, antibody 3B12 precipitated a molecule of molecular weight of approximately 205,000 (Fig. 5).

Hybridoma 6.10. Antibody 6.10 bound to the surface of intact cultured cells of mouse bladder carcinoma in experiments similar to those carried out for antibody 3B12; one such experiment is included in Table 3.

When tested on frozen sections with the PAP technique, antibody 6.10 stained all bladder carcinomas strongly (Figs. 1 and 2) but did not stain any other tumors (Table 1). The staining of the bladder carcinomas were uniform, with few if any cells that were not stained. Normal adult tissues were entirely negative, except for a very weak staining of scattered (less than 25%) epithelial cells from esophagus, endothelial cells in the brain, and perineural and epithelial cells from the tail (Table 2). It is noteworthy that antibody 6.10 did not stain any cells at all from normal bladder, while antibody 3B12 did (Fig. 3). Epidermal and periosteal cells of 10- to 19-day-old mouse embryos were uniformly stained, the latter strongly (Fig. 4).

Radioimmunoprecipitation followed by gel analysis (Fig. 5) demonstrated one band of molecular weight of approximately 175,000.

DISCUSSION

We have generated 2 rat monoclonal antibodies, 3B12 and 6.10, which are specific for antigens expressed by cells from chemically induced mouse bladder carcinomas, both in vitro and
MOUSE BLADDER TUMOR ANTIGENS

in vivo. This expands upon the available antibodies to such tumors (4, 13). The antigens are localized at the cell surface according to binding (Table 3) and immunofluorescence assays on living cells, radioimmunoprecipitation tests with surface-labeled bladder tumor cells (Fig. 5), and immunohistological studies (Fig. 1, b and c; Fig. 2, b and c). It is significant that they could be detected on both primary and transplanted bladder tumors and also that a FANFT-induced bladder carcinoma shared antigens with MCA-induced bladder carcinomas. The FANFT-induced tumor appeared in mice given a diet containing the carcinogen, and, like other FANFT-induced bladder carcinomas (14), is histologically similar to transitional cell carcinomas in humans.

Using immunoprecipitation followed by polyacrylamide gel analysis, we have identified target molecules from cell-surface-labeled lysate bound by each of the two antibodies. Both antibodies precipitate putative antigens which migrate on SDS-polyacrylamide gel electrophoresis with apparent molecular weights between 175,000 and 205,000 under reducing conditions. Medium control was used in Track A. Supernatant from 3B12 was used in Track B; 6.10 was used in Track C. Apparent molecular weights were calculated from parallel standards on the same gel.

Since our reason for obtaining monoclonal antibodies to mouse bladder carcinoma was to develop a model to study questions of clinical relevance, our screening was carried out toward defining antigens which fulfill 3 criteria: bladder carcinoma specificity, presence at the cell surface, and expression by tumor tissue in vivo. The specificity testing was primarily done by immunohistology using tissue sections, since this approach, in our opinion, provides the best information about antigen expression and specificity in vivo (3, 6, 15). The virtue of the immunohistological techniques is demonstrated by the fact that the antigens defined by 2 hybridomas reported previously by us, 2H5 and IE6, were relatively specific for bladder carcinoma when tested in binding assays with cell extracts (4) but were found to be expressed rather strongly in sections of normal bladder and some other organs when studied by immunohistology (data not shown).

Like most human tumor-associated antigens identified by xenogeneic monoclonal antibodies (3), the antigens defined by antibodies 3B12 and 6.10 can be detected in embryonic tissues. Their degree of tumor specificity equals that of the more specific antigens so far defined by monoclonal mouse antibodies in human neoplasms (3); the antigens are present on certain normal cells, but the differences in antigen expression, between normal and neoplastic cells, are large. Entirely tumor-specific antibodies (or nonspecific ones) would have been undesirable for our purpose, which is to develop a "model."

The presence of monoclonal antibody-defined cell surface antigens shared by mouse bladder carcinomas is in keeping with the demonstration that such tumors share tumor-associated antigens which can induce cell-mediated antitumor reactivity in immunized mice (1); however, the relationship, if any, between those antigens and the ones detected by antibodies 3B12 and 6.10 remains to be established. It is interesting to note that no monoclonal antibody has yet been obtained to any individually unique antigen of bladder carcinomas, in spite of the fact that such antigens were the ones detected as tumor-specific transplantation antigens in vivo (2); there has been a similar difficulty in obtaining antibodies to individually unique tumor antigens of chemically induced mouse sarcomas (16). It is also noteworthy that, when mice rather than rats were immunized, we were unable, in spite of the screening of more than 10,000 hybridomas, to obtain any hybridomas defining antigens with bladder carcinoma specificity (data not shown). It is not known why the shared mouse bladder carcinoma antigens failed to immunize mice under conditions used successfully with rats. Maybe the antigens are regarded as "self" components within the same species.

Although much work aimed toward developing monoclonal antibody-based therapy for human tumors is probably best performed directly in humans, (17–19), it is often hard to justify and evaluate clinical trials in patients with little tumor load, who may be the ones most likely to benefit from the therapy. Studies in animal models are not hampered by such problems or by the influence of unrelated forms of therapy. Nude mice xenotransplanted with human tumors are often used for such studies. We believe, however, that conventional normal mice carrying primary or transplanted syngeneic bladder carcinomas have advantages over nude mice for many investigations relating to immunotherapy and immunoprevention. The tumor antigens defined by antibodies 3B12 and 6.10 are expressed, at low levels, by normal mouse cells, while the normal tissues of nude mice (unlike normal human tissues) completely lack the target antigen of the human tumor. Even more important is the fact that effects of active immunization can only be studied in the conventional normal mice. In particular, it should be possible, by using the model described here, to investigate whether "vaccination" with an antigen defined by a monoclonal antibody or with an "internal image" antidiotypic antibody (prepared against a binding site of a monoclonal antibody), will induce antitumor immunity and, if immunity is induced, what its effects would be on both the tumor and the host. Work is in progress using mice transplanted with...
MOUSE BLADDER TUMOR ANTIGENS

bladder carcinoma and antiidiotypic antibodies raised against antibody 6.10.¹

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Bladder carcinoma 1660. a, hematoxylin stain, monoclonal antibody treatment with; b, 3B12; c, 6.10; d, control antibody treatment.
Fig. 2. Bladder tumor 1660-15 with carcinoma/sarcoma mixture. a, hematoxylin stain, monoclonal antibody treatment with; b, 3B12; c, 6.10; d, control antibody treatment.
Fig. 3. Normal mouse bladder. a, hematoxylin stain, monoclonal antibody treatment with; b, 3B12; c, 6.10; d, control antibody treatment.
Fig. 4. Normal mouse embryo (19 days). a, hematoxylin stain; monoclonal antibody treatment with; b, 3B12; c, 6.10; d, control antibody treatment.
Monoclonal Antibodies to Cell Surface Antigens Shared by Chemically Induced Mouse Bladder Carcinomas

Ingegerd Hellström, Karl Erik Hellström, Nicola Rollins, et al.

*Cancer Res* 1985;45:2210-2218.

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