Monoclonal Antibodies to a Rat Colon Carcinoma: Model for Monoclonal Antibody Therapy of Solid Tumors

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ABSTRACT

Tumor progression, lung metastasis, and death occur in tumor-bearing BD IX syngeneic rats in a fashion similar to the course of patients with metastatic colon cancer. In an effort to establish a relevant model for monoclonal antibody (MoAb) therapy of tumors, we generated murine MoAb against DHD/TR, a dimethylhydrazine-induced rat colon carcinoma which has been adapted to cell culture. Murine MoAb 17B10 E4 (E4) reacts with the TR tumor and shows weak immunoperoxidase reactivity with normal rat tissues. Murine MoAb 5F7 D3 (D3) reacts with the tumor and a variety of normal rat epithelia. Both are IgG2a and do not mediate cytotoxicity by rat peripheral blood mononuclear cells. 1D5 F6 (F6) also reacts with the tumor and normal tissues but is an IgG2b and does not mediate cytotoxicity in the presence of rat effector cells. Indicated E4 and D3 antibodies retained their immunoreactivity. E4 revealed 9.8 × 10^9 antigenic sites per TR cell, while D3 demonstrated 2.5 × 10^9 antigenic sites and an affinity constant of 4.2 × 10^9 M^-1. Immunoblotting showed that the antigens recognized by D3 and E4 are glycoproteins with molecular weights of 27,000 and 66,000, respectively. F6 failed to react with its antigens present in the blot. This rat colon carcinoma and the monoclonal antibodies described here may provide experimental data useful for implementing monoclonal antibodies in cancer therapy.

INTRODUCTION

Antibody therapy of tumors has evoked renewed interest due to the availability of mouse monoclonal antibodies to tumor-associated antigens. Unfortunately the practicality of this approach remains problematic. Numerous fundamental questions are unanswered, including dose of antibody, schedule and route of administration, mechanisms of action, possible side effects, and long-term impact of xenogeneic mouse antibodies on the immune system of the host. So far, criteria to select monoclonal antibodies for clinical use have been based on in vitro cytotoxic properties and in vivo tumor growth inhibition in athymic mice. Data generated with the use of murine monoclonals in the nude mouse may not be directly relevant to the human situation of a syngeneic tumor in an immune competent host treated with xenogeneic antibodies. Therefore, more appropriate animal models are needed. The DHD/TRb tumor is a dimethylhydrazine-induced colon adenocarcinoma which has been selected for resistance to trypsin (1). This tumor has been adapted to cell culture and is tumorigenic in syngeneic BD IX rats. Inoculation s.c. of tumor cells results in tumor growth at the site of injection, metastatic spread to lymph nodes and lung, and death from tumor progression in 3 to 4 months. This model has been extensively studied in terms of growth characteristics and for its possible suppressive effect on the rat immune response (2). We report here the production and characterization of mouse MoAb5 to this rat colon adenocarcinoma for use in a model system of solid tumor immunotherapy.

MATERIALS AND METHODS

Animals. Inbred BD IX rats were purchased from Centre National de la Recherche Scientifique (Orleans, France), housed, and bred at NIH. Animals were 3 to 6 months old when used. BALB/c mice, 6 to 8 weeks old, were obtained from NIH.

Cell Lines. Rat colon adenocarcinomas DHD K12 TRb (trypsin-resistant) and TSb (trypsin-sensitive) were generously provided by F. Martin (Dijon, France). Rat virus-induced lymphomas CS8NTD and G1-TC and rat breast carcinoma MAD B 106 were a gift from C. Reynolds (Frederick Cancer Research Facility, National Cancer Institute, NIH). Rat fetal lung cell line RFL-6 was purchased from the American Type Culture Collection (Rockville, MD). Mouse myeloma NS1 was used as a fusion partner. All cell lines were cultured in RPMI 1640 enriched with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), and 1% penicillin-streptomycin (GIBCO, Grand Island, NY). For immunization of mice, adherent cell lines were detached from flasks by a 1 min incubation with EDTA disodium salt, 1 mg/ml (Sigma, St. Louis, MO), followed by 3 min of incubation with trypsin, 1 mg/ml (Sigma) in Ca^2+ -Mg^2+-free Hanks' balanced salt solution.

Immunization and Fusion. BALB/c mice were immunized i.p. with 5 × 10^6 TS cells in complete Freund's adjuvant, followed by reimmunization 21 and 28 days later by the same route, with the same number of cells, in incomplete Freund's adjuvant. Mouse sera were tested periodically during the immunization course for their reactivity with TR cells. A final boost without adjuvant was given i.v. on day 35 to the mouse with the highest serum titer. Its splenocytes were fused with NS1 on day 38. Fusion was performed with polyethylene glycol (Sigma) at a ratio of 5 spleenocytes to 1 NS1 cell. After fusion, cells were seeded in 96-well plates at 5 × 10^4 spleen cells/200 μl of hypoxanthine-aminopterin-thymidine enriched culture medium (GIBCO). Growth was visible at day 6 after the fusion.

Screening. Screening was performed by ELISA on glutaraldehyde-fixed cells as described previously (3).

Indirect immunofluorescence was also performed with supernatants of selected cell lines. Adherent cells (TR, TS, MADB 106, and RFL 6) were detached by using EDTA and trypsin. Cell suspensions were incubated for 30 min in 10% horse serum at 37°C and washed once in PBS. Two million cells were incubated for 1 h on melting ice with 100 μl undiluted culture supernatants from hybrids. Three additional washes were performed and cells were incubated for 30 min on melting ice with a fluoresceinated goat anti-mouse immunoglobulin (Cappel, Cochranville, PA) diluted 1:40. After three more washes, fluorescence was analyzed in a fluorescence-activated cell sorter (Ortho FC 400 Cytofluorograph).

Cloning. Hybrids selected by the ELISA as described above were cloned in soft agar. Two suspensions of soft agar (1.25 and 0.9%) were used to establish cell lines. Indirect immunofluorescence was used to select cell lines. Adherent cells (TR, TS, MADB 106, and RFL 6) were detached by using EDTA and trypsin. Cell suspensions were incubated for 30 min in 10% horse serum at 37°C and washed once in PBS. Two million cells were incubated for 1 h on melting ice with 100 μl undiluted culture supernatants from hybrids. Three additional washes were performed and cells were incubated for 30 min on melting ice with a fluoresceinated goat anti-mouse immunoglobulin (Cappel, Cochranville, PA) diluted 1:40. After three more washes, fluorescence was analyzed in a fluorescence-activated cell sorter (Ortho FC 400 Cytofluorograph).

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mm Petri dishes and allowed to solidify at —20°C for 10 min. Six ml of top layer agar containing 1000 hybrid cells/ml were poured over the solid bottom layer and incubated at 37°C in 5% CO₂. Five Petri dishes per selected hybrid were seeded. Macrophagic growth was visible after 12 days. The 12 largest colonies of each Petri dish were then transferred to 96-well plates and later to 25-cm² culture flasks. Supernatants of growing clones were tested for their reactivity with the panel of cell lines by ELISA and immunofluorescence.

**Immunoglobulin Subclass Determination.** Subclass of IgG produced by cloned hybrids was determined from culture supernatants by immunodiffusion following instructions provided in a monoclonal antibody typing kit purchased from Miles Scientific (ICN Immunobiologics, Lisle, IL).

**Ascites Production and Immunoglobulin Purification.** BALB/c mice were primed with 0.5 ml of 2,6,10,14-tetramethylpentadecane (pristane; Aldrich, Milwaukee, WI) 2 weeks before i.p. injection of 5 x 10⁵ cloned hybrid cells. Ascites was collected by tapping the mice approximately 10 days afterward. Ascitic fluid was centrifuged at 4000 x g for 15 min immediately after collection. IgG from pooled ascites was purified by using a recombinant Protein A purification kit (Beckman, Fullerton, CA), following the manufacturer's instructions. The concentration of purified immunoglobulins was measured by radial immunodiffusion with a commercially available kit for each subclass (ICN Immunobiologics).

**Iodination of Monoclonal Antibodies.** Monoclonal antibodies were labeled with ¹²⁵I (Na¹²⁵I, Amersham, Arlington Heights, IL) by the procedure described by Haisma et al. (4).

**Immunoreactivity.** To analyze if the iodination of the MoAb had modified their binding abilities, we performed an immunoreactivity assay as described by Lindmo et al. (5).

**Analysis of Affinity Constants and Number of Antigenic Sites.** To determine the amount of antigen expressed on the membrane of TR cells and the affinity constant of the antibodies, 10⁶ cells were incubated, in duplicate, with increasing concentrations of radiolabeled antibodies for 3 h at 4°C. The value of antibody bound at saturation was calculated by incubating 5 x 10⁶ cells in a large excess of labeled antibody. After incubation, cells were washed three times with HBSS at 4°C, and the cell-bound radioactivity was determined. The amount of nonspecifically bound radioactivity was measured by incubating the cells with the radiolabeled antibodies in the presence of a 100-fold excess of the same nonlabeled antibody. Rat myeloma cells were also used as a control to estimate the nonspecifically bound radioactivity. The data of the specifically bound radioactivity, calculated by the previous procedure, were analyzed by using the Sips equation (6). Linear regression was used to fit the experimental data. The extrapolation of this line to the point log [F] (mol/l) = 0 was used to estimate the affinity constants. The amount of antigen expressed on the membrane was calculated from the data of antibody bound at saturation, assuming a bivalent binding of the antibody.

**Biochemical Characterization of the Antigens Recognized by MoAb.** Confluent TR or TS cells were detached from culture flasks with a 0.1 M EDTA solution and washed twice with cold PBS containing 1 mM PMSF. Cells were suspended at 200 x 10⁶/ml in NP-40 buffer (0.5% NP-40, 140 mM NaCl, 10 mM Tris, 5 mM EDTA solution and washed twice with cold PBS containing 1 mM PMSF. Cells were suspended at 200 x 10⁶/ml in NP-40 buffer (0.5% NP-40, 140 mM NaCl, 10 mM Tris, 5 mM EDTA, 0.5 M KCl, 1 mM MgCl₂, 1 mM CaCl₂, 100 lU/ml kallikrein inactivator (Calbiochem, San Diego, CA), 1 mM PMSF, pH 7.5). Fifty µl (10⁵) of this suspension was treated with 5 volumes of ice-cold hypotonic buffer (1 mM MgCl₂, 1 mM KCl, 10 mM Tris, pH 8.1; Ref. 7). After 15 min, the cell suspension was quickly frozen to —20°C, followed by rapid thawing after 20 min. This process yielded a rather homogeneous preparation of cell ghosts that were pelleted at 12,000 x g for 10 min, washed twice with PBS/PMSF, and extracted for 1 h at 4°C with NP-40 buffer. Following extraction, the suspension was clarified by centrifugation for 1 h at 105,000 x g.

Proteins in the extract were separated by SDS electrophoresis. Western blotting was performed according to the method of Towbin et al. (8) with BA85 nitrocellulose paper (Schleicher & Schuell, Inc.). Blocking was achieved with 6% bovine serum albumin (BSA) in PBS. Antigens were identified by incubation of the blot with 10 µg/ml of D3 or E4 in PBS-6% BSA for 1 h at room temperature. After washing twice, the blot was incubated under the same conditions of time and temperature with rabbit 125I-F(ab)γ; anti-mouse IgG (Bioproducts for Science, Inc.) was iodinated by the procedure already described. After extensive washing, the nitrocellulose paper was dried and autoradiography was performed for 1 h at —70°C by using X-R film (Kodak, Rochester, NY). To determine if D3 and E4 antigens were glycoproteins, NP-40 extracts of TR cells were passed through Con A-Sepharose columns (Sigma) and the bound-membrane material eluted with a solution of 1 M NaCl and -methylmannoside 0.5 M. Con A bound and unbound fractions were dialyzed with water, lyophilized, resuspended in NP-40 buffer, and analyzed as described above.

**Immunohistochemistry.** To determine the in vitro specificity and cross-reactivity of monoclonal antibodies, rat primary tumors and lung metastases were examined, along with a panel of normal rat tissues. To ascertain the best technique for optimal antigen preservation and visualization with immunoperoxidase staining, tissue samples were prepared in a variety of ways. First, paraffin-embedded tissues, fixed with each of the following: Clark's, Carnoy's, Bouin's fluid, formalin, cold acetone, methanol, and ethanol were examined. Next, fresh-frozen (~70°C) cryostat tissue sections fixed with the same variety of fixatives were studied. Lastly, fresh-frozen cryostat sections left unfixed were either stained immediately or kept at —20°C from 1 to 14 days, then stained. The avidin-biotin complex immunoperoxidase technique as described by Hsu et al. (9) and modified by Nuti et al. (10) was used (Mouse IgG Vectostain ABC kits, Vector Laboratories, Burlingame, CA). To reduce nonspecific cross-reactivity between anti-mouse antibodies and rat tissues, a rat-adsorbed, biotinylated anti-mouse IgG (Vector Laboratories) was substituted for the kit's secondary antibody.

**Antibody-dependent Cell Cytotoxicity.** TR cells were detached by trypsinization as described. Ten µCi of ¹¹¹In-oxyquinoline solution at 1 mCi/ml (Amersham) were added per million TR cells, and incubated at 37°C for 30 min. Cells were then washed four times in 50 ml medium and transferred to a 96-well plate at a concentration of 2000 cells in 100 µl. Monoclonal antibodies were added to the well at a final concentration of 10 µg/ml. An irrelevant antibody of same isotype (IgG1, 1A), generously provided by Centocor, Inc., Malvern, PA) was used as a negative control (11). Rat PBMNC were isolated by Ficoll-Hypaque (Pharmacia-LKB, Uppsala, Sweden) gradient centrifugation and were used as effector cells at different ratios. Spontaneous release was measured by replacing antibodies and PBMNC by a similar volume of medium; maximum release was determined by replacing medium by Triton X-100. TR cells, PBMNC, and antibodies were incubated for 18 h at 37°C. The supernatant was then harvested by using the Skatron Supernatant Collection System (Skatron, Sterling, VA) and counted in a gamma counter (LKB 1282 Compugamma).

**RESULTS**

**Immunization.** Among the three mice immunized with TS cells, one died at the third injection on day 28. Spleen cells from one of the two surviving mice with the more reactive sera were fused and produced the antibodies described here. Among 14 mice immunized with the TR cells under a similar protocol, 8 died during the immunization course. In addition, the 6 surviving animals whose spleenocytes were fused with NS1 myeloma did not generate antibodies with specificity restricted to the TR cell line but yielded immunoglobulins, mostly of the

<p>| Table 1 Fluorescence-activated cell sorter analysis of E4, D3, and F6 binding to rat cell lines |
|---------------------------------------------|-----------------|----------------|-----------------|-----------------|----------------|</p>
<table>
<thead>
<tr>
<th>TR</th>
<th>TS</th>
<th>NTD</th>
<th>G1TC</th>
<th>RFL/6</th>
<th>MAD B106</th>
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<td>2.7</td>
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<tr>
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<td>1.1</td>
<td>0.5</td>
<td>5.2</td>
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<tr>
<td>F6</td>
<td>85.0</td>
<td>98.5</td>
<td>1.0</td>
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<td>12.5</td>
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Results are expressed as percentage of fluorescent cells in the gate defined by the positive control (serum of mouse immunized with TS). Negative control for nonspecific binding was supernatant of the cultured parental myeloma NS1. (TR: TS: rat colon carcinomas; NTD, G1TC: rat lymphomas; RFL/6: rat fetal lung cell line; MAD B106: rat mammary carcinoma.)
Fig. 1. Immunoreactivity of radiolabeled E4 and D3. Increasing number of cells were incubated with a fixed amount of each antibody. The ratio of total radioactivity versus specifically bound was plotted against the inverse of the cell concentration. The extrapolation of the fitted line to the point 1/cell concentration = 0 gives the value of immunoreactivity, which was close to 100% for both antibodies after labeling.

Fig. 2. Sips analysis of E4 and D3 binding to TR tumor cells. TR cells (10⁶) were incubated for 3 h at 4°C with increasing concentrations of labeled antibodies. After incubation, the free and bound radioactivities were determined. The amount of antibody bound at saturation, and therefore the number of antigenic sites per cell, was calculated by incubating the cells with an excess of antibody. Data were analyzed by linear regression (r > 0.99). The extrapolation of the line to the point log [F] (molar units) = 0 was used to calculate the affinity constants.

IgM isotype, with broad reactivity to numerous rat normal tissues (data not shown).

Screening. ELISA screening on glutaraldehyde-fixed cells was performed on day 13. Seven % of the wells were considered positive and tested against TR cells on day 26. Seven wells remained consistently positive on repeated ELISA with TR cells, and were tested for their reactivity on the panel of rat cell lines described above. Two of them reacted with all the cell lines tested, and one was weakly reactive with both TR and MAD B 106. The antibodies produced by these three cell lines presumably reacted with rat major histocompatibility antigens and were not further expanded. The remaining four hybridomas were cloned in soft agar and an additional screening was performed. Among these four clones, one lost its reactivity 4 months later, and therefore three antibodies were available. They were called 5F7 D3 (later referred to as D3), 17B10 E4 (E4) and 18D5 F6 (F6). Binding of these selected antibodies was also studied in immunofluorescence with a fluorescence-activated cell sorter. As shown in Table 1, E4 MoAb reacts with the two rat colon carcinomas and the rat breast cancer cell line; it also binds to the normal fetal rat cell line RFL-6, but remains negative with the two lymphomas. D3 MoAb binds to both TR and TS colon carcinomas but does not react with all the other rat cell lines tested. F6 MoAb is positive on the colon and breast adenocarcinomas and displays a weak reactivity with the fetal lung cells.

Immunoglobulin classes and subclasses determined by the immunodiffusion technique proved to be IgG2a for D3 and E4, and IgG2b for F6.
Immunoreactivity of Labeled D3 and E4 Antibodies. As shown in Fig. 1, the value of the immunoreactivity (reactive/not reactive antibody) for the two radiolabeled antibodies D3 and E4, calculated by extrapolation at the point 1/C = 0, was near 1. This indicates that the labeling of the antibodies with iodine by the iodogen procedure, up to a specific activity of 4–5 μCi/μg, does not modify the affinity of these antibodies for their antigens.

TR Antigen Sites and Affinity Constants of D3 and E4. The incubation of TR cells with an excess of radiolabeled antibodies gave 980,000 antigenic sites/cell for E4 and 2,500,000 sites/cell for D3. These values were used to calculate the affinity constant of each antibody by the Sips equation. Fig. 2 shows the Sips analysis for D3 and E4. The regression coefficient (r) was greater than 0.99 in both cases. Heterogeneity indices (A) were close to 1, indicating the homogeneity of antibody population, as expected in an antigen-monoclonal antibody reaction. The values of the affinity constants, calculated by extrapolating the straight line to log [F] (molar) = 0, were 4.2 x 10^7 M^-1 for D3 and 9.35 x 10^7 M^-1 for E4.

Biochemical Nature of D3 and E4 Antigens. Upon Western blotting of NP-40 extracts of TR and TS cell membranes, and incubation of these blots with the three MoAbs (D3, E4, F6), TR and TS extracts gave identical results. Only two antibodies (D3 and E4) gave a distinct pattern (Fig. 3). D3 MoAb gave an extremely intense band at M₆, 27,000 and one minor band at M₆, 25,000. The E4 MoAb bound to a protein with a molecular weight of 66,000, giving a band of an intensity somewhat weaker than the major band of D3. Although cytofluorimetric analysis indicated that F6 also strongly stained TR and TS cells (Table 1), F6 failed to react with its antigen present on the blot.

Immunohistochemical Distribution. Antigen expression detected with the monoclonal antibodies E4 and D3, was demonstrated on unfixed cryostat sections of fresh frozen tissues and on sections kept at −20°C for no longer than 2 days. As shown in Table 2, the two monoclonals stained both primary tumor and lung metastasis with similar intensity, ranging from moderate to strong. Monoclonal antibody reaction with normal tissues varied significantly. E4 MoAb reacted moderately to strongly with the TR tumor (Fig. 4A), lightly stained the bronchial epithelium and alveolar walls as compared to lung metastasis (Fig. 4B); the gastrointestinal tract epithelia were only slightly stained (Fig. 4D), and no staining was observed with other normal tissues tested. In contrast, D3 MoAb moderately stained bronchial epithelium and alveolar walls (Fig. 4C), bladder epithelium and mesothelium, and leptomeningeal cells, and reacted with gastrointestinal tract epithelia as strongly as with...
Fig. 4. Immunoperoxidase reaction of tumor cells and normal colon epithelium with E4 and D3 monoclonal antibodies. Unfixed, fresh-frozen cryostat sections were stained using the avidin-biotin complex technique. Original magnification, × 210. A, positive staining of TR tumor cells with E4; B, lung metastasis with positive reaction to E4; C, lung metastasis treated with D3 antibody; positive staining of the tumor cells and light staining of the alveolar wall (arrows); D, normal colon mucosa section treated with E4; epithelial cells reacted lightly (arrows); E, normal colon epithelium cross-reacts strongly with D3 (arrows).

In contrast to D3 and E4, the F6 MoAb was detected only on sections of Bouin’s fixed, paraffin-embedded tissues. The intensity of immunoperoxidase staining of tumor cells with F6 was moderate. Small intestinal and colon epithelium, kidney tubular and bronchial epithelium stained moderately as well. F6 also moderately stained hepatocytes, while D3 and E4 did not.

Antibody-dependent Cell Cytotoxicity. Weak ADCC was observed at 50:1 and 100:1 rat PBMNC:TR cell ratios for both E4 and D3 antibodies. F6 did not mediate ADCC under similar experimental conditions as compared to an irrelevant antibody (Fig. 5).

DISCUSSION
Since monoclonal antibodies with human tumor specificity have become available (11), a number of clinical trials have been undertaken in patients with advanced tumors (12–14). Empirical use of such possible therapeutic agents has demonstrated some efficacy in a very limited number of cases. Numerous questions remain as to why this therapy has worked in only a few patients. In order to better understand the mechanism(s) of action of antibody in therapy, animal models with characteristics close enough to the human situation are needed.
The rat model and the antibodies described here might be more suitable for the study of mouse monoclonal antibody therapy of syngeneic tumors.

The E4 antibody seems to have a preferential specificity for the TR tumor and only weak reactivity with normal rat tissues. Its binding to a rat fetal lung cell line and much weaker expression in adult rat lung, as documented in immunoperoxidase staining, might indicate the oncofetal character of its antigen. In addition, E4 mediates ADCC by rat peripheral blood mononuclear cells; levels of ADCC observed are low, but might reflect the binding of mouse monoclonal antibodies to only a fraction of rat blood mononuclear cells in vitro. Its affinity constant and antigenic site number on tumor cells should also make it a likely candidate for experimental therapeutic use. D3 and F6 show reactivity with the tumor, but also cross-react with a variety of normal rat tissues. Both antibodies do not react with rat fetal lung cells but do bind to adult rat lung epithelium. D3 is strongly expressed on the TR tumor cell but has a lower reactivity with the tumor cell population. It is to be noted that both E4 and D3 are mouse IgG2a and mediate ADCC with rat PBMNC whereas F6, IgG2b, does not. D3 and F6 might be of interest in studying the side effects resulting from their reaction with rat effector cells. It is to be noted that both E4 and D3 are mouse IgG2a and mediate ADCC with rat PBMNC whereas F6, IgG2b, does not. D3 and F6 might be of interest in studying the side effects resulting from their reaction with rat effector cells. It is to be noted that both E4 and D3 are mouse IgG2a and mediate ADCC with rat PBMNC whereas F6, IgG2b, does not. D3 and F6 might be of interest in studying the side effects resulting from their reaction with rat effector cells.

Preliminary results of pharmacokinetics for E4 and D3 antibodies could be produced against the mouse monoclonals described here to measure the effect of rat immunization with anti-idiotypic (Ab2) on tumor development.

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