ABSTRACT

A monoclonal antibody is described that specifically detects the ganglioside antigens GD2 and GD3, binding preferentially to GD3 in melanoma. Antibody specificity was demonstrated with solid-phase radioimmunosay and enzyme-linked immunosorbent assay as well as by immunostaining on thin-layer chromatography plates using structurally characterized gangliosides. Binding of both the IgG3 antibody and its IgG2a switch variant were assayed on live cells by cytofluorography and by immunoperoxidase staining on frozen tissue sections. The binding patterns correlated with antitumor activity in antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity assays with human effector cells and complement and in an 111In-release assay using cell lines derived from the same individual. The significant level of killing in all tumor cells tested that express GD2, GD3, or both, suggests the importance of multiple specificity towards tumor antigens, i.e., binding of a monoclonal antibody to two or more tumor-associated antigens.

INTRODUCTION

mAbs2 have been produced that specifically detect human melanoma cells (3), and several recent studies have pointed to the ganglioside antigens, especially GD3,3 as potential targets for immunotherapy in human melanoma (4–10). Although these ganglioside antigens are present in central nervous tissue, they are greatly enriched in melanoma. We have produced a mAb of the IgG3 isotype, and an IgG2a switch variant, which bind to both GD2 and GD3 gangliosides. Earlier studies identified GD2 as a melanoma-associated antigen as well as a possible human immunogen (11–13). The present experiments illustrate the immunotherapeutic potential of the GD2-GD3-specific mAb, based on its ability to mediate tumoricidal activity with human effector cells and complement. The results suggest the significance of selecting mAbs that recognize multiple-tumor antigens.

MATERIALS AND METHODS

Tissues and Cells. The majority of the melanoma cell cultures used in this study were established in our laboratory. The origin and maintenance of these and other cells have been described (14–16). Table 1 shows the origins and ganglioside content of the cell cultures used. Tissues were obtained and prepared as described (9). Lymphocytes and monocytes were obtained from heparinized human peripheral blood by centrifugation on a Ficoll-Hypaque density gradient whereafter separation of adherent cells (monocytes) and nonadherent cells (lymphocytes) using plasma-gelatin-coated flasks was performed as described (17). Natural killer cells were removed from the monocyte preparations by treatment with anti-human Leu-11b mAb (Becton & Dickinson, Mountain View, CA), using a concentration of 0.5 μg/ml and rabbit complement.

Glycolipids. Preparation of total nonalkali-treated melanoma cell ganglioside fractions was as described (21). Gangliosides were purified and characterized essentially as described (9). TLC was performed on glass-backed and alumina-backed high-pressure TLC plates (Bodman Chemicals, Gibbstown, NJ). The solvent system used for developing plates was chloroform/methanol/0.2% CaCl2 in H2O (60/40/9, v/v/v), and detection was made with the resorcinol reagent (22).

Antibody Binding Assays. Solid-phase ELISA and RIA, using purified antibody ME 361 (1 μg/μl) of both isotypes and serially diluted glycolipid antigens, were performed on 96-well microtiter plates (Linbro/Titertek; Flow Laboratories, McLean, VA, for ELISA and Dynatech Laboratories Inc., Alexandria, VA, for RIA). Glycolipids were serially diluted in methanol and applied to the plate in a volume of 50 μl/well. Four samples of each dilution were tested in ELISA (9) after evaporating the methanol in 22°C and in RIA using 125I-labeled goat anti-mouse F(ab')2 (1500 cpm/μl) as the second antibody (23). Standard deviation was less than 7% for all values.

The TCL binding assay was performed essentially as described (23, 24), using hybridoma culture supernatant as antibody source with the same second antibody as above. Results from negative reference glycolipids (>500 ng/band) were obtained from the chromatogram binding assay, except GM1, which was run serially diluted in ELISA, and plotted in Fig. 1 for the sake of simplicity.

Reactivity of mAbs with cryostat sections of freshly frozen melanocytic lesions, after 10 min acetone fixation as described (25), was determined using the peroxidase-antiperoxidase procedure for immunoperoxidase assays (Ortho Diagnostic Systems Inc., Raritan, NJ). Binding of mAb to antigen in supplemented serum-free culture supernatants was determined in indirect solid-phase RIA (9). Western blotting and immunoprecipitation were performed as described (26–28).

For flow cytometry of the melanoma cell cultures, cells from one 75-cm² flask were trypsinized on the day before assay and transferred to a new flask. Cells were removed from the flask on the day of assay by a short incubation with 0.1% EDTA in phosphate-buffered saline and resuspended in Hanks' medium (GIBCO Laboratories, Grand Island, NY) containing 10% heat-inactivated human serum. Viability was assessed by trypan blue exclusion and was >90%. Cells were then diluted to 4 × 10⁶ cells/ml, and 250 μl (1 × 10⁶ cells) were placed in a 1.5-ml Eppendorf tube for each assay. Primary antibody (50 μl) was then added and incubated for 30 min on ice. Undiluted culture supernatant of the mouse myeloma P3X63 Ag8 was used as negative control. Cells were then washed twice, resuspended, and incubated for 30 min in 50 μl of fluorescein-labeled goat anti-mouse F(ab')2 (diluted 1/100) (Cappel, Worthington, PA) in Hanks' medium as above. Cells were then washed twice and resuspended in 0.5 ml of Hanks' medium and kept on ice for

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2 The abbreviations used are: mAb, monoclonal antibody; TCL, thin-layer chromatography; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity.

3 Gangliosides were named according to the nomenclature of Svennerholm (1); otherwise, the nomenclature used follows the IUPAC-IUB convention (2).
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less than 2 h before flow cytometry. An Ortho Cytofluorograf 50 HH connected to a Data General MP/200 microprocessor was used (Ortho Instruments, Westwood, MA). Cells were considered positive when their fluorescence intensity exceeded the threshold at which 99% of the cells treated with control antibody (negative control) had lower fluorescence intensity.

ADCC and CDC Assays. An 18-h 111In-release assay was used to measure antibody-dependent, monocyte-mediated cytotoxicity. Ascites was used at a 1/100 dilution, the highest dilution giving significant lysis in the assay, as titrated on melanoma cell culture WM 164. No attempts were made to compare the killing efficiency of the two different isotypes, since the purified γ2a antibody was difficult to solubilize at the necessary concentrations. However, purified γ2a antibody at a concentration of 10 μg/ml showed the same effect as the 1/100 ascites dilutions. Target cells (1 × 10⁶) were labeled with 10 μCi of [111In]indium oxide (Medi-Physics Inc., Emerville, CA) for 15 min in 5 μl of saline at 21°C. The cells were washed three times in medium and were added at 1 × 10⁶ cells/well in round-bottom microtiter plates (Linbro; Flow Laboratories, McLean, VA). ADCC assay, effector cells and various concentrations of mAbs were then added in triplicate and incubated for 18 h at 37°C in 5% CO₂. Plates were then centrifuged at 80 × g for 2 min. The supernatants were harvested and analyzed in a gamma counter. The percentage cytotoxicity was calculated by the following formula:

\[
\% \text{ cytotoxicity} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{input cpm} - \text{spontaneous cpm}} \times 100
\]

The values in Table 2 have control values subtracted (percentage of 111In-release without mAb). The standard deviation was below 10% for all values. For CDC assay, the 111In-labeled target cells were incubated in microtiter plates with 40% autologous plasma from human donors and various concentrations of mAbs.

RESULTS

Identification of the Antigen Detected by Antibody ME 361. Western blotting and immunoprecipitation, using extracts and cells, respectively, from melanoma cell cultures WM 75, WM 266-4, WM 115, WM 373, WM 164, and SK MEL 23, revealed no glycoprotein antigen forms associated with antibody ME 361 (data not shown). The detailed specificity of the antibody was tested by ELISA (summarized in Fig. 1), and by RIA (data not shown) using pure glycoliber references as well as total glycolipid fractions from human brain and erythrocytes. The results of ELISA indicated highest binding levels for ganglioside GD2 (GalNACβ1→4Gal[3→α2NeuNAcβ1→3Galβ1→1Cer]) followed by GD3 (NeuNAcα2→8NeuNAcα2→3Galβ1→4Glcβ1→1Cer). No other gangliosides present in human brain and erythrocytes showed binding reactivity in the chromatogram binding assay (data not shown). In Fig. 2B, antibody ME 361 was shown to bind to the GD2 ganglioside (lower arrow) and the GD3 ganglioside (upper arrow), consistent with the ELISA data. The fastest migrating bands, seen most clearly in lanes 1, 4, and 10–11 in Fig. 2B, were found to disappear after alkali treatment (Fig. 2C) and therefore most probably represented GD2 lactones that also were reactive with the mAb ME 361. The antibody bound readily to total ganglioside fractions from all 11 melanoma cell cultures studied so far, using a TLC binding assay (Fig. 2, B and C).

Binding of Antibody ME 361 to Shed Material. The hybridoma was initially screened for binding to serum-free culture supernatants from the cell cultures indicated in Fig. 3. As shown in this figure, all cultures tested released the antigen into the medium. The cell cultures established from metastatic melanoma released slightly higher amounts than those established from primary melanoma. Nonmelanoma cell cultures which did not release any of the antigens were: SW 1783 and SW 1088 astrocytoma; SW 620, SW 707, SW 1116, and SW 1345 colorectal carcinoma; KATO III gastric carcinoma; Capan-2 pancreatic carcinoma; 2774 and CaOV 3 ovarian carcinoma; T-24 bladder carcinoma; SW 684 sarcoma; Tera 1 teratocarcinoma; and WI 38 fibroblasts.

Cell Binding of Antibody ME 361. The results obtained from antibody binding assay using indirect flow cytometry indicated binding to all six cell cultures studied here (Table 3). The cells with the highest extractable amount of the GD2 ganglioside, i.e., WM 75, WM 115, WM 266-4, and SK MEL 23 (Fig. 2A, lower arrow, lanes 1, 3, 4, and 11, respectively, and Table 1), showed higher levels of antibody binding than cultures WM 373 and WM 164, which contain relatively small amounts of GD2 (Fig. 2A, lower arrow, lanes 2 and 9). However, WM 373 and WM 164 cells had high amounts of the GD3 ganglioside (Fig. 2A, upper arrow, lanes 2 and 9), so that despite weak antibody binding.
**Table 3** Binding of antibody ME 361 to melanoma cell cultures in indirect flow cytometry

<table>
<thead>
<tr>
<th>Cells</th>
<th>Antibody</th>
<th>Mean % total binding (number of determinations)</th>
<th>MCF*</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM 75</td>
<td>P3</td>
<td>2.7 (2)</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td>30.1 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>36.0 (2)</td>
<td>76</td>
</tr>
<tr>
<td>WM 373</td>
<td>P3</td>
<td>3.8 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td>11.2 (3)</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>16.1 (2)</td>
<td>138</td>
</tr>
<tr>
<td>WM 115</td>
<td>P3</td>
<td>1.6 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td>74.2 (2)</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>80.0 (1)</td>
<td>90</td>
</tr>
<tr>
<td>WM 266-4</td>
<td>P3</td>
<td>2.5 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td>91.6 (3)</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>96.4 (2)</td>
<td>172</td>
</tr>
<tr>
<td>WM 164</td>
<td>P3</td>
<td>1.2 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td>5.6 (2)</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>16.2 (2)</td>
<td>57</td>
</tr>
<tr>
<td>SK MEL 23</td>
<td>P3</td>
<td>2.3 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td>80.6 (4)</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>93.9 (2)</td>
<td>162</td>
</tr>
</tbody>
</table>

* P3, culture supernatant from mouse myeloma P3X63-Ag8; 2a, purified ME 361 γ₂a antibody; and 3, γ₁-containing ascites diluted 1/100.
* MCF, mean channel fluorescence.

**Table 4** Immunoperoxidase staining on frozen sections

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Number of cases</th>
<th>Number of positive cases</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocytes/keratinocytes, nerves, and Langerhans cells</td>
<td>71</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nevii</td>
<td>7</td>
<td>4</td>
<td>57</td>
</tr>
<tr>
<td>Dysplastic nevi</td>
<td>51</td>
<td>19</td>
<td>37</td>
</tr>
<tr>
<td>Primary melanoma</td>
<td>10</td>
<td>6</td>
<td>60</td>
</tr>
<tr>
<td>Metastatic melanoma</td>
<td>23</td>
<td>22</td>
<td>96</td>
</tr>
<tr>
<td>Lymphocytes*</td>
<td>126</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>Brain</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Liver, kidney, and testis</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Lymphocytes in sections of melanocytic lesions.
* One case each.

Reactivity to G3 (Fig. 1), these cultures still showed significant binding (Table 3). Other melanoma cell cultures reactive with the antibody were WM 9, WM 46, and the neuroblastoma cell culture IRM-5. No binding was detected to the same human cell cultures that were mentioned above as negative for binding to shed cell material.

**Tissue Specificity of Antibody ME 361.** The binding of antibody ME 361 to tissues representing all stages of melanoma tumor progression (16) was determined using immunoperoxidase staining of frozen cryostat sections (Table 4). Staining was diffuse throughout the cytoplasm of the cells. In general, the reactivity was moderate to strong in intensity, involving 50% or more of the lesional cells in most instances. The reactivity to brain was mainly confined to myelin and peripheral nerves were nonreactive as seen throughout the tissues studied. The binding of ME 361 to all ganglioside fractions in Fig. 2 and all cells in Table 3 was consistent with the high binding to primary and metastatic melanoma.

**Cytotoxicity in ADCC and CDC.** Six melanoma cell cultures were selected that showed different ganglioside patterns in TLC (Table 1). All cells were significantly lysed in ADCC with human monocytes and lymphocytes, using an effector-to-target cell ratio of 50:1, as well as in CDC with human complement in an 18-h 111In-release assay (Table 2). The killing efficiency of ME 361 was significantly higher ($P \leq 0.01$; Student's $t$ test, using all four values) in WM 75 cells than in WM 373 cells, consistent with the higher amount of G32 and higher antibody binding levels in the WM 75 cells. However, no significant
difference was found between the lysis of WM 115 and WM 266-4 cells (also derived from a single individual), which correlated well with the similar ganglioside patterns of these cultures. Antibody binding in the indirect flow cytometry assay was also similar for WM 115 and WM 266-4 cells, i.e., 73 and 83% (Table 3), respectively. The distribution of the major gangliosides GM3, GM1, GD2, and GD1 in these cell cultures was similar, in sharp contrast with the patterns seen from WM 75 cells, in which GD2 was the major ganglioside, and for WM 373 cells, in which GD1 was the major ganglioside and GD2 was found only in low amounts (Fig. 2, A and B, lanes 1–4). In the case of two cell cultures from different individuals, mAb binding and killing of cells expressing both GD2 and GD1 (SK MEL 23) was even larger than in cells expressing only GD1 (WM 164) (Tables 2 and 3, respectively).

**DISCUSSION**

The selection of mAbs with potential immunotherapeutic use in the treatment of human melanoma has necessitated criteria for antibody screening in order to identify those that are effective mediators of cytotoxicity. Antibody isotype is one important parameter, and it has been shown that mouse antibody isotypes \( \gamma_2a \) (17, 29) and \( \gamma_3 \) (19) are most effective. In the present study, we have produced an IgG3-secreting hybridoma, ME 361, and selected from it an IgG2a-secreting switch variant. Antibodies of \( \gamma_2a \) isotype are generally easier to handle in experiments, although the \( \gamma_3 \) antibodies are better able to fix human complement (data not shown). The latter property might be an important factor in determining the *in vivo* effect of \( \gamma_3 \) antibodies (6). Thus, the combined use of two different isotypes directed to the same antigen(s) might mediate killing *in vivo* more efficiently because of optimization of two cytotoxic systems, *i.e.*, ADCC and CDC. Verification of this hypothesis awaits results of ongoing clinical trials. The number of available antigen binding sites on the tumor cells is also a factor in determining an antibody’s usefulness (30). In this respect, the major ganglioside antigens of human melanoma cells are logical targets since they are expressed at high levels on the cell surface.

The popular criterion of tissue specificity, *i.e.*, that antibody reactivity should be confined to tumor tissue only, probably should be reevaluated in light of recent encouraging results using the anti-GD2 antibody R24 (6). It appears to be more important to select an antigen system having a high ratio of antigen in tumor *versus* antigen in normal tissue, which, despite its binding to several normal tissues, induced only minimal side effects confined to the skin. ME 361 detects both GD2 and GD3 gangliosides (Fig. 1), but binds at lower levels than antibody R24 to GD2. Moreover, because the tissue distribution of GD2 (the primary antigen for ME 361) was even more restricted than that of GD3 (ME 361 bound to brain tissue but not to melanocytes and fibroblasts; Table 4), any side effects of ME 361 *in vivo* might be expected to be even less than for antibody R24. However, antibody binding to antigens present only as glycolipids in tissue sections must be interpreted with caution. Since it is difficult to find preparation methods of tissue sections that do not involve organic solvents at some point, there is always a risk of some loss of reactivity due to antigen elution. Furthermore, since the antibody reacts with both GD2 and GD3, although at different levels, it is not clear which antigen is involved in the observed reactivity.

The GD2 and GD3 antigens are biosynthetically linked, *i.e.*, GD2 is formed from GD3 by the addition of N-acetylgalactosamine (31). An antibody that recognizes both antigens is desirable, especially since melanoma cell cultures can be empirically divided into groups based on the differential expression of GD2 and GD3; those that express mainly GD2 (WM 75), those expressing GD2 and GD3 (WM 115, WM 266-4, and SK MEL 23), and those expressing mainly GD3 (WM 373 and WM 164). To correlate the presence of antigen with antibody cell binding and antibody-dependent cytotoxicity, we first ruled out the possibility of any other antigen forms such as glycoproteins that might carry the antibody-binding epitope. It was then possible to calculate that the cytotoxicity on WM 75 cells (expressing mainly GD2) was significantly higher than that on WM 373 cells (expressing mainly GD3) (Table 2), as was the binding of antibody ME 361 (Table 3). The highest binding levels and cytotoxic activity was observed with the cell cultures containing both antigens (WM 115, WM 266-4, and SK MEL 21). No correlation between amounts of extractable gangliosides and amount expressed on the cell surface as measured by fluorescence-activated cell sorting was found. In the absence of information on the extent of “crypticity” and the extent of antigen expression within the cell, such a correlation may remain elusive. On the other hand, comparison of cell cultures established from one individual, *i.e.*, WM 75 with WM 373 and WM 115 with WM 266-4, revealed such a correlation, arguing for individual differences in expression versus total amount of ganglioside present.

ME 361-mediated cytotoxicity was observed in all cell cultures studied by us so far, even in WM 164 and WM 373 cells, which express mainly GD3. Thus, antibodies that detect only GD2 or only GD3 might not affect cell cultures that do not express one of the gangliosides and therefore might also be ineffective against certain cell clones *in vivo*. The use of antibody mixtures could also circumvent this problem, provided that they do not sterically block each other on the tumor cell surface.

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ANTI-GD2 AND -GD3 MELANOMA ANTIBODY


Monoclonal Antibody-defined Correlations in Melanoma between Levels of GD₂ and GD₃ Antigens and Antibody-mediated Cytotoxicity

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