Disialoganglioside $G_{D2}$ on Human Neuroblastoma Cells: Target Antigen for Monoclonal Antibody-mediated Cytolysis and Suppression of Tumor Growth

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ABSTRACT

A murine monoclonal antibody 14.18 specifically recognizes disialoganglioside $G_{D2}$, the major ganglioside expressed on the surface of human neuroblastoma cells. This monoclonal antibody (Mab) is of immunoglobulin G3 isotype, has an affinity constant ($K_d$) of $3.5 \times 10^7$ M$^{-1}$, and reacts preferentially with tumor cells and fresh frozen tumor tissues of neuroectodermal origin in enzyme-linked immunosorbent assay and immunoperoxidase assays, respectively. Mab 14.18 effectively lyses a number of human neuroblastoma cell lines by two distinct mechanisms, i.e., antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. There is a good correlation between the average number of antibody-binding sites per neuroblastoma cell and the amount of cell lysis observed in complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity. In addition, Mab 14.18 suppresses establishment as well as growth of progressively growing, established human neuroblastoma tumors in nude mice when injected 24 h and 9 days, respectively, after the initial s.c. inoculation of tumor cells. These data suggest that Mab 14.18 can mediate tumor cell killing in vivo and in vitro and may thereby prove useful for immunotherapy of human neuroblastoma.

INTRODUCTION

Neuroblastoma is a neoplasm of the peripheral autonomic nervous system that represents the second most common malignancy of childhood. Fifty-five % of patients have widespread metastatic disease at the time of the diagnosis (1). More than one-half of the children present with the disseminated disease and are incurable by currently known methods. There has been relatively little improvement in outcome during the last 25 years, in contrast to the considerable advances made in most other childhood cancers. It is apparent that new therapeutic modalities are needed for the patients with disseminated neuroblastoma (Stage III and IV).

Initial studies by several investigators that evaluated a series of Mabs for new immunotherapy modalities to be applied to neuroblastoma were largely unsuccessful since these reagents failed to react with all neuroblastoma cells and were unable to kill tumor cells in the presence of human complement even when used as a panel (2). In addition, a number of these Mabs lacked specificity since they reacted with megakaryocyte precursors and with some normal blood and bone marrow cells (3). When injected into tumor-bearing animals, these Mabs localized largely in the organs of the reticuloendothelial system, i.e., liver, spleen, and bone marrow (4). Most importantly, the target antigens of most of these antibodies were not extensively characterized and thus their biological function is unknown (5).

However, more progress was made in other studies investigating disialoganglioside $G_{D2}$ with both human (6) and murine (7, 8) Mabs directed to this chemically defined target antigen uniformly expressed on human neuroblastoma cells. In this regard, anti-$G_{D2}$ murine Mabs of IgG3 isotype were used to demonstrate that $G_{D2}$ redistributes into microprocesses making direct contact with a fibronectin substrate and being substantially involved in attachment of melanoma and neuroblastoma cells to this substrate (9, 10). Most recently, another anti-$G_{D2}$ Mab of IgG3 isotype was reported to produce a prolonged remission in a neuroblastoma patient (11).

We report here on the reactivity of anti-$G_{D2}$ Mab 14.18 with a variety of normal and neoplastic cells and tissues as well as on the functional role of this antibody with emphasis on its potential for immunotherapy of neuroblastoma.

MATERIALS AND METHODS

The following human cell lines were obtained from the American Type Culture Collection, Rockville, MD: MOLT-4; HPB-ALL (lymphoblastoid); and U-138MG (glioblastoma). The neuroblastoma cell lines LAN-1 and LAN-5 were kindly provided by Dr. R. C. Seeger, UCLA. Additional neuroblastoma cell lines SK-N-AS, SK-N-RA, and SK-N-LE were a gift from Dr. H. H. N. Mest, Memorial Sloan-Kettering Cancer Center, New York. SMS-KCN, SMS-KAN, and CHP-134 were kindly provided by Dr. P. Reynolds, Naval Medical Center, Bethesda, MD, and the IMR-6 and NMB-7 neuroblastoma cell lines were a gift from Dr. S. K. Liao, McMaster University, Hamilton, Ontario, Canada. L-14 (lymphoblastoid), M21, and M-14 (melanoma) lines were kindly provided by Dr. D. L. Morton, UCLA. FM8 and FM9-Met melanoma cell lines are available from our laboratory and the Melanoma melanoma cell line was kindly provided by Dr. U. Koldovsky, University of Dusseldorf, West Germany. The melanoma A375P line and its A375 Met mix spontaneous metastatic variant were a gift from Dr. I. J. Fidler, M. D. Anderson Hospital and Tumor Institute, Houston, TX. The LG-2 lymphoblastoid cell line is available in our laboratory. The T293 (small cell lung carcinoma) line was provided by Drs. H. Masue and G. Sato, University of California, San Diego. The NCI H-69, H-82, and N-417 small cell lung carcinoma were made available by Drs. D. Minna and A. Gazdar, NIH, Bethesda, MD. The pancreatic carcinoma cell lines FG and SG were provided by Dr. S. Kajji at our institution.

Monoclonal Antibodies. Monoclonal antibody W6/32 (IgG2a anti-HLA) is maintained in our laboratory as are Mabs 126-4 (IgM; anti-GD2); C281 (IgG3; anti-GD2); 5G3 (IgG2a anti-M, 215,000 glycoprotein antigen).

Animals. BALB/c athymic (nu/nu) mice were obtained from the nude mouse colony at the University of California, San Diego, La Jolla, CA.

Production of Monoclonal Antibody. mab 14.18 was produced against the human neuroblastoma cell line LAN-1 by standard hybridoma technology (12). Briefly, $5 \times 10^6$ LAN-1 cells were injected i.p. into BALB/c mice, once every week for 4 weeks. On the third day after the last injection, the animals' splenocytes were removed and fused with the M-5 variant of the nonsecretor murine myeloma cell line SP2/0. Hybridoma 14.18 was selected by growth in Dulbecco's modified Eagle's medium containing 10% horse serum, hypoxanthine, aminopterin, and thymidine and subcloned by limiting dilutions.

Ascites Preparation. BALB/c mice were primed by injecting 0.5 ml pristane two weeks after priming, $10^{-5} \times 10^6$ hybridoma cells were
injected i.p. into the mice and ascites fluid was collected repeatedly every 2–3 days. The fluid was centrifuged at 2000 × g for 20 min to remove cells and other debris and quickly frozen at −20°C.

Purification of MAb. The ascites fluid was clarified at −10,000 × g for 20 min. The supernatant was then titrated on ELISA and only ascites with high titer was selected for the purification of MAb. Purification was achieved by Protein A-Sepharose affinity chromatography as described previously (13). Antibody was eluted with 0.1 M sodium citrate buffer, pH 4.5, and dialyzed extensively at 4°C against PBS, pH 7.4. The purified antibody was stored in small aliquots at −70°C.

Isotyping. The isotype of the MAb was determined by ELISA, using affinity-purified rabbit antiserum specific for different murine light chains (Southern Biotechnology Associates, Birmingham, AL) that were dried into 25-mm microtiter plates (Dynatech). Prior to ELISA, the dried plates were rehydrated with washing them twice with 10 mM PBS, pH 7.4, containing 0.1% Tween 20 and 0.02% thimerosal (washing buffer). Supernatants were diluted 1:2 in washing buffer containing 0.1% BSA as diluent. Diluted test supernatant (50 μl) was added to each well and plates were incubated for 1 h at 4°C. Following three washes, 50 μl of horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Richmond, CA) were added to each well and incubated for 1 h at 4°C. After two final washes, 50 μl of substrate solution (400 μg/ml o-phenylenediamine in 0.12% H2O2) were added to each well. The reaction was stopped after 15 min by addition of 25 μl of 4 N H2SO4 to each well. Absorbance at 492 nm was measured with a microtiter plate reader.

Tissues. Fresh frozen normal and malignant tissue were obtained from the surgical pathology department of the Ida M. Green Hospital of the Clinical, La Jolla, CA. Additional tumor specimens were kindly provided by Drs. F. Kung and A. Yu, Department of Pediatrics, University of California at San Diego. Specimens were embedded in tissue Tek-II O.C.T. (Miles, Naperville, IL) frozen in blocks in isopentane at −176°C and stored at −70°C.

Immunoperoxidase Staining of Frozen Tissues. Two- to 4-μm-thick sections were cut from frozen tissue blocks on a cryostat/microtome (Damon Biotech, Needham Heights, MA). These sections were mounted on gelatin-coated glass slides, air dried briefly, and tested immediately in an indirect immunoperoxidase assay essentially as described earlier (15). Briefly, after washing them twice in HBSS and once in PBS, we preincubated the sections for 15 min with PBS containing 10% goat serum and 0.1% BSA. Excess buffer was then pipetted off and sections were overlaid with appropriately diluted hybridoma supernatants and incubated for 1 h at room temperature. After two washes in HBSS and one wash in PBS, the tissue sections were overlaid with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Bio-rad), diluted 1:50 with dilution buffer (PBS containing 10% goat serum and 0.1% BSA). Excess buffer was then pipetted off and sections were overlaid with appropriately diluted hybridoma supernatants and incubated for 1 h at room temperature. Finally, the tissue sections were washed twice in HBSS and once in PBS and incubated for 15 min at room temperature with immunoperoxidase substrate buffer (10 mM Tris, pH 7.6, containing 0.6 mg/ml 3,3’-diaminobenzidine and 0.015% H2O2). The sections were counterstained briefly in 1% methylene blue, dehydrated through graded ethanol, washed in HistoClear (National Diagnostics Somerville, NJ), mounted with Pro-Tex (Lerner Laboratories, New Haven, CT), and examined by microscopy.

Ganglioside Extraction. Packed neuroblastoma cells (1 ml) were washed extensively with PBS and homogenized in chloroform:methanol (2:1). The residue was reextracted with chloroform:methanol (1:1) and passed through a sintered glass funnel, and the remaining residue was again extracted with this same solvent. The extracts were combined, dried under nitrogen, and partitioned in diisopropyl ether:n-butyl alcohol (6:4) and 50 mM NaCl as described by Laidish and Gillard (16).

TLC. Plastic-backed silica gel TLC plates (E. M. Merck, Darmstadt, Germany) were activated by heating at 110°C for 1 h. Glycolipids were dissolved in chloroform:methanol (1:1) and spotted 1.5 cm from the bottom of the TLC plates. These plates were then placed in a developing tank presaturated with 100 ml of chloroform:methanol:H2O (55:45:10) containing 0.02% CaCl2. Chromatograms were developed for 1.5 h at room temperature, after which the TLC plates were allowed to dry. The chromatograms were then sprayed with resorcinol reagent to visualize the gangliosides.

Immunostaining of Gangliosides Separated by TLC. The reactivity of MAb 14.18 with gangliosides separated by TLC was determined by using the immunostaining method described by Cheresh et al. (17).

Lipid ELISA. The lipid ELISA was performed essentially as described earlier (15), except that the plates were incubated for 1 h with PBS containing 5% BSA before the addition of monoclonal antibody. The remaining assay was essentially the same as that described for the regular ELISA, except washing buffer consisted of PBS containing 1% BSA. For preparation of lipid plates, a total lipid extract was prepared from 2 ml of packed M21 melanoma cells which are known to extensively express G2O ganglioside. These cells were homogenized in 40 ml chloroform:methanol (2:1), followed by filtration through a sintered glass filter. The residue was reextracted with chloroform:methanol (1:1) and refiltered. The combined filtrates were then dried down. Finally, the glycolipids were resuspended in methanol. Routinely, glycolipids from 50 μl packed cell volume were plated per well in flat-bottomed, polyvinyl microtiter plates (Dynatech).

125I-Labeling of MAb 14.18. The antibody was labeled with 125I by the chloramine-T method, as described previously by us (18). Briefly, 100 μl of antibody (1 mg/ml) were mixed with 400 μl of 0.2 M sodium phosphate buffer, pH 7.5. 125I (carrier-free, 17 Ci/mg) (0.5 μCi) and chloramine-T (10 μg; Sigma) were added to this solution. The sample was incubated for 5 min and the reaction was stopped by the addition of 10 μg of sodium metabisulfite (1 mg/ml in H2O) and 200 μl of PBS containing 3% Trasylol (Sigma). After completion of the reaction, a Sephadex G-25 column (Pharmacia), was used to separate the bound and free antibody. The radiolabeled protein from free 125I. This column was washed with 0.1% BSA/PBS and fractions were collected with the same buffer. The percentage of incorporation of radioiodine was determined by trichloroacetic acid precipitation.

Scatchard Plot Analysis. The specific activity of the radiolabeled antibody was 2 × 104 cpm/μg. The association constant (Kd) of MAb 14.18 was determined from Scatchard plot analysis of saturation binding data (19). Briefly, NMB-7 neuroblastoma cells (105/0.1 ml of RPMI 1640 containing 2% fetal calf serum and 0.01% NaN3) were incubated for 1 h at 4°C with increasing amounts of radiolabeled antibody (5–600 ng), diluted in the same media. Fractions containing bound and free antibody were separated by a dibutyl phthalate and dinonyl phthalate (1:1) oil layer as described by Beaufour et al. (20). Radioactivity in each fraction was determined in a LKB 1270 gamma counter. The amount of nonspecific binding of MAb 14.18 was estimated by adding a 100-fold excess of cold antibody to a cell suspension and subtracting bound cpm from total cpm. The data were subjected to linear regression analysis.

FACS Analysis. Viable cells were stained at 4°C and used for quantitation of cell surface expression of antigens by FACS as described earlier (21). Briefly, the cells were washed twice with HBSS containing 1% BSA before the addition of primary antibody, SP2/0 culture supernatant served as negative control for the primary antibody binding assay. After 1 h of incubation at 4°C, the cells were washed twice with HBSS/1% BSA and allowed to react with fluorescein isothiocyanate-conjugated goat anti-mouse sera for 1 h after which the percentage of reactive cells and the mean intensity of fluorescence was determined by FACS analysis.

CDC. Neuroblastoma cells suspended in 1 ml of growth media were labeled with 100 μCi of (Na)125CrO4 (New England Nuclear, 1 Ci = 37 GBq) for 2 h at 37°C. After 2 h of incubation, the cells were washed twice with tissue culture medium RPMI 1640 and 25-μl aliquots containing 104 cells were plated in 96-well microtiter plates (Costar, Cambridge, MA) with various concentrations of MAb 14.18. Each plate was incubated for 1 h at 37°C after which 150 μl of fresh human serum diluted 1:3 were added as the source of complement. Each plate was allowed to incubate for an additional 90 min at 37°C and was then centrifuged at 400 × g for 5 min and the radioactivity in the supernatant
was measured in a gamma counter. The total count was assessed by
adding 1% Nonidet P-40. Percentage of lysis was calculated as

\[
\frac{\text{(Experimental } ^{51}\text{Cr release}) - \text{(Spontaneous } ^{51}\text{Cr release})}{\text{(Maximal } ^{51}\text{Cr release})} \times 100
\]

ADCC. Neuroblastoma cells (SK-N-AS, IMR-6, SMS-KCNR) were
labeled in 1 ml of growth media with 300 \(\mu\)C of (Na)\(_2^{51}\text{CrO}_4\) for 2 h at
37°C. After the cells were washed twice with RPMI 1640, 5 \(\times\) 10^5 cells
in 25 \(\mu\)l were plated in 96-well microtiter plates. Mab 14.18 (5 \(\mu\)g in
50 \(\mu\)l) was added to each well containing target cells. Mononuclear
human effector cells were isolated by subjecting heparinized blood of
healthy individuals to centrifugation on a Ficoll-Hypaque gradient.
These cells were added to microtiter wells at the indicated target:effector
cell ratios and the plates were incubated for 4 h at 37°C. The plates
were then centrifuged at 1000 \(\times\) g for 5 min and radioactivity was
measured in a 100-\(\mu\)l aliquot of each supernatant. Spontaneous \(^{51}\text{Cr}
release was determined in wells that contained only labeled target cells.
Total cpn release was determined by lysing tumor cells with 1%
Nonidet P-40. Percentage of lysis was calculated as

\[
\frac{\text{Experimental } ^{51}\text{Cr release}}{\text{Maximal } ^{51}\text{Cr release}} \times 100
\]

The percentage of specific lysis was calculated by subtracting the
percentage of lysis obtained with effector cells in the absence of anti-
body, i.e., natural killer cell lysis, from each value obtained.

RESULTS

ELISA Reactivity of Mab 14.18 with Human Tumor Cell Lines. Table 1 summarizes the ELISA reaction patterns of Mab 14.18 with various human tumor cell lines. This antibody is of IgG3 isotype and has an association constant of \(3.5 \times 10^8\) M\(^{-1}\), as determined by Scatchard plot analysis. The antibody reacted very strongly with eight neuroblastoma cell lines tested. In
addition, Mab 14.18 also reacted to varying degrees of intensity
with a number of melanoma and small cell lung carcinoma cell
lines. This is not too surprising since neuroblastoma, mela-
noma, and possibly tumor cells of small cell lung carcinoma are of
neuroectodermal origin. Mab 14.18 also reacts with one glioma cell line. However, the antibody failed to react with cell lines derived from pancreatic carcinoma and adenocarcinoma
of lung and with lymphoblastoid cell lines of either T- or B-cell
origin.

Tissue Specificity of Mab 14.18. When Mab 14.18 was tested
to a number of frozen normal and tumor tissues, the antibody reacted very strongly with neuroblastoma, melanoma, glioblastoma, and small cell lung carcinoma tissues (Table 2).
Since reactivity was particularly pronounced with these tissue
sections obtained from tumors of neuroectodermal origin, it is
not surprising that this anti-GD2 antibody also reacted with normal adult cerebellum and fetal brain. A variety of other
tumor tissues showed negative staining. All normal tissues
tested, with the exception of cerebellum and fetal brain, failed
to react with Mab 14.18. Fig. 1 illustrates the strong positive
staining obtained when Mab 14.18 was reacted with fresh frozen
neuroblastoma tissue and contrasts with the lack of staining
observed when the same tissue was reacted with a negative
control, i.e., the myeloma fusion partner SP2/0 culture super-
natant (Fig. 1B).

Immunohistochemical Identification of the Target Antigen of Mab
14.18. The pattern of gangliosides in an extract obtained from
neuroblastoma cell line, IMR-6 is revealed by TLC. GD2 is the
major ganglioside present in these cells as demonstrated by the
resorcinol sprayed total ganglioside profile (Fig. 2A). By im-
munochemistry, Mab 14.18 was clearly shown to bind to a single
component that comigrated with authentic GD2 on the TLC
plate (Fig. 2B). In addition, results from lipid ELISA indicate
that Mab 14.18 binds only to GD2 and not to other gangliosides
standards tested, i.e., GD3, GM1, and GM3 (26).

Binding of Mab 14.18 to Viable Human Tumor Cells. The GD2
antigen was detected by FACS analysis on viable cells of six
table 1 ELISA reactivity of Mab 14.18 with cultured cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroblastoma</td>
<td>++++</td>
</tr>
<tr>
<td>LAN-1</td>
<td>++++</td>
</tr>
<tr>
<td>LAN-5 Duke</td>
<td>++++</td>
</tr>
<tr>
<td>SMS-KCNR</td>
<td>++++</td>
</tr>
<tr>
<td>CHP-134</td>
<td>++++</td>
</tr>
<tr>
<td>SMS-KAN</td>
<td>++++</td>
</tr>
<tr>
<td>NMB-7</td>
<td>++++</td>
</tr>
<tr>
<td>IMR-6</td>
<td>++++</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>++++</td>
</tr>
<tr>
<td>Melanoma</td>
<td>+</td>
</tr>
<tr>
<td>Melu</td>
<td>+</td>
</tr>
<tr>
<td>A375 P</td>
<td>+++</td>
</tr>
<tr>
<td>A375 Met</td>
<td>+++</td>
</tr>
<tr>
<td>FM8</td>
<td>+++</td>
</tr>
<tr>
<td>FM9 Met</td>
<td>+++</td>
</tr>
<tr>
<td>M-14</td>
<td>+++</td>
</tr>
<tr>
<td>M-21</td>
<td>+++</td>
</tr>
<tr>
<td>Glioma</td>
<td>++++</td>
</tr>
<tr>
<td>U138 M6</td>
<td>++++</td>
</tr>
<tr>
<td>Small cell lung carcinoma</td>
<td>++++</td>
</tr>
<tr>
<td>T293</td>
<td>++++</td>
</tr>
<tr>
<td>NIH-N417</td>
<td>++</td>
</tr>
<tr>
<td>NIH-H-82</td>
<td>++</td>
</tr>
<tr>
<td>NIH-H-69</td>
<td>++</td>
</tr>
<tr>
<td>Other tumor cell lines</td>
<td></td>
</tr>
<tr>
<td>Pancreatic carcinoma (fast growing)</td>
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</tr>
<tr>
<td>Pancreatic carcinoma (slow growing)</td>
<td>-</td>
</tr>
<tr>
<td>Pancreatic carcinoma (metastatic)</td>
<td>-</td>
</tr>
<tr>
<td>Adenocarcinoma of lung (UCLA-P3)</td>
<td>-</td>
</tr>
<tr>
<td>Lymphoblastoid cell lines</td>
<td></td>
</tr>
<tr>
<td>L-14 (B-cell)</td>
<td>-</td>
</tr>
<tr>
<td>L-2 (B-cell)</td>
<td>-</td>
</tr>
<tr>
<td>MOLT-4 (T-cell)</td>
<td>-</td>
</tr>
<tr>
<td>HPB-ALL (T-cell)</td>
<td>-</td>
</tr>
</tbody>
</table>

*Absorbance at 492 nm: 0.15-0.3 (+); 0.3-0.6 (++; 0.6-0.9 (+++); 0.9->2.0
(++++).
Fig. 1. Indirect immunoperoxidase staining of frozen human neuroblastoma tissues with Mab 14.18 (top) or with SP2/0 culture supernatant (bottom) as a negative control.

Table 3 Surface binding of Mab 14.18 to human neuroblastoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% of positive cells</th>
<th>MIF*</th>
<th>Av. no. of binding sites/cell (x 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMR-6</td>
<td>94</td>
<td>70</td>
<td>2.1</td>
</tr>
<tr>
<td>SMS-KCNR</td>
<td>92</td>
<td>101</td>
<td>2.7</td>
</tr>
<tr>
<td>CHP-134</td>
<td>96</td>
<td>87</td>
<td>1.6</td>
</tr>
<tr>
<td>NMB-7</td>
<td>79</td>
<td>73</td>
<td>2.7</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>82</td>
<td>57</td>
<td>1.4</td>
</tr>
<tr>
<td>SMS-KAN</td>
<td>86</td>
<td>52</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* MIF, mean index of fluorescence in FACS analyses.

were incubated with 5 μg/well of Mab 14.18 together with human peripheral blood mononuclear effector cells at various effector:target cell ratios. As depicted in Fig. 5, Mab 14.18 effectively lysed 49% SMS-KCNR, 35% of IMR-6, and 20% SK-N-AS neuroblastoma cells at effector:target cell ratios of 200:1 (open bars). Natural killer lysis of the cells in the absence of Mab 14.18 ranged from 5 to 8% (hatched bars).

Mab 14.18 Suppresses the Establishment of Human Neuroblastoma Tumors in Athymic (nu/nu) Mice. When 18 athymic (nu/nu) BALB/c mice were given s.c. injections of 9 x 10⁶ SK-N-AS neuroblastoma cells, variable tumor growth was observed in all animals. One control group consisted of six mice given i.p. injections of 200 μl of PBS on days 2, 4, 6, 8, 13, and 18 after the initial tumor inoculum. All of these animals exhibited large tumors that started to develop very rapidly, 9 days after the initial tumor cell inoculation. The tumor volumes ranged
DISialoganglioside GD1a ON HUMAN NEUROBLASTOMA CELLS

Fig. 3. Complement-dependent cytotoxicity of Mab 14.18. Specific lysis was determined for 6 neuroblastoma cell lines that were labeled with (Na)251CrO4 and challenged with Mab 14.18 and human complement as described in "Materials and Methods." Cell lines: CHP-134 (O); SMS-KAN (O); SK-N-AS (O); SMS-KCNR (O); NMB-7 (A); IMR-6 (A).

Fig. 4. Kinetics of complement-dependent cytotoxicity. Neuroblastoma cells SMS-KCNR (O) and NMB-7 (O) were labeled with (Na)251CrO4 and incubated at various time points with Mab 14.18 and human complement.

Fig. 5. Antibody-dependent cellular cytotoxicity mediated by monoclonal antibody 14.18 and normal human peripheral blood mononuclear cells as effector cells. The percentage of specific lysis was determined at different effector:target ratios for 3 human neuroblastoma cell lines labeled with (Na)251CrO4 and incubated with human PBMCs in the presence (O) or absence (A) of Mab 14.18.

Fig. 6. Suppression of neuroblastoma tumor establishment in athymic (nu/nu) mice. A, PBS (200 µl); B, irrelevant Mab C281 (anti-GD1a); C, Mab 14.18 (anti-GD1a), and antibodies were injected i.p., at 200 µg/dose, 24 h after the initial tumor cell inoculum, on days indicated by the arrows. Growth suppression of progressively growing, established neuroblastoma tumors in athymic (nu/nu) mice. D, irrelevant Mab C281 (anti-GD1a); E, Mab 14.18 (anti-GD1a). Tumor cells were inoculated s.c. and antibodies were injected i.p. at 200 µg/dose, 9 days after the initial tumor cell inoculum on days indicated by the arrows. The tumor volume in each group of mice was analyzed by the two-sided Student's t test.

in volume between 1600 and 6800 mm³ after 23 days (Fig. 6A). Another control group of six mice received 200 µg each of irrelevant Mab C281. Similar to the specific Mab 14.18 (anti-GD1a), Mab C281 is of IgG3 isotype but is, however, directed to GD3, an antigen not expressed on neuroblastoma cells (26). All animals in this group received the antibody at the same time intervals as the PBS control animals, and after 9 days rapidly developed large tumors ranging in volume from 3000 to 7500 mm³ when measured on the last day of the experiment (Fig. 6B). Finally, six mice received Mab 14.18 (anti-GD2) at the same dose level and time schedule as Mab C281 (anti-GD3). In contrast to the two control groups, starting on day 20 these animals slowly developed relatively small tumors ranging in volume from 200 to 900 mm³ when measured on the last day of the experiment (Fig. 6C). Tumor volumes of control groups receiving either PBS or irrelevant Mab C281 showed differences that were not statistically significant (P > 0.1). However, there was statistical significance (P < 0.01) when tumor volumes, measured on days 8 to 23, were compared between the PBS control group and the group of animals given injections of Mab 14.18. The mean tumor volume of each animal was measured at various times after tumor inoculation as indicated by the arrows by measuring three diameters (d1, d2, and d3) with graduated calipers, and tumor volume was calculated by the formula

\[ \text{Volume} = \frac{4}{3} \pi (d1d2d3) \]
DISIALOGLANGLIOSIDE GD2 ON HUMAN NEUROBLASTOMA CELLS

comparing between tumor volumes of the control group re-
ceiving irrelevant Mab C281 with those of the group of animals
given Mab 14.18 injections showed statistical significance ($P < 0.01$) when tumor volumes were compared between days 10 and 23.

**DISCUSSION**

There has been an ever increasing interest in evaluating the
efficacy of monoclonal antibodies to a variety of human tumor-
associated antigens for diagnosis and therapy of cancer. These
reagents have been extensively used as effective molecular
probes to delineate structural and functional properties of their
target antigens and in some cases as agents mediating cancer
therapy. During the last few years, Mabs directed to the car-
bohydrate moieties of glycolipids, particularly those containing
one or more sialic acid residues, i.e., gangliosides, have become
available to define these antigens on melanoma, neuroblastoma,
small cell lung carcinoma, and colon carcinoma as summarized
in several recent reviews (22–26). Furthermore, Mabs of IgG3
isotype directed to disialoganglioside GD2 were shown previ-
ously by this laboratory (27) and others (28–30) to mediate
both complement-dependent cytotoxicity and antibody-depend-
ent cellular cytotoxicity as well as to suppress tumor growth in
athymic mice (27) and in melanoma patients (30). As far as
disialoganglioside GD2 is concerned, a murine Mab of IgM
isotype was initially found to be useful in our laboratory for the
detection of GD2 in serum and monitoring of tumor progression
in neuroblastoma patients (7). More recently, this same anti-
body bound to immunobeads was used successfully for bone
marrow clearance of such patients.\(^5\) Others reported several
murine Mabs to GD2, i.e., three of IgM and one of IgG3 isotype
(8). One of the IgM antibodies was used successfully together
with complement to purge tumor cells from autologous bone
marrow of neuroblastoma patients (31) while in a recent prelimi-
nary study, an anti-GD2 Mab of IgG3 isotype was reported to
produce a complete tumor regression in one of four neuroblas-
toma patients (11).

Although these results are encouraging because they suggest
that GD2 may be an effective target for immunotherapy of
neuroblastoma, it is apparent that additional trials involving
more patients and possibly also more sophisticated treatment
modalities making use of this target antigen will be required to
optimize therapy regimens and to render them more uniformly
successful. To achieve this goal, a necessary first step is to
determine the specificity and functional properties of monoclo-
nal antibodies directed to GD2.

Following this general line of reasoning, we determined first
the specificity and second some of the functional properties of
our anti-GD2 murine Mab 14.18 of IgG3 isotype. In initial
experiments, we observed a lack of expression of GD2 on neo-

rublastoma cells in spite of evidence indicating that GD2 is a
precursor of GD2 (26). This may be due to the fact that GD2 is
synthesized very rapidly in these cells so that it is difficult to
detect reasonable amounts of GD2 on their surface. It is also
apparent from the results reported here that Mab 14.18 reacts
strongly on ELISA with all cell lines of neuroectodermal origin,
*i.e.*, neuroblastoma, melanoma, glioma, and possibly small cell
cancer of the lung. The results from immunoperoxidase assays
of fresh frozen normal and tumor tissues corroborate these
results as Mab 14.18 reacted solely with tissues of neuroecto-
dermal origin, including among normal tissues only cerebellum
and fetal brain. However, it is relatively unlikely that this Mab
will actually react to any extent with brain tissues in vivo because
of the blood brain barrier. The complete lack of reactivity of
Mab 14.18 with other normal adult and fetal tissues is advan-
tageous, particularly when one considers using this antibody
for immunotherapy. It is likely that this antibody may also
prove useful for adjuvant type therapy of melanoma and small
cell lung carcinoma. In this regard, another advantage of GD2
is that this ganglioside, when shed into the circulation, does
not form immune complexes with antibody. In fact, GD2 present
in serum must be extracted with organic solvents and dried
down before it can react with anti-GD2 antibody (7).

As far as the functional properties of Mab 14.18 are con-
cerned, it is evident from the data presented here that this
antibody effectively mediates CDC in the presence of human
complement (Fig. 3) and that this activity varies to some degree
among different neuroblastoma cell lines. This is probably
caused by the difference in average number of binding sites per
cell for Mab 14.18 as indicated in Table 3. In 5 of 6 neuroblas-
toma cell lines analyzed, there is good correlation between the
number of binding sites per cell and the percentage of CDC
shown in Fig. 3. Mab 14.18 (IgG3) is also effective in mediating
ADCC with human peripheral blood mononuclear cells as
effectors against several neuroblastoma cell targets. Again
there is some variability in this reactivity among the neuroblas-
toma cell lines tested. Similar to CDC, the degree of ADCC-
mediated tumor cell lysis also correlates with the average
number of binding sites expressed per neuroblastoma cell (Table 3).
Taken together, data on the functional properties of Mab 14.18
indicate that this Mab can effectively mediate neuroblastoma
cell lysis *in vitro* by two distinct mechanisms, *i.e.*, CDC and
ADCC.

Results from our *in vivo* studies in athymic (nu/nu) mice,
inoculated s.c. with SK-N-AS human neuroblastoma cells, dem-
emonstrated that repeated i.p. injections of Mab 14.18 clearly
produced a measurable suppression of tumor growth. This was
evident when a definite suppression of tumor establishment was
observed that was statistically significant ($P < 0.01$) whenever
Mab 14.18 was repeatedly injected i.p. into mice, starting 24 h
after they were inoculated s.c. with human neuroblastoma cells.
It is apparent from the data that this suppression of tumor
establishment is specifically achieved with Mab 14.18 (anti-
GD2) since injection of animals with Mab C281 (anti-GD3), an
irrelevant antibody of identical IgG3 isotype, is ineffective since
its GD3 target is not expressed to any measurable extent on SK-
N-AS human neuroblastoma cells. In terms of a possible clinical
applicability of Mab 14.18, it is relevant that in comparison to
the irrelevant Mab C281 (anti-GD3), Mab 14.18 (anti-GD2) at
least partially suppressed the growth of progressively growing
human neuroblastoma tumors that were allowed to establish
themselves as palpable 40- to 100-mm\(^3\) lesions for 9 days before
any antibody was injected into the nude mice. This partial
suppression of tumor growth was statistically significant ($P <
0.01$). It would seem reasonable to assume that *in vivo*, both
CDC- and/or ADCC-like mechanisms may be involved in the
suppression of tumor growth, especially in view of data pub-
lished on other human tumor systems (31–33).

One can only speculate at this time whether monoclonal
antibodies are required to evoke CDC and/or ADCC to be
useful in the treatment of neuroblastoma; however, from the
relatively few data available from phase I clinical trials with
melanoma (30) and neuroblastoma (11) patients, one would
assume this to be the case, especially when the injection of
monoclonal antibody *per se* is the sole therapy regimen. In this

\(^5\) R. C. Seeger, personal communication.
ACKNOWLEDGMENTS

The authors wish to thank Regina A. Derango, Marc Allen, Lillian Ramos, and David Lauffer for their expert technical assistance. The preparation of the manuscript by Bonnie Pratt Filiault is gratefully acknowledged.

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Disialoganglioside $G_D^2$ on Human Neuroblastoma Cells: Target Antigen for Monoclonal Antibody-mediated Cytolysis and Suppression of Tumor Growth

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