Disialoganglioside GD2 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 GD2, the major ganglioside expressed on the surface of human neuroblastoma cells. This monoclonal antibody (Mab) is of immunoglobulin G3 isotype, has an affinity constant (Kd) of 3.5 x 10^9 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 cell of 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 Mabs4 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 GD2 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-GD2 murine Mabs of IgG3 isotype were used to demonstrate that GD2 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-GD2 Mab of IgG3 isotype was reported to produce a prolonged remission in a neuroblastoma patient (11).

We report here on the reactivity of anti-GD2 Mab 14.18 (IgG3) 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. Nelson, Memorial Sloan-Kettering Cancer Center, New York. SMS-KCNR, 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 Melur 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. Didler, 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. J. Minna and A. Gazdar, NIH, Bethesda, MD. The pancreatic carcinoma cell lines FG and SG were provided by Dr. S. Kaji 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-O3); 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 x 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' spleens 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, amaminopterin, and thymidine and subcloned by limiting dilutions.

Ascites Preparation. BALB/c mice were primed by injecting 0.5 ml pristane. Two weeks after priming, 10-15 x 10^6 hybridoma cells were
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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 bound to 96-well microtiter plates (Dynatech, Alexandria, VA). The murine monoclonal antibody 14.18 is of IgG3 isotype as determined by analysis with these murine isotype standards.

ELISA. Screening of Mab by ELISA was done essentially as described previously (14). Briefly, 5 × 10^4 target cells/well were plated in polyvinyl microtiter plates (Dynatech). Prior to ELISA, the dried plates were rehydrated by washing them twice with 10 mM PBS, pH 7.4, containing 1.0% 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 at Scripps Clinic, 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.

Immonoperoxidase 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 at room temperature, after which the TLC plates were allowed to dry. The chromatograms were then sprayed with resorcinol reagent to visualize the gangliosides.

Inmunostaining 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 Chereshe et al. (17).

Lipid ELISA. The lipid ELISA was performed essentially as described earlier (17), 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 G\textsubscript{D\textsubscript{3}} 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 2 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, the Sephadex G-25 column (Pharmacia), was used to separate the bound, 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 radiiodine was determined by trichloroacetic acid precipitation.

Scatchard Plot Analysis. The specific activity of the radiolabeled antibody was 2 × 10^4 cpm/ng. The association constant (Kₐ) of Mab 14.18 was determined from Scatchard plot analysis of saturation binding data (19). Briefly, NBMB-7 neuroblastoma cells (10⁶/0.1 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 Beaumier et al. (20). Radioactivity in each fraction was determined in an 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)²⁵CrO₄ (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 10⁶ 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 were 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 μCi of (Na)251CrO4 for 2 h at
37°C. After the cells were washed twice with RPMI 1640, 5 × 10^5 cells
in 25 μl were plated in 96-well microtiter plates. Mab 14.18 (5 μg in
50 μl) 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 × g for 5 min and radioactivity was
measured in a 100-μl aliquot of each supernatant. Spontaneous 51Cr
release was determined in wells that contained only labeled target
cells. Total cpm release was determined by lysing tumor cells with 1%
Nonidet P-40. The percentage of lysis was calculated by the same
formula as that used to compute complement-dependent cytotoxicity.
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 × 10^6 M⁻¹,
as determined by Scatchard plot analysis. The antibody reacted
very strongly with all 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
against 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. 14 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).

Immunological 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-
munostaining, 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 ganglioside
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

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<th>Absorbance*</th>
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<tr>
<td>Neuroblastoma</td>
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<td>LAN-1</td>
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<td>LAN-5 Duke</td>
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<tr>
<td>SMS-KCNR</td>
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<td>CHP-134</td>
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<td>SMS-KAN</td>
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<td>NMB-7</td>
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<td>IMR-6</td>
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<td>SK-N-AS</td>
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<td>A375 P</td>
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<td>M-14</td>
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<td>Glioma</td>
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<td>U138 M6</td>
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<td>Small cell lung carcinoma</td>
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<td>T293</td>
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<tr>
<td>NIH-N417</td>
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<tr>
<td>NIH-H-82</td>
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<td>NIH-H-69</td>
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<td>Other tumor cell lines</td>
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<td>Pancreatic carcinoma (fast growing)</td>
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<td>Pancreatic carcinoma (slow growing)</td>
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<td>Adenocarcinoma of lung (UCLA-P3)</td>
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<td>Lymphoblastoid cell lines</td>
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<td>LG-2 (B-cell)</td>
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<td>MOLT-4 (T-cell)</td>
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<td>HPB-ALL (T-cell)</td>
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Table 2 Tissue reactivity of Mab 14.18

<table>
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<th>Reactivity*</th>
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<tbody>
<tr>
<td>Tumor tissues</td>
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<td>Neuroblastoma</td>
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<td>Melanoma</td>
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<td>Glioblastoma</td>
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<td>Small cell lung carcinoma</td>
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<td>Ewing's sarcoma</td>
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<td>Lung adenocarcinoma</td>
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<tr>
<td>Stomach carcinoma</td>
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<td>Squamous lung carcinoma</td>
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<tr>
<td>Breast carcinoma</td>
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<tr>
<td>Fibroblastoid</td>
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<td>Colon cancer</td>
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<td>Oral squamous cancer</td>
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<td>Normal tissues</td>
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<td>Lung</td>
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<td>Colon</td>
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<td>Liver</td>
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<td>Kidney</td>
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<td>Cerebellum</td>
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<td>Fetal tissues</td>
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<td>Lung</td>
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<td>Kidney</td>
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<td>Brain</td>
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* Absorbance at 492 nm: 0.15-0.3 (+); 0.3-0.6 (++); 0.6-0.9 (+++); 0.9->2.0
(++++); 0-0.050 (−).

* ++, strongly positive; +++, positive; +, weak/positive; −, negative.
neuroblastoma cell lines by measuring their binding reactivity with Mab 14.18. Table 3 summarizes the results obtained indicating the percentage of positive cells and the mean index of fluorescence, a measure of relative antigen density as well as the number of binding sites of Mab 14.18 per cell in different neuroblastoma cell lines. It is apparent that these cultured neuroblastoma cells differ considerably in antigen density as revealed by mean indices of fluorescence ranging from 52 to 101 as well as in the number of actual antigen sites varying from 110,000 to 267,000 per neuroblastoma cell.

CDC Mediated by Mab 14.18. In order to assess whether GD2 serves as an effective target antigen for complement-mediated cytolysis, six neuroblastoma cell lines, each labeled with (Na)251CrO4, were incubated with Mab 14.18 together with human serum as a source of complement. The anti-GD2 antibody 14.18 mediated CDC ranging from 50 to 95% among the six cell lines tested at antibody concentration ranging from 5 to 10 μg/ml (Fig. 3). A separate experiment, designed to study the kinetics of Mab 14.18-induced CDC, indicated that significant killing occurs as early as 30 min; however, maximum killing (>95%) was observed after 2 h incubation (Fig. 4). Lysis was not detected with either active human complement, combined with irrelevant (nonbinding) antibody of IgG3 isotype or active antibody (14.18) used in conjunction with heat-inactivated human complement (data not shown).

Mab 14.18 Mediates ADCC of Human Effector Cells with Human Neuroblastoma Cells. To determine whether Mab 14.18 can induce ADCC of normal human peripheral blood mononuclear effector cells, three neuroblastoma cell lines (SMS-KCN, IMR-6, and SK-N-AS), each labeled with (Na)251CrO4, 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-KCN, 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 × 10^6 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...
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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 (O); IMR-6 (O).

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 (O) 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-GD1); C, Mab 14.18 (anti-G02). Tumor cells were inoculated s.c. 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-GD1); E, Mab 14.18 (anti-G02). 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-G02), Mab C281 is of IgG3 isotype but is, however, directed to GD1, 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-G02) at the same dose level and time schedule as Mab C281 (anti-GD1). 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 indicated by the arrows by measuring three diameters (d1, d2, and d3) with graduated calipers, and tumor volume was calculated by the formula

\[ \frac{\pi}{6} (d_1 d_2 d_3) \]

Mab 14.18 Suppresses the Growth of Established Human Neuroblastoma Tumors in Athymic (nu/nu) Mice. Two groups of 6 athymic (nu/nu) mice were given s.c. injections of \( 9 \times 10^6 \) SK-N-AS neuroblastoma cells. At day 9 after the initial tumor cell inoculum, these animals were treated as follows. One group of mice received i.p. 200 µg each of irrelevant Mab C281 (anti-GD1) on days 10, 13, 15, and 18. Starting on day 10, all animals in this group rapidly began to develop large tumors that ranged in volume from 4500 mm³ when measured at the end of the experiment on day 23 (Fig. 6B). Finally, six mice received Mab 14.18 (anti-G02) at the same dose level and time schedule as Mab C281 (anti-GD1). 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 indicated by the arrows by measuring three diameters (d1, d2, and d3) with graduated calipers, and tumor volume was calculated by the formula

\[ \frac{\pi}{6} (d_1 d_2 d_3) \]

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comparison between tumor volumes of the control group receiving 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 carbohydrate 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 previously by this laboratory (27) and others (28–30) to mediate both complement-dependent cytotoxicity and antibody-dependent 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 antibody bound to immunobeads was used successfully for bone marrow clearance of such patients. 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 preliminary study, an anti-GD2 Mab of IgG3 isotype was reported to produce a complete tumor regression in one of four neuroblastoma 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 monoclonal 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 neuroblastoma 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 neuroectodermal 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 advantageous, 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 concerned, 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 neuroblastoma 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 effector cells against several neuroblastoma cell targets. Again there is some variability in this reactivity among the neuroblastoma 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, demonstrated 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-mm³ 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 published 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
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regard, additional modalities may prove to be useful for the treatment of neuroblastoma, such as those recently reported from nude mouse model studies for the treatment of melanoma, where human peripheral blood mononuclear cells, augmented ex vivo with recombinant interleukin 2, and then admixed and targeted with anti-Gd diluted antibodies, proved effective in suppressing the growth of established, progressively growing human melanoma tumors in nude mice (34). One may predict that future treatment of human neuroblastoma may involve this type of regimen combined with modalities involving conjugates of chemotherapeutic drugs and radionuclides with monoclonal antibodies directed to an effective tumor target on neuroblastoma cells such as disialo ganglioside Gd2.

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