ABSTRACT

A complete family of IgG isotype switch variant hybridomas was generated from the anti-GD2 monoclonal IgG3-producing hybridoma, 14.18, with the aid of the fluorescence-activated cell sorter. The IgG1, IgG2a, and IgG2a monoclonal antibodies (Mabs) produced by respective isotype switch variant hybridomas 14G1, 14G2a, or 14G2b, have binding activities for the biochemically defined GD2 antigen and GD2-expressing neuroblastoma target cell lines identical to that of IgG3 Mabs produced by the 14.18 parent cell line. This permitted us to examine the relative in vitro and in vivo cytotoxic capacities of each of the anti-GD2 antibodies for GD2-expressing neuroblastoma cells independent of antibody binding affinity or specificity. Mabs produced by 14.18, 14G2a, or 14G2b, but not 14G1, can direct efficient complement-dependent cytotoxicity against neuroblastoma tumor cells in the presence of human complement. Mabs produced by the parent 14.18 or by 14G2a are more efficient in directing antibody-dependent cell-mediated cytotoxicity than Mabs produced by 14G2b, and Mabs of 14G1 are inactive. However, despite these in vitro differences, antibodies produced by each member of this switch variant family suppress the growth of human neuroblastoma tumor cells in BALB/c athymic nu/nu mice. These studies suggest that a mechanism(s) other than Fc-directed complement-dependent cytotoxicity or antibody-dependent cell-mediated cytotoxicity may account for the in vivo antitumor effects of these particular antibodies.

INTRODUCTION

Earlier, we described a murine IgG3 monoclonal antibody, 14.18, that reacts with the GD2 ganglioside present on the cell surface of human neuroblastoma and melanoma. This Mab is highly specific for cell lines and tumor tissues of neuroectodermal origin that express GD2. That 14.18 may have clinical utility in the treatment of neuroectodermal neoplasms is indicated by our observations that this Mab can direct both ADCC and CDC against GD2-expressing cell lines (1). Furthermore, this Mab also can suppress the growth of human neuroblastoma and melanoma cells in athymic nu/nu mice (1, 2). The mechanism(s) involved in the suppression of tumor growth by this antibody is presently unknown.

In other tumor model systems, several investigators have demonstrated that the efficiency of such antibody-mediated in vitro and in vivo cytotoxic activities depends, in part, on the particular IgG subclass of the specific antitumor antibody. For example, antitumor antibodies of the IgG1 isotype, in contrast to antibodies of the IgG2a or IgG2b subclass, frequently are inactive in ADCC (3, 4) and CDC (5), when tested with human effector cells and human complement, respectively. Antibodies of IgG1 isotype are also ineffective in suppressing tumor growth in vivo (6, 7). In this regard, Mab 14.18 (IgG3), although highly effective in directing ADCC and CDC with human effector cells and human complement against human tumor cell lines expressing GD2 (1), failed to show any such activity in the presence of murine spleen cells or murine complement. However, this same antibody effectively suppressed human tumor growth in nude mice (1). These findings raised the question as to whether the antigen-binding domain of Mab 14.18 is more critical for this antitumor effect than its Fc portion. It is quite difficult to address this issue with (Fab')2 fragments, since it is impossible to prepare them from murine antibodies of IgG3 isotype (8), and also because the in vivo half-life of these fragments is considerably less than that of whole IgG.

Consequently, in order to investigate mechanism(s) whereby anti-GD2 antibodies mediate in vivo suppression of tumor growth, we prepared an isotype switch variant family of hybridomas from 14.18. Switch variant hybridomas express a heavy chain isotype different from that of the parent hybridoma, but retain expression of the same heavy chain variable region and light chains (9). Mabs produced by members of such switch variant families have identical reactivities with antigens and differ only in isotype, thus permitting a comparative study of the biological activities of each immunoglobulin isotype independent of other antibody variables (10). This report describes our study of the relative in vitro and in vivo cytotoxic potentials of IgG3, IgG1, IgG2b, and IgG2a Mabs produced by members of a switch variant family derived from 14.18.

MATERIALS AND METHODS

Cell Lines and Hybridomas. The human neuroblastoma cell line, SMS-KCNR, was provided by Dr. P. Reynolds, UCLA Medical Center. The IMR-6 and NMB-7 human neuroblastoma cell lines are from Dr. S. K. Liao, McMaster University, Hamilton, Ontario, Canada. IgG3-producing hybridomas 14.18 and MB3.6 were generated in our laboratory (1, 2). Hybridoma MB3.6 produces a Mab of IgG3 isotype, specific for a ganglioside, GD2 that is not expressed by human neuroblastoma cells (11).

Isolation of Isotype Switch Variants. A family of isotype switch variants was selected from the IgG3-producing hybridoma, 14.18, with a fluorescence-activated cell sorter, as described (12). One isotyping kit was from Southern Biotechnology Associates, Birmingham, AL, which was used to test spent culture fluids. The other isotyping kit was from ICN Immunobiologicals, Costa Mesa, CA, and consisted of radial immunodiffusion plates which were used to test ascites and purified antibody. Initially, 14.18-derived hybridomas expressing surface IgG1

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were selected by sorting for cells labeled with fluorescein-conjugated goat antibodies specific for the mouse IgG1 isotype. After two rounds of sorting, the 14.18-derived population was enriched sufficiently in IgG1 variants to allow for single cell cloning by limiting dilution. Culture supernatants of individual clones were assayed by enzyme-linked immunosorbent assays for IgG1 anti-GD3 activity. Through this, we isolated the IgG1 switch variant hybridoma, 14G1. After 5 weeks, culture to allow for accrual of spontaneous IgG2 switch variants, 14G1-derived hybridomas expressing surface IgG2a or IgG2b were selected by sorting for cells labeled with fluorescein-conjugated goat antibodies specific for mouse IgG2. From IgG2-enriched populations, hybridomas expressing exclusively IgG2a or IgG2b were cloned and designated 14G2a or 14G2b, respectively. Estimated frequencies at which spontaneous isotype switch variant hybridomas arose within the 14.18 or 14G1 population were comparable to those described previously (12).

Animals. BALB/c athymic (nu/nu) mice were obtained from the nude mouse colony at the National Cancer Institute, Bethesda, MD.

Radio labeling of Monoclonal Antibodies. Antibodies were labeled as described (13). Briefly, 150 μg antibody were incubated for 25 min on ice with 2.5 μCi of [125I]NaI in polyethylene tubes coated with 50 μg of Iodo-Gen reagent (Pierce Chemical Co., Rockford, IL). Unincorporated [125I] was removed by gel filtration on PD 10 columns (Pharmacia, Piscataway, NJ). The specific activity was generally 2 x 10^6 cpm/ng antibody.

Scatchard Plot Analysis. NMB-7 neuroblastoma cells (1 x 10^6) were suspended in ice-cold RPMI 1640 medium containing 1% bovine serum albumin and [125I]-labeled antibody at concentrations ranging from 25-1300 ng. Following a 2-h incubation at 4°C cells were washed in RPMI 1640 medium containing 1% bovine serum albumin and nonspecific binding was determined in the presence of 500-fold excess of cold antibody. All analyses were done in duplicate in a γ scintillation counter as described (13) and the data were analyzed by the method of Scatchard (14). In this regard, we considered in this study not only the steep slope of the binding curve to calculate the number of high affinity GD3-binding sites as was done previously (1), but also took into account the more extended slope of this curve. This resulted in the calculation of both high and low affinity GD3-binding sites on NMB-7 neuroblastoma cells.

Ganglioside Extraction. The method is as described (1). Briefly, 1 ml of packed neuroblastoma cells were washed extensively with PBS and homogenized in chloroform:methanol (2:1). The residue was reextracted with chloroform:methanol (1:1), passed through a glass scintillation funnel, and then reextracted with this same solvent. The extracts were combined, dried under nitrogen, and partitioned in diisopropl ether: normal butanol (6:4) containing 50 mM NaCl as described by Ladish and Gillard (15).

Thin Layer Chromatography. Plastic-backed silica gel TLC plates (E. 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:0.2% aqueous CaCl2 (55:45:10). 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. The reactivity of Mab 14.18 and its isotype switch variants with gangliosides separated by TLC was determined by the immunostaining method described by Chereshef et al. (16).

Suppression of Tumor Growth in Vivo. NMB-7 neuroblastoma cells (1.2 x 10^7) were injected s.c. into 35 BALB/c athymic (nu/nu) mice. These animals were subdivided into 7 groups which included 2 control groups in which mice were given injections i.v. of either PBS or 200 μg of anti-GD3 monoclonal antibody MB3.6 (negative control). The other 5 groups of animals were given injections of either 200 μg of anti-GD3 monoclonal antibody 14.18 (IgG3) or its isotype switch variants, namely 14G1, 14G2b, and 14G2a. The last group of mice received a combination of monoclonal antibodies 14G2a, 14G2b, and 14.18. All mice receiving monoclonal antibodies were given injections of 200 μg Mabs each of days 2, 5, 8, 11, 14, 17, 23, 26, 29, 32, 35, 38, 41, and 44. Tumor volumes were measured every 4 days with graduated callipers by measuring three dimensions (d1, d2, and d3) and tumor volume was calculated by the formula: V = (d1 x d2 x d3) / 2. The mice in both control and experimental groups were sacrificed 83 days after the initial s.c. injection with neuroblastoma cells. These animals were dissected and examined for residual tumors.

Complement-dependent Cytotoxicity. Neuroblastoma cells suspended in 1 ml of growth media were labeled with 100 μCi of [51Cr] (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, each containing 10^5 cells, were plated in 96-well microtiter plates (Costar, Cambridge, MA) with various concentrations of Mab 14.18 and its isotype switch variants. The plates were 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 x g for 5 min. The cpm of [51Cr] in the supernatant were measured in a γ scintillation counter. The total counts were assessed after adding 1% Nonident P-40. Percentage of specific lysis was calculated as follows:

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

Antibody-dependent Cellular Cytotoxicity. Neuroblastoma cells NMB-7 were labeled with 300 μCi of [51Cr] in ml of RPMI 1640 growth medium for 2 h at 37°C. After washing the cells twice with this medium, 5 x 10^5 cells in 25 μl were plated in 96-well microtiter plates. Mab 14.18 and its isotype switch variants (5 μg in 50 μl) were each added to respective wells containing tumor target cells. Human peripheral blood mononuclear effector cells were isolated by density gradient centrifugation on Ficoll-Hypaque. These cells were added to the respective microtiter wells at the effectortarget cell ratios of 1:200, 1:100, and 1:50. The plates were incubated for 4 h at 37°C and then were spun at 1000 x g for 5 min. A 100-μl aliquot of each supernatant was measured for radioactivity. Spontaneous [51Cr] release was determined in wells that contained only labeled target cells. Total cpm release was determined by lysing tumor cells with 1% Nonident P-40. The percentage lysis was calculated by the same formula as that used to compute complement-dependent cytotoxicity. Percentage of specific lysis was derived by subtracting the percentage of lysis obtained with effector cells in the absence of antibody, i.e., natural killer cell lysis, from each value obtained.

RESULTS

Generation of Isotype Switch Variants. The isotype switch variants were selected from the original, parental IgG3-producing hybridoma 14.18. Each of these switch variants expressed only one subclass of surface immunoglobulin as determined by fluorescence-activated cell sorter analysis with fluorescein conjugated antibody specific for each of the various subclasses. Each hybridoma population expressed only the immunoglobulin subclass corresponding to the isotype of the antibody it secreted. Consistent with this purity of each antibody population, ascites produced from each hybridoma contained >90% of the murine immunoglobulin corresponding to the isotype of the hybridoma injected into mice to produce ascites. The isotype switch variants when tested repeatedly with the two isotyping kits were found stable without any significant reversion of the original subclass during passage and antibody production in tissue culture for more than 1 year.

Immunological and Immunochimical Characterization of Mab 14.18 (IgG3) and Its Isotype Switch Variants. The association constant (kA) of Mab 14.18 and its isotype switch variants, 14G1, 14G2b, and 14G2a were 3.5, 3.4, 1.0, and 1.5 x 10^8 M^-1, respectively, and the average number of binding sites per cell for the NMB-7 human neuroblastoma cell line were in the order of 10-12 x 10^6 per cell. A typical binding analysis and Scat-
To confirm that the isotype switch variants of this antibody are directed against the GD2 antigen, we immunostained the gangliosides separated on TLC with Mabs 14.18, 14G1, 14G2b, and 14G2a or Mab MB3.6. The TLC pattern of gangliosides found within an extract obtained from neuroblastoma cell line NMB-7 is shown in Fig. 2. GD2 appears to be the major ganglioside present in the extracts of these cells as demonstrated by visualization of the total ganglioside profile with resorcinol. Mab 14.18 and its isotype switch variants are shown to bind to a component that comigrates with authentic GD2 (Fig. 2, Lanes B, C, D, and E), whereas the TLC plate immunostained with anti-GD3 Mab MB3.6 (negative control) was without bands (data not shown). The NMB-7 neuroblastoma cells express Go2 as a doublet. It has been shown previously that such doublets in gangliosides are due to identical sugar moieties on ceramides containing fatty acids of variable chain length (17). Alternatively, these doublets could also be explained by the formation of lactones (18).

Suppression of Neuroblastoma Tumor Growth in Athymic (nu/nu) Mice by Mab 14.18 and Its Isotype Switch Variants. When nude mice were given injections s.c. of 1.2 × 10⁷ NMB-7 neuroblastoma cells, tumor growth was observed in both control groups. All control mice that received PBS had large tumors that were detected grossly 16 days after the initial tumor inoculation (Fig. 3A). Similarly, all animals that received 200 µg of anti-GD2 Mab MB3.6 (IgG3) developed gross tumors that were detected 18 days after the initial tumor inoculation (Fig. 3B).

However, all animals in the 4 groups of 5 mice each that received IgG3, IgG1, IgG2b, or IgG2a anti-GD2 antibodies were without detectable tumor 83 days after the initial tumor cell inoculation (Fig. 3C). The experimental results depicted in Fig. 3, A, B, and C were repeated in two separate experiments. In another experimental group given injections of a cocktail of all anti-GD2 Mabs, only 1 of 5 mice developed a tumor. This tumor was detected 28 days after the initial tumor cell injection (Fig. 3D). The volume of this tumor measured 11,000 mm³ on the 73rd day of the experiment when the animal was sacrificed. These observations indicate that Mabs of each member of the 14.18 switch variant family can suppress the growth of human NMB-7 neuroblastoma tumors in nude mice.

Analysis of CDC of Monoclonal Antibodies. Neuroblastoma
cell lines NMB-7, SMS-KCNR, and IMR-6 were labeled with Na$_2$CrO$_4$ and incubated with various concentrations of monoclonal antibodies 14.18, 14G1, 14G2b, and 14G2a in the presence of human complement (150 μl of 1:3 diluted fresh human serum) (Fig. 4). At antibody concentrations ranging from 5–60 μg/ml, Mabs 14.18, 14G2b, and 14G2a each direct efficient CDC ranging from 60–95%. In contrast, Mab 14G1 directs CDC poorly, ranging from 4–37%. The value of 37% was only reached with the NMB-7 cell line and then only at the highest concentration of Mab 14G1, i.e., 80 μg/μl. It is apparent from the data shown in Fig. 4 that antibody added in excess of 20 μg/ml apparently saturated the systems and thus it is likely that in the case of Mab 14G1, the 37% lysis reached at an antibody concentration of 80 μg/ml is nonspecific. There is no evidence of any tumor cell lysis by these Mabs when the assay is performed in the presence of inactivated human complement (data not shown).

Antibody-dependent Cellular Cytotoxicity with Human PMBCs as Effector Cells. Neuroblastoma cells (NMB-7), labeled with Na$_2$CrO$_4$ were incubated with 1 μg/well of Mab 14.18, 14G1, 14G2b, or 14G2a. Human PBMC were added at E:T ratios ranging from 50:1 to 200:1. Mab 14.18 directs lysis of 48% of NMB-7 neuroblastoma target cells with an E:T ratio of 200:1 (Fig. 5). With the same E:T ratio and antibody concentration, Mabs 14G1, 14G2b, or 14G2a direct lysis of 8, 37, and 54% of NMB-7 cells, respectively. With lower E:T ratios of 100:1 or 50:1, Mabs 14.18 and 14G2a direct 25–37% specific lysis. These data indicate that 14G2a directs ADCC with efficiency comparable to the parent Mab 14.18 (IgG3). Mab 14G2b is less effective at the lower E:T ratios of 100:1 and 50:1, whereas Mab 14G1 is inactive.

**DISCUSSION**

In this study we compared some of the functional properties of murine anti-GD$_2$ Mab 14.18 (IgG3) and its isotype switch variants 14G1, 14G2b, and 14G2a. Our major aim was to elucidate possible mechanisms of action involved in the antitumor effects produced by these antibodies. Our initial approach was to determine whether any correlation exists between the antitumor effects of these antibodies and their in **vitro** functions such as directing ADCC or CDC.

First, we established that Mabs produced by each member of the 14.18 switch variant family recognized the same chemically defined GD$_2$ antigen and bound a similar number of sites on the human neuroblastoma cell line NMB-7.

Next, we examined the in **vivo** antitumor effects of Mab 14.18 and its three isotype switch variants. We found that all four monoclonal antibodies were effective in suppressing neuroblastoma tumor establishment in nude mice (Fig. 3). Thus, we could not detect a correlation between the efficiencies of these Mabs in directing ADCC or CDC and their relative capacities to suppress the growth of human neuroblastoma xenografts in nude mice.

Since these in **vivo** experiments indicated that Mab 14.18 (IgG3) and its isotype switch variants are all equal in suppressing neuroblastoma tumor establishment in nude mice, we were interested to examine some of the possible mechanisms that may be involved in this phenomenon. To this end, we compared the capacities of the different isotype switch variants to direct CDC in the presence of added human complement. Despite identical binding activities, only Mabs 14.18, 14G2b, and 14G2a can direct efficient CDC with human complement. Mab 14G1 directs at most 37% CDC of only NMB-7 cells at the highest antibody concentration (80 μg/ml) tested. By comparison, the anti-GD$_2$ antibodies bearing the other three IgG isotypes can effectively mediate CDC at all concentrations and with all three cell lines tested, ranging in specific lysis from 60 to 95% (Fig. 4). These findings are not completely unexpected since of the various murine Mabs against human tumor-associated antigens tested, only those with isotypes of IgG3 (1, 2, 19), IgG2b, and IgG2a (20) were reported effective in directing CDC with human complement.

We also compared the different Mabs in their ability to direct ADCC with human peripheral mononuclear effector cells. Again, Mabs 14.18, 14G2a, and 14G2b direct this activity effectively; however, 14.18 and 14G2a Mabs may be more efficient than 14G2b at lower E:T ratios. Mab 14G1, on the other hand, is ineffective in directing significant ADCC with human effector cells. This is essentially in agreement with findings reported previously by Hellstrom et al. (3), Imai et al. (4), and Kipps et al. (9), who noted far greater effectiveness of murine monoclonal antibodies of IgG2a isotype than those achieved with Mabs of IgG1 isotype directed against the same antigen when using human PBMCs as effector cells in ADCC against human cell lines.

Although the in **vivo** assays were performed with human effector cells, this should not detract from the discrepancies between our in **vitro** and in **vivo** results. Human complement and effector cells were used because murine complement and murine effector cells are not efficient in mediating CDC or ADCC directed by Mabs 14.18, 14G2b, and 14G2a and are ineffective in these assays with Mab 14G1 (data not shown). It
is also known from the work of Hellström et al. (3) that human peripheral lymphocytes function better than mouse splenocytes as a source of effector cells in the presence of murine Mabs. Our data on CDC and ADCC substantiate the functional purity of the isotype switch variants used in this study since it is well known that mouse immunoglobulins of IgG1 isotype usually fail to mediate CDC (5) and ADCC (3, 4).

Functional properties of antitumor Mabs other than isotype may play a key role in determining their in vivo antitumor activities. Key among these may be the type of target antigen and its biological activity. Ganglioside GD2 and GD3 may be important for the attachment of neuroblastoma and melanoma cells to extracellular matrix proteins since anti-GD2 monoclonal antibodies inhibit attachment of neuroblastoma tumor cells to several substrate adhesive proteins (21). Moreover, GD2 exists in a divalent cation-dependent functional complex with arginine-glycine-aspartic acid-directed receptors on the surface of human melanoma and neuroblastoma cells (21, 22). It is possible that anti-GD2 Mabs may suppress the establishment of neuroblastoma tumors in nude mice by inhibiting cell-substratum interactions.

Clinically, Mabs against GD3 and GD2 gangliosides have produced partial regressions in malignant melanoma (19, 23) and neuroblastoma (24) tumors in patients. Perhaps such partial regressions are secondary to the administered Mab affecting tumor cell adhesion to extracellular matrix proteins. However, mechanisms of CDC and ADCC also may partially account for some of these responses as reported by Hersey et al. (25, 26). Studies testing the relative efficacy of some members of the 14.18 switch variant family in the treatment of neuroblastoma and melanoma are in progress that ultimately may resolve this issue.

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Functional Properties and Effect on Growth Suppression of Human Neuroblastoma Tumors by Isotype Switch Variants of Monoclonal Antiganglioside GD2 Antibody 14.18

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