Binding Activities and Antitumor Properties of a New Mouse/Human Chimeric Antibody Specific for GD2 Ganglioside Antigen

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Abstract

Purpose: We previously generated a mouse monoclonal antibody (mAb) specific for the tumor-associated GD2 ganglioside antigen. Here, we describe the development of a chimeric anti-GD2 mAb for more effective tumor immunotherapy.

Experimental Design: We cloned the cDNA encoding the immunoglobulin light and heavy chains of the 60C3 anti-GD2 mAb, and constructed chimeric genes by linking the cDNA fragments of the variable regions of the murine light and heavy chains to cDNA fragments of the human κ and γ1 constant regions, respectively.

Results: The resultant chimeric anti-GD2 mAb, c.60C3, showed identical binding affinity and specificity to that of its murine counterpart. Both c.60C3 and 60C3 were rapidly internalized by tumor cells at 37°C. When human serum and human natural killer cells were used as effectors in complement-mediated cytotoxicity and antibody-dependent cell cytotoxicity, respectively, c.60C3 was more effective in killing GD2-expressing tumor cells. However, c.60C3 was ineffective at inducing cell death by apoptosis, although binding of 60C3 induced apoptotic death in vitro. In an in vivo, GD2-expressing, syngeneic tumor model, i.v. injection of c.60C3, but not 60C3, significantly suppressed tumor growth in mice (P < 0.0005).

Conclusion: Immune effector functions mediated by this antibody and its potentially reduced immunogenicity make chimeric c.60C3 a promising therapeutic agent against neuroectodermic tumors.

Gangliosides are sialic acid–bearing glycosphingolipids that are expressed in variable amounts on the surface of all mammalian cells (1). Human neuroectodermal tumors, such as melanoma, glioma, neuroblastoma, and small cell lung carcinoma express large amounts of the GD2 ganglioside (2–5), which, in contrast, is only expressed at very low levels in the peripheral nervous system (6) and the cerebellum (7). Ganglioside GD2 also seems to be involved in several biological functions (reviewed in ref. 8), such as cell recognition (9), cell matrix attachment (10), and cell growth and cell differentiation (11), suggesting that tumor-associated GD2 gangliosides may play a significant role in the tumorigenic phenotypes of these cells.

Several murine and mouse/human chimeric anti-GD2 monoclonal antibodies (mAb) have been generated (12–18); two of which, the murine 3F8 and the mouse/human chimeric Ch14.18 antibodies, are under evaluation in clinical trials (19–21), with significant measurable regressions of neuroblastoma and melanoma in several patients (19, 21–23). Ganglioside GD2 on tumor cell surfaces was shown to be a relevant target antigen for antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC; refs. 16, 17, 24, 25). Other mechanisms contributing to the antitumor effect of passive immunotherapy with GD2 tumor-specific mAbs involve the anti-idiotypic network (26), and direct apoptotic cell death induction (11, 27). These findings suggest the usefulness of anti-GD2 antibodies or their derivatives in therapy of human tumors of neuroectodermal origin. However, at present, there is relatively little information regarding analysis at the cellular level of the interaction between anti-GD2 antibodies and target cells. In particular, the fate of anti-GD2 antibody-antigen complexes on the cell surface remains to be investigated in order for efficient application of anti-GD2 antibody to be achieved.

We previously generated several murine anti-disialogangliosides mAbs and detailed the fine specificity and the immune repertoire used in anti-disialoganglioside responses (12). One of the anti-disialoganglioside IgG3 isotype mAbs generated was...
specific for ganglioside GD2 and has potential for passive immunotherapy of human cancer. However, mouse mAbs are highly immunogenic in humans, and for this reason, their therapeutic potential in patients is greatly limited (28). In order to reduce the immunogenicity, humanization by genetic engineering, producing mouse/human chimeric antibodies for example, has been one solution to this problem (29). Therefore, we decided to generate the mouse/human chimeric antibody, c.60C3, by linking cDNA sequences encoding the light and heavy chain–variable region of 60C3, with the cDNA encoding human κ and γ1 constant regions. The antitumor effects of chimeric c.60C3 and mouse 60C3 were analyzed in vitro in terms of CDC, ADCC, and apoptosis induction. We also investigated the internalization of these antibodies and finally showed that chimeric c.60C3 showed antitumor activity against GD2-expressing tumors in a syngeneic murine model.

Materials and Methods

Cell lines and hybridomas. The murine 60C3 mAb (IgG3, κ) specific for GD2 established in our laboratory was used for the isolation of c.60C3 heavy and light chains (12). Chinese hamster ovary (CHO) cells were used for all transfectants with chimeric antibody constructs (American Type Culture Collection). GD2-expressing cells, IMR32, a human neuroblastoma cell line, and EL4, a murine T-lymphoma cell line, were purchased from American Type Culture Collection. A murine neuroblastoma cell line, not expressing GD2, Neuro-2a (American Type Culture Collection), was used as a negative control. A hybridoma producing a human antibody against P1 antigen, P3Non2 (IgM, κ) kindly provided by Dr. F. Vérité, EFS, Rennes, France) was used for the isolation of human Cκ-chain cDNA. A human myeloma LP1 cell line producing human Cγ1-chain (ref. 30; kindly provided by Dr. R. Bataille, U601 INSERM, Nantes, France), was used for the isolation of human Cγ1-chain cDNA. All cell lines (60C3, IMR32, Neuro-2a, LP1, and P3Non2) were grown in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (Sigma) and 2 mmol/L of L-glutamine (Sigma); EL4 and CHO cell cultures were maintained in DMEM supplemented with 4.5 g/L of glucose (Sigma), 10% heat-inactivated FCS (Sigma), and 2 mmol/L of L-glutamine (Sigma).

Cloning and sequencing of the murine VH and Vκ cDNAs. The VH- and the Vκ-region cDNAs were isolated from the 60C3 hybridoma cells by reverse transcription-PCR as already described (12). The primers used for the VH and the Vκ cDNA amplification were Vκ BamHI BACK (5’-AGGGATCCAAATTCAAAAGAAGATTGAT) and Vκ XhoI FOR (5’-TTCAGCTGAGCTTGTGCCCAGCCAC), VH BamHI BACK (5’-TCCGATCCGAACACGTCTGCTAACCAGT) and Vκ NheI FOR (5’-TCTAGCTGAGAGACAGCAGGACTGAG). An XhoI site was created near the 5’ side of the murine Vκ cDNA for the light chain fusion. The human Vκ- and VH-chain cDNAs were cloned in pBlueScript SK II(+) (Stratagene) and sequenced. The nucleotide sequences of the cloned cDNAs were identical to the one previously reported (12).

Cloning and sequencing of the human Cγ1 and human Cκ region cDNAs. The human Cκ-chain cDNA was generated from the P3Non2 hybridoma cells, and the human Cγ1-chain cDNA was obtained from the human myeloma LP1 cell line by reverse transcription-PCR using primers with the following sequences: Cκ NheI BACK (5’-AGGGTCTCAGGCTGAAACGAACTGTGGCAG) and Vκ XhoI FOR (5’-TTCAGCTGAGCTTGTGCCCAGCCAC), Cκ XbaI FOR (5’-CGTCTTCATTATTTATCCTCGGACAGGAGAGACAG). For the heavy chain fusion, an NheI site was created at the 5’ side of the human Cγ1 cDNA. The human Cκ- and Cγ1-chain cDNAs were cloned in pBlueScript SK II(+) (Stratagene) and sequenced. The nucleotide sequence of the cloned human Cκ was identical to that of human Cκ cDNA (31). The nucleotide sequence of the cloned human Cγ1 cDNA was identical to that of human Cγ1 cDNA (32).

Construction of the chimeric genes. The Vκ and the human Cκ cDNAs were cloned into the pcDNA3 expression vector (Invitrogen Life Technologies) using specific restriction sites BamHI-XhoI and XhoI-XbaI, respectively. The resulting expression vector contained an 0.8 kb BamHI-XbaI fragment containing the L chain and was named pcDNA3/c.60C3-L. The VH and human Cγ1 cDNAs were cloned into the plBlueScript SK II(+) (Stratagene) plasmid using specific restriction sites BamHI-NheI and NheI-XbaI, respectively. The resultant cloning
vector was named pBluescript SK II (+)/c.60C3-H. The 1.6-kb Immuneeffector functions of chimeric c.60C3 and its mouse 60C3. Fig. 3. described in Materials and Methods and Results. of c.60C3 and 60C3 mAbs. Specifically, lysis was determined for the IMR32 cell line as mAbs with IMR32 cell line at an E/T ratio of 20:1 (n irrelevant mAb. Chimeric c.60C3 (n give the pcDNA3.1-Zeo/c.60C3-H. In these constructions, none of the the pcDNA3.1-Zeo expression vector (Invitrogen Life Technologies) to Xba construction was confirmed SDS-PAGE. c.60C3 was determined by measuring the absorbance at 280 nm and filtered (0.22 column (GE Healthcare) for affinity purification of mAbs. The eluted purified from culture supernatants by using a HiTrap Protein A FF effects were screened by ELISA. The chimeric antibody was affinity- dilution, and the supernatants of G-418- and Zeocin-resistant trans- genic-HCl buffer (pH 2.5), whereas the other was treated with PBS (pH 7.4) at 4 °C for 5 min. After centrifugation, total antibody binding was determined from the pellet of cells treated with PBS, whereas the membrane-bound and internalized fractions were determined, respectively, from the supernatant and pellet of cells treated with the glycine-HCl buffer.

Flow cytometric analysis. In indirect immunofluorescence, the tumor cells (1 × 10^3 cells) were incubated with purified antibodies (10 μg/mL) for 45 min at 4 °C. After reaction with FITC-labeled goat anti-human IgG F(ab)² fragments as a second antibody (Jackson ImmunoResearch), cell fluorescence was analyzed in a FACScan flow cytometer (BD Biosciences). C2C12 cells were used as a negative control.

CDC. A CDC assay was done as reported previously (33). Briefly, cancer cells (IMR32 cells) were labeled with 100 μg (3.7 MBq) of Na₂¹²⁵I (Perkin-Elmer) for 1 h at 37 °C and kept at 4 °C for 30 min to remove loosely bound ¹²⁵I after washing. Aliquots of the labeled cells were divided into a 96-well microtiter plate (3,000 cells/25 μL) and incubated with 25 μL of antibody at various dilutions in the presence of 50 μL diluted human serum as complement for 1 h at 37 °C. After centrifugation, ¹²⁵I released in the supernatant was counted. The percentage of specific lysis was calculated from the experimental ¹²⁵I release, the total release, and the spontaneous release.

ADCC. ADCC assays were done using a standard ¹²⁵I release assay as previously reported (34). Briefly, aliquots of the ¹²⁵I-labeled tumor cells were used in 96-well microtiter plates (3,000 cell/well) and incubated with 25 μL antibodies with various dilutions in the presence of 50 μL effector cells at the indicated effector-to-target (E/T) ratio for 4 h at 37 °C. After centrifugation, releasing ¹²⁵I in the supernatant was counted with a scintillation counter (Perkin-Elmer). The percentage of specific lysis was calculated from the experimental ¹²⁵I release, the total release, and the spontaneous release.

Measurement of cell viability and apoptosis induction by anti-GD2 antibodies. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (35). Briefly, 60C3 and c.60C3 antibodies were diluted in 100 μL of DMEM and added to each well of 96-well microplates to give the final concentrations of 200, 100, and 50 μg/mL. EL4 cells (1 × 10⁵ cells) in 100 μL antibodies with various dilutions in the presence of 50 μL of 10% FCS and kept at 4 °C for 5 min. After centrifugation, total antibody binding to CHO cells was determined by using a HitTrap Protein A FF column (GE Healthcare) for affinity purification of mAbs. The eluted chimeric c.60C3 was diazylated against PBS for buffer exchange, sterile-filtered (0.22 μm), and stored at 4 °C. The concentration of chimeric c.60C3 was determined by measuring the absorbance at 280 nm and construction was confirmed SDS-PAGE.

Binding assay and internalization. Labeling of anti-GD2 antibodies with iodine-125 was done using an iodogen reagent (Sigma). For internalization studies, cells were equilibrated at 4 °C with labeled antibodies, and the temperature was rapidly switched to 37 °C. At selected time intervals, two aliquots of the cell suspensions were washed and centrifuged. One of the cell pellets was treated with 0.2 mol/L of glycine-HCl buffer (pH 2.5), whereas the other was treated with PBS (pH 7.4) at 4 °C for 5 min. After centrifugation, total antibody binding was determined from the pellet of cells treated with PBS, whereas the membrane-bound and internalized fractions were determined, respectively, from the supernatant and pellet of cells treated with the glycine-HCl buffer.

Flow cytometric analysis. In indirect immunofluorescence, the tumor cells (1 × 10^3 cells) were incubated with purified antibodies (10 μg/mL) for 45 min at 4 °C. After reaction with FITC-labeled goat anti-human IgG F(ab)² fragments as a second antibody (Jackson ImmunoResearch), cell fluorescence was analyzed in a FACScan flow cytometer (BD Biosciences). C2C12 cells were used as a negative control.
Statistics. An analysis of covariance taking into account the antibody concentration was used to compare survival rates and nonparametric ANOVA was used to compare the delay of tumor onset among the three and four groups, respectively, followed by post hoc multiple comparisons with the Tukey honestly significant difference test ($\alpha = 0.05$). A McNemar test was done in order to compare the internalization rates of antibodies.

Results

Expression of the chimeric mouse/human anti-GD2 antibody c.60C3. The heavy and light V-region cDNAs of the murine anti-GD2 mAb 60C3 were cloned as described in Materials and Methods. Chimeric light and heavy chain cDNAs were constructed by linking the cloned Vn and VH cDNAs to the appropriate human C-region cDNA. The chimeric antibody expression vectors were constructed by insertion of chimeric light and heavy chain cDNA constructs into the plasmids pcDNA3 and pcDNA 3.1 Zeo, respectively. The two expression vectors were transfected into CHO cells. Transfected cells were initially selected by G418 and Zeocin and screening was done by ELISA against GD2-expressing cells. One of the stable clones secreted 2 $\mu$g/mL of chimeric antibody. We designated the chimeric anti-GD2 antibody as c.60C3 to distinguish it from the mouse counterpart 60C3.

Chimeric c.60C3 was purified from culture medium using protein A affinity chromatography. SDS-PAGE under reducing conditions showed that the molecular weights of chimeric light and heavy chains were ~25,000 and 50,000, respectively. From analysis by nonreducing SDS-PAGE, the molecular weight of chimeric antibodies was ~150,000 (data not shown). These results indicated that the chimeric light and heavy chains were assembled as correct tetrameric molecules. The amino-terminal amino acid sequences of chimeric light and heavy chain V regions were identical to those of the murine counterparts (data not shown).

Direct binding of anti-GD2 mAbs to cell surface antigen. Scatchard plot analysis of data from saturation binding studies ($n = 3$) with EL4 cells indicates that the average number of binding sites per cell for the EL4 murine T lymphoma cell line were 1.1 $\times$ 10$^6$ for c.60C3 and 60C3. $K_d$ values were found to be 206 nmol/L for c.60C3 and 212 nmol/L for 60C3, indicating that c.60C3 and 60C3 bind to GD2 to the cell surface with equal affinity. Binding of iodinated c.60C3 was inhibited by unlabeled 60C3 (data not shown).

Indirect immunofluorescence assay. To further analyze surface binding to GD2-expressing tumor cells, human neuroblastoma IMR32, mouse neuroblastoma Neuro-2a, and mouse lymphoma EL-4 cells were incubated with c.60C3 and 60C3 under saturating conditions. The cells were stained with the appropriate secondary antibody-FITC conjugate and analyzed in a flow cytometer. As shown in Fig. 1A and B, both antibodies stained virtually all IMR32 and EL4 GD2-expressing cells, respectively, whereas neither c.60C3 nor 60C3 stained non–GD2-expressing Neuro-2a cells (Fig. 1C). Thus, indirect immunofluorescence analysis reveals the same staining pattern for IMR32, EL4, and Neuro-2a cells. Chimeric c.60C3 specificity for GD2 ganglioside was also confirmed by immunostaining gangliosides separated on TLC (data not shown).

Internalization of the anti-GD2 antibodies by GD2-expressing tumor cells. To investigate the kinetics of internalization of the mAbs, $^{125}$I-labeled c.60C3 and 60C3 mAbs were incubated with GD2-positive EL4 cells. The ratio of intracellular counts to cell surface counts (ratio of internalized to surface-bound anti-GD2 antibody) shows the internalization of anti-GD2 antibodies by...
GD2-expressing tumor cells and provides a general indication of the internalization rate. The ratio of intracellular counts to cell surface counts of 125I-labeled c.60C3 was gradually changed from 10:90 at 0 min to 70:30 at 10 min (Fig. 2). The internalization was examined until 60 min and was not found to increase markedly; the ratio was 60:40 at both 20 and 60 min. The ratio of intracellular counts to cell surface counts of 125I-labeled 60C3 was enhanced from 20:80 at 0 min to 70:30 at 20 min and 60:40 at 60 min.

**Human complement-mediated cytolsis.** The ability of c.60C3 and 60C3 to trigger complement-mediated cytolsis of GD2-expressing tumor cells was tested in a ²¹Cr release assay. Antibody concentrations ranging from 0.01 to 10 μg/mL were tested with GD2-expressing IMR32 cells (Fig. 3A). Amounts of c.60C3 and 60C3, as low as 0.1 μg/mL, were able to mediate maximum lysis of IMR32 cells. There is no apparent difference in the capability of murine 60C3 and chimeric c.60C3 mAbs to bind human complement and to mediate CDC. Rituxan, used as a negative control, did not show any significant CDC activity.

**Antibody-mediated cytotoxicity of human effector cells.** The ADCC of c.60C3 and 60C3 was tested with ⁵¹Cr-labeled IMR32 cells. Antibody concentrations ranging from 0.01 to 10 μg/mL were tested at an E/T ratio of 20:1. As shown in Fig. 3B, specific antibody-dependent lysis occurred in a dose-dependent fashion for c.60C3 and 60C3 antibodies. However, c.60C3 mediated a higher specific ⁵¹Cr release than 60C3 and the amount of c.60C3 that was necessary to mediate specific lysis was almost 10 times less than that of 60C3. The control chimeric antibody Rituxan did not show any significant ADCC activity. Effector/target ratios ranging from 1:1 to 30:1 were also tested at 1 μg/mL of antibody. Chimeric 60C3 showed ADCC against IMR32 cells, which was dependent on the E/T ratio and significant ADCC was observed at an E/T ratio of 20:1. Chimeric c.60C3 also mediated a higher specific release than its murine counterpart 60C3 under these conditions and the amount of effector cells that was necessary to mediate specific lysis by c.60C3 was 10 times less than that by 60C3.

**Suppression of tumor cell growth by anti-GD2 antibodies.** The effects of 60C3 and c.60C3 antibodies on tumor cell growth were then examined by adding antibodies to the EL4 culture medium. Microscopic observation of cells incubated with antibodies indicated that 60C3 antibody induced EL4 cell aggregation, whereas c.60C3 antibody and Rituxan did not aggregate EL4 cells, respectively (data not shown). The inhibiting effects of 60C3 mAb were dependent on the concentration of the antibody and became significant at concentrations >50 μg/mL after 24 h (Fig. 4A). At this concentration, chimeric c.60C3 did not show any significant effect compared with the Rituxan control mAb (P < 0.006). However, both 60C3 and c.60C3 mAbs showed significant inhibitory effects compared with the control mAb at saturating concentrations in which the greatest inhibitory effect were observed (growth inhibition after treatment at 200 μg/mL, 36.7% for 60C3 and 13.85% for c.60C3 versus control; P < 0.006). Nonspecific Rituxan mAb showed no effects. Anti-GD2 60C3 and c.60C3 antibodies did not suppress the growth of the non–GD2-expressing Neuro2a cell line (data not shown).

**Apoptosis induction by anti-GD2 antibodies.** To examine if apoptosis could be considered a major event involved in growth inhibition by GD2 antibodies, EL4 cells were incubated with different concentrations of 60C3 and c.60C3 antibodies, fixed with paraformaldehyde at different time points, and stained with bisbenzimide trihydrochloride. Incubation of EL4 cells with 60C3 resulted in the appearance of typical morphologic changes of apoptosis upon staining the DNA-specific fluorochrome bisbenzimide trihydrochloride. This included condensation of chromatin, its compaction along the periphery of the nucleus and segmentation of the nucleus (data not shown). Treatment of the EL4 cells with 60C3 induces apoptosis in a dose- and time-dependent manner (Fig. 4B and C). Apoptosis at 24 h reached 27% when cells were treated with 200 μg/mL of 60C3 (P < 0.001 versus Rituxan). As compared with 60C3, c.60C3 treatment induced a lower amount of apoptotic cells (treatment with 200 μg/mL of c.60C3, 10%; P = 0.054 versus 60C3). However, this amount was still relevant as compared with the one induced after Rituxan (P < 0.001). These results indicate that mouse 60C3 was effective in inhibiting GD2-positive tumor cell proliferation with apoptosis, whereas chimeric c.60C3 was far less potent.

**Inhibition of GD2-expressing tumor outgrowth in the syngeneic C57BL/6 model.** When C57BL/6 mice were given s.c. injections of 5 × 10⁵ EL4 lymphoma cells, tumor growth was observed in all animals. In control mice that received PBS, tumors became detectable by palpation on day 12.9, and the animals rapidly developed large tumors (Fig. 5A). According
to good practices in animal experiments, when the tumor volume exceeded 3,000 mm³ the animals were sacrificed. Similarly, all animals that received 200 µg of Rituxan developed gross tumors that were detected 12.5 days after inoculation. All animals in the group of mice that received 60C3 mAb were without detectable tumors 20.5 days after initial inoculation. However, the delay of the time of tumor appearance was not significant compared with the control groups. Mice treated with mAb c.60C3 were tumor-free until day 28. Furthermore, 25% of the mice challenged with EL4 cells were still tumor-free on day 90, the last day of the experiment (Fig. 5B). The difference between the day of tumor detection in control groups and that of the group of animals given the mAb c.60C3 injection was statistically significant (P < 0.0005). These results indicate that chimeric c.60C3 had a strong antitumor activity in vivo, whereas mouse 60C3 did not have any significant effect.

Discussion

In this study, we established a mouse/human chimeric antibody specific for GD2 ganglioside and analyzed some of its functional properties compared with its mouse counterpart. Passive immunotherapy against GD2 has been proposed for the treatment of human tumors of neuroectodermal origin, especially neuroblastomas and melanomas (2, 3). Results from early phase clinical trials suggest that anti-GD2 antibodies may have an effect on the course of the disease based on the reported response rate (20, 22, 37). In order to target the GD2 antigen in melanomas and neuroblastomas, and to reduce potential immunogenicity in patients, we developed a mouse/human chimeric anti-GD2 antibody, c.60C3, from the mouse mAb 60C3.

Chimeric antibody c.60C3 was made by joining cDNA sequences encoding the V regions, with their respective leader regions, of the light and heavy chain of mouse mAb 60C3 to cDNA sequences encoding the human κ chain and γ1 C regions, respectively. The expression vectors were transfected into CHO cells and a complete immunoglobulin molecule of chimeric c.60C3 was detected in the culture supernatant. Chimeric c.60C3 was found to react with GD2 with binding specificity identical to that of the original murine 60C3 mAb, which had high specificity and strong binding for GD2 (12). Scatchard analysis with [125I]-labeled antibodies showed that chimeric c.60C3 had the same affinity as murine 60C3. The equilibrium dissociation constant (Kd) of murine 60C3 and chimeric c.60C3 were 212 and 206 nmol/L, respectively. These Kd values are within the range of antiganglioside antibodies reported by others (5, 16, 18, 38) but the relatively low affinity of antiganglioside antibodies is compensated by high antigen density. Effector functions of antibodies such as CDC and ADCC, which are dependent on the isotype of the C regions, are critical in the passive immunotherapy of cancer patients (25). Steplewski et al. (39) showed that chimeric IgG1 had superior CDC and ADCC activities than mouse/human IgG2a, IgG3, and IgG4 chimeric antibodies. In another study, Zeng et al. (24) showed the superior ADCC of the mouse/human chimeric IgG1 CHO cells over the ch14.18 manufactured from SP2/0 and NSO cells at low concentration. Thus, we developed the chimeric c.60C3 with a human IgG1 isotype in the CHO cell line. Whereas both the chimeric c.60C3 and its mouse counterpart 60C3 triggered CDC and ADCC activity against GD2-expressing tumor cells, chimeric c.60C3 was more potent and displayed ADCC (~ 60%) at a lower mAb concentration of 0.1 µg/mL when human effector cells were used (Fig. 3).

Another mechanism contributing to the antitumor effect upon passive immunotherapy with anti-GD2 mAbs involves cell death induction without immune effector mechanism (11, 40). Apoptosis induced by anti-GD2 mAbs is very interesting because this mechanism may be very important in the treatment of solid tumors that have evolved complex mechanisms to protect themselves from CDC and ADCC (41). Direct apoptotic induction with anti-GD2 mouse mAbs has thus far been reported with GD2-expressing small cell lung carcinoma cell lines (11). Anti-GD2 mAbs binding on the cell surface are thought to cause conformational changes in integrin molecules, leading to the disruption of cell-matrix interactions with subsequent dephosphorylation of focal adhesion kinases (40). In this study, EL4 lymphoma cell apoptosis induced by anti-GD2 mAbs was mainly associated with the murine IgG3 anti-GD2 60C3 antibody, whereas chimeric c.60C3 had a weak effect at the highest concentration (Fig. 4). Although the cell death mechanisms initiated by mouse anti-GD2 60C3 antibodies have not been elucidated, our results suggest that the cross-linking of the anti-GD2 mAb molecules at the tumor cell surface may be critical for apoptosis induction. It is well known that at high antigen densities, cross-linking of mouse IgG3 specific for ganglioside antigens occurs and leads to the formation of strong homophilic networks while bound to a ganglioside antigen-bearing tumor cell surface (42). This in agreement with Yoshida et al. (11, 40) who used a mouse IgG1 anti-GD2 antibody in their studies, and Durrant et al. (43) who reported that a mouse IgG1 specific for a tumor-associated glycolipid antigen with homophilic binding properties induced apoptosis in colorectal cancer cells.

We found that chimeric c.60C3 was effective in its ability to inhibit the growth of murine EL4 lymphoma tumors in syngeneic mice, whereas murine 60C3 did not significantly inhibit the tumor growth (Fig. 5). Chimeric c.60C3 inhibiting tumor growth effect was specific because treatment with Rituxan at an equivalent dose was ineffective in this regard. ADCC is considered to be a major mechanism for suppression of tumor growth. On the other hand, mouse 60C3 was ineffective in suppressing tumor growth in this study. This is in agreement with findings reported previously by Miyaza et al. (16). Bergman et al. (44) reported that mAb 14.18, an anti-GD2 mouse IgG3, was less effective with murine than human effectors in ADCC against tumor cells. Furthermore, the in vitro antiproliferative effect and apoptosis induction of mouse 60C3 were only significant at high concentrations (Fig. 4).

The amount and the ganglioside expression pattern on tumors that had escaped chimeric c.60C3 treatment were identical to that of untreated tumors (data not shown). Therefore, it was considered that this tumor outgrowth was caused by the rapid internalization of GD2, as shown in Fig. 2. The fate of mAbs after binding to the cell surface is important in antibody therapy. Internalization is one of the critical steps that determine the effect of mAb because it may be detrimental to immune effector mechanisms such as ADCC and CDC. Only a few results have thus far been reported on anti-GD2 antibody...
internalization. Our findings that c.60C3 and its 60C3 are intensively incorporated into the cytoplasm of GD2-positive tumor cells is in contrast with a previous report in which no internalization of anti-GD2 mAb (45) or slow internalization (46) were reported. Although in vitro experiments showed that c.60C3 was very effective in killing GD2-positive target cells (Fig. 3), in this assay, the relatively small amount of antibody used was continuously bathing the cells. In the in vivo situation, the high injected dose of antibody and the relatively dense antigen present on the tumor cell surface, might provide sufficient time for the surface-bound antibody to interact with the immune system to kill cancerous cells and induce antitumor activity despite the rapid internalization. Lee et al. (47) have shown, using immunohistochemical techniques, that injected unlabeled antibody for ganglioside GD3 can be detected in xenograft sections despite the internalization process of anti-GD3 antibodies (48).

In conclusion, the mouse/human chimeric anti-GD2 antibody c.60C3, having enhanced antitumor activities and lower immunogenicity than its mouse counterpart, should be a useful agent for the passive immunotherapy of human cancer. However, this study showed the rapid internalization of GD2 antibody and indicates that anti-GD2 mAbs are also suitable for immunoneutralization with toxins and cytotoxic drugs. Thus, the anti-GD2 mAbs should be suitable to deliver toxins and cytotoxic drugs. For radioimmunotherapy applications, this study strongly suggests that c.60C3 antibody should be labeled with a residualizing radionuclide for in vivo targeting of GD2-expressing tumors.

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References


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