

Anti-ganglioside antibody-induced tumor cell death by loss of membrane integrity

Lourdes Roque-Navarro,¹
Krittalak Chakrabandhu,⁴ Joel de León,¹
Sandra Rodríguez,² Carlos Toledo,³
Adriana Carr,¹ Cristina Mateo de Acosta,¹
Anne-Odile Hueber,⁴ and Rolando Pérez¹

¹Antibody Engineering Department, Centre of Molecular Immunology; ²Electronic Microscopy Department, National Centre of Scientific Investigations; ³Criminality Central Laboratory, Havana, Cuba and ⁴Institute of Signaling, Developmental Biology and Cancer Research, CNRS UMR 6543, Nice France

Abstract

Gangliosides have been involved in multiple cellular processes such as growth, differentiation and adhesion, and more recently as regulators of cell death signaling pathways. Some of these molecules can be considered as tumor-associated antigens, in particular, *N*-glycolyl sialic acid-containing gangliosides, which are promising candidates for cancer-targeted therapy because of their low expression in normal human tissues. In this study, we provided the molecular and cellular characterization of a novel cell death mechanism induced by the anti-NGcGM3 14F7 monoclonal antibody (mAb) in L1210 murine tumor cell line but not in mouse normal cells (B and CD4⁺ T lymphocytes) that expressed the antigen. Impairment of ganglioside synthesis in tumor cells abrogated the 14F7 mAb cytotoxic effect; however, exogenous reincorporation of the ganglioside did not restore tumor cell sensitivity to 14F7 mAb-induced cytotoxicity. 14F7 F(ab')₂ but not Fab fragments retained the cytotoxic capacity of the whole mAb. By contrary, other mAb, which recognizes *N*-glycolylated gangliosides, did not show any cytotoxic effect. These mAbs showed quite different capacities to bind NGcGM3-positive cell lines measured by binding inhibition experiments. Interestingly, this complement-

independent cell death mechanism did not resemble apoptosis, because no DNA fragmentation, caspase activation, or Fas mediation were observed. However, NGcGM3 ganglioside-mediated 14F7 mAb-induced cell death was accompanied by cellular swelling, membrane lesion formation, and cytoskeleton activation, suggesting an oncosis-like phenomenon. This novel mechanism of cell death lets us to support further therapeutic approaches using NGcGM3 as a molecular target for antibody-based cancer immunotherapy. [Mol Cancer Ther 2008;7(7):2033–41]

Introduction

One or more sialic acid residues distinguish gangliosides, the prominent glycosphingolipids of the plasma membrane. Gangliosides are responsible for much of the negative charge at the cell surfaces and they are involved in biological processes such as pathogen adhesion, cell-cell interaction, inflammation, and tumor progression (1). Different tissues display different ganglioside expression patterns, which can undergo drastic changes during development or malignant transformation, such as the presence of unusual sialic acid species (2, 3). *N*-glycolyl sialic acid differs by an additional oxygen atom to the *N*-acetyl moiety and is not detected in healthy human tissues due to a genetic deletion in the CMP-*N*-acetyl hydroxylase enzyme that catalyze the biosynthesis conversion of *N*-acetyl to *N*-glycolyl sialic acid (4). However, the *N*-glycolyl variant can be detected in some human tumors and fetal tissues (3, 5), where is known to be immunogenic (6, 7). Free *N*-glycolyl sialic acid can be metabolically incorporated into human carcinoma cells by fluid pinocytosis and lysosomal sialic acid transporter (8). Also, tumor hypoxia can increase membrane expression of a sialic acid transporter in human cancer cells (9). Our group has described the expression of NGcGM3 as the major *N*-glycolylated ganglioside in human breast cancer (5), where the NGcGM3/VSSP vaccine was able to elicit a strong antibody response in cancer patients (10). This tumor marker expression leads us to consider the use of NGcGM3 ganglioside as an attractive target antigen for cancer immunotherapy.

We have reported previously the generation and characterization of 14F7 monoclonal antibody (mAb), which recognized specifically NGcGM3 (11). This antibody reacted with breast carcinoma and melanoma tissue sections (11). Recently, we have obtained clinical immunogammagraphic evidences of 14F7 ^{99m}Tc-labeled mAb uptake in breast primary tumors (12). Moreover, 14F7 mAb elicited tumor rejection in a murine myeloma model of P3X63 cells (13). Surprisingly, *in vitro* studies showed that 14F7 mAb induced a complement-independent cell death in these myeloma cells (13).

Received 3/4/08; accepted 4/2/08.

Grant support: Boehringer Ingelheim Fonds and International Union Against Cancer fellowships and the Cuban Government (L. Roque-Navarro) and Susan G. Komen Breast Cancer Foundation postdoctoral fellowship (K. Chakrabandhu).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Rolando Pérez, Antibody Engineering Department, Centre of Molecular Immunology, 216 Street and 15th Avenue, Atabey, Playa, Havana, 11600 Cuba. Phone: 53-7-2716810; Fax: 53-7-2720644. E-mail: rolando@cim.sld.cu

Copyright © 2008 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-08-0222

Some of the biological functions of gangliosides in cell death mechanisms have been described previously (14). However, the possible activation of these signaling processes by anti-ganglioside mAbs is still unknown. There are only evidences of apoptosis induction in melanoma and small cell lung cancer cells by anti-GM2 and anti-GD2 antibodies, respectively (15, 16). In the present report, we described for the first time that an anti-NGcGM3 antibody induced a novel cell death mechanism. This cytotoxicity differed from apoptosis but resembled oncosis, involving loss of membrane integrity and cytoskeleton molecules. These results contribute to the therapeutic concept based on this NGcGM3 ganglioside target.

Materials and Methods

Murine Lymphocytes and Cell Lines

Lymph node cells were isolated from C57BL/6 mice, between 6 and 12 weeks of age, and purchased from the Centre for Laboratory Animal Production. These lymph nodes were smashed and made into a single-cell suspension in PBS.

Murine cell lines L1210 (lymphocytic leukemia) and P3-X63.Ag8.65 (myeloma) were purchased from the American Type Culture Collection. L1210 cell line transfected with murine Fas (L1210-Fas) was provided by A.O. Hueber (CNRS UMR 6543). Cells were maintained in DMEM supplemented with 5% heat-inactivated FCS (Hyclone), antibiotic mixtures of penicillin (100 units/mL) and streptomycin (100 mg/mL), and 2 mmol/L L-glutamine (all from Life Technologies).

Antibodies and Reagents

NGcGM3 was isolated from horse erythrocytes (17). D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP; Matreya) was added to the culture medium at 10 μ mol/L for 3 days. Vinblastine was purchased from Lemery. Cycloheximide and actinomycin D (BDH Chemicals) were added 24 h before the 14F7 mAb treatment. Cytochalasin B was purchased from Sigma.

14F7 mAb (murine IgG1, κ ; ref. 11), 14F7 anti-idiotypic 4G9 mAb (murine IgG1, κ ; ref. 18), iorC5 mAb (murine IgG1, κ) that recognizes a glycoprotein expressed in colorectal cancer cells (19) and chimeric P3 (human IgG1, κ), an anti-NGc-containing gangliosides and sulfatides mAb (20), were purified by Protein A Affinity Chromatography (Pharmacia) and analyzed by SDS-PAGE under reducing conditions. F(ab')₂ and Fab fragments antibodies were obtained using a standard procedure described previously (21).

Generation of Chimeric 14F7 Antibody

Chimeric 14F7 antibody was generated as described previously (22). Briefly, 14F7 mAb variable heavy and light chains were amplified by PCR using the corresponding oligonucleotides (23) and cloned into pAH4604 and pAG4622 expression vectors containing human IgG1 and κ constant domains, respectively (24). Non-antibody-producing murine myeloma cell line (NSO) was transfected by electroporation and positive clones were selected using L-histidinol at 10 mmol/L.

Flow Cytometry Analysis

The following FITC-conjugated rat anti-mouse mAbs were used to analyze surface molecule expression by flow cytometry assays in murine lymphocytes: CD4 (RM4-5), CD8 (53-6.7), and CD45R/B220 (RA3-6B2) from BD PharMingen. Expression of NGcGM3 was detected with biotin-conjugated 14F7 mAb followed by RPE-conjugated streptavidin (BD PharMingen). Mean fluorescence intensity and percentage of NGcGM3-positive stained cells were determined in a FACScan instrument (Becton Dickinson). The WinMDI 2.8 program was used to analyze a total of 10⁴ cells acquired on every fluorescence-activated cell sorting assay.

Cytotoxic Assays

For induction of cell death, cells were suspended in culture medium with 1% FCS at 10⁶/mL and were incubated with 14F7 mAb in an atmosphere of 5% CO₂ at 37°C for the appropriated incubation time. Cells were washed, resuspended in PBS with propidium iodide (PI; 10 μ g/mL; Sigma), and analyzed by flow cytometry. Dead cells were determined by scatter measurement (forward scatter and side scatter) and PI internalization. All those cells that gate out of live cells and were PI stained were considered as dead cells. A quantitative measurement of dead cells was done by PI labeling histogram.

Western Immunoblot Analysis

L1210 cells were gently sonicated in Laemmli buffer to get protein lysates. Equal amounts of protein lysate were fractionated by SDS gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked 1 h at room temperature in 0.05% Tween 20/5% nonfat dried milk in TBS and incubated with the appropriate primary antibodies at the same conditions. After washing with 0.05% Tween 20 in TBS, the membranes were incubated with secondary antibodies (diluted 1:500-1:1,000) for 1 h at room temperature followed by four washes. The immunoreactive proteins were visualized using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The membranes were exposed to Hyperfilm (Amersham). mAbs against caspase-8 from Alexis, caspase-9, cleavage caspase-3, ezrin, phosphorylated ezrin, and CHC from Santa Cruz Biotechnology, and anti-poly(ADP-ribose) polymerase antibody from Biomol International were used. The relative intensities of these bands were calculated using an Image Scanner (Amersham Bioscience) and the spots were analyzed using UMAX (Amersham Pharmacia Biotech) and TotalLab 120 software (Nonlinear Dynamics).

DNA Fragmentation

Aliquots of 10⁶ L1210 cells were incubated in the presence or absence of drugs or mAbs, and DNA was extracted using a salting-out procedure (25). In brief, cells were lysed at 37°C in 10 mmol/L Tris-HCl (pH 8.0) containing 2 mmol/L EDTA, 400 mmol/L NaCl, 1% SDS, and proteinase K (0.5 mg/mL). Cell lysates were vortexed for 15 s with 0.1 mL of 6 mol/L NaCl and centrifuged followed by DNA ethanol precipitation. Then, RNase treatment (0.1 mg/mL) was done followed by another round of precipitation and centrifugation. Equal amounts of DNA

samples were electrophoresed in a 1.8% agarose gel prepared in TAE buffer [40 mmol/L Tris, 2 mmol/L EDTA (pH 7.5)] containing 0.3 $\mu\text{g}/\text{mL}$ ethidium bromide and visualized by UV light. As size marker, 100-bp DNA ladder from Amersham were used.

Scanning Electron Microscopy

L1210 cells in medium with 1% FCS were incubated with 14F7 or 4G9 mAb as isotype control for 3 h at 37°C, washed three times with PBS, and then fixed with 3.2% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) at 4°C for 1 h. These cells were postfixed for 1 h in 1% OsO_4 , washed three times with PBS, and dehydrated in ethyl alcohol. Cells were mounted onto metallic stub and gold coated for 2 min before analyze by TESCAN Vega TS 5130 SB Scanning Electron Microscope.

Results

NGcGM3 Mediated 14F7-mAb-Induced Cell Death in Tumor Cells but Not in Primary Cells

The NGcGM3 expression was examined by flow cytometry analysis using 14F7 mAb, which is able to distinguish between *N*-glycolyl and *N*-acetyl sialic acid-containing GM3 ganglioside (11). In this study, we found that lymphoid tumor cell lines, L1210 and P3X63, showed a high percentage of positive cells (Fig. 1A). Ganglioside extraction also showed that NGcGM3 is the major ganglioside in L1210 cells (NGcGM3/NAcGM3, 85:15 ratio; data not shown) as was reported previously for P3X63 myeloma (26). However, NGcGM3 ganglioside showed a differential expression at the cell membrane of murine lymphocyte subsets and lymphoid tumor cell lines. 14F7 mAb was able to label $68 \pm 6\%$ of total primary lymphocytes (Fig. 1A). However, all subpopulations did not exhibit the same ganglioside expression pattern. CD4^+ T and B lymphocytes showed a high percentage of positive cells ($82 \pm 12\%$ and $97 \pm 1\%$, respectively), but only $19 \pm 7\%$ of CD8^+ T cells were stained. It is remarkable that similar mean fluorescence intensity were found for lymphoid tumor cell lines and CD4^+ T and B lymphocytes, although CD8^+ T lymphocytes showed a lower mean fluorescence intensity.

We described previously that 14F7 mAb exhibited a direct cytotoxicity on P3X63 myeloma cells by an unusual complement-independent pathway (13). Now, we are reporting similar results with L1210 cells. Using PI internalization as a measurement of cell death, low concentrations of 14F7 mAb induced a cytotoxic effect in both L1210 and P3X63 tumor cells (Fig. 1B). Surprisingly, such complement-independent cytotoxicity was not observed in normal B and CD4^+ T cell subsets that also expressed the antigen, incubated under the same conditions. Moreover, no cell death was observed on mitogen-activated normal lymphocytes (data not shown).

Expression of NGcGM3 Ganglioside at the Plasma Membrane Was Necessary but Not Sufficient for 14F7 mAb-Induced Cell Death

To elucidate how much relevant NGcGM3 ganglioside would be for 14F7 mAb-induced cell death, cellular

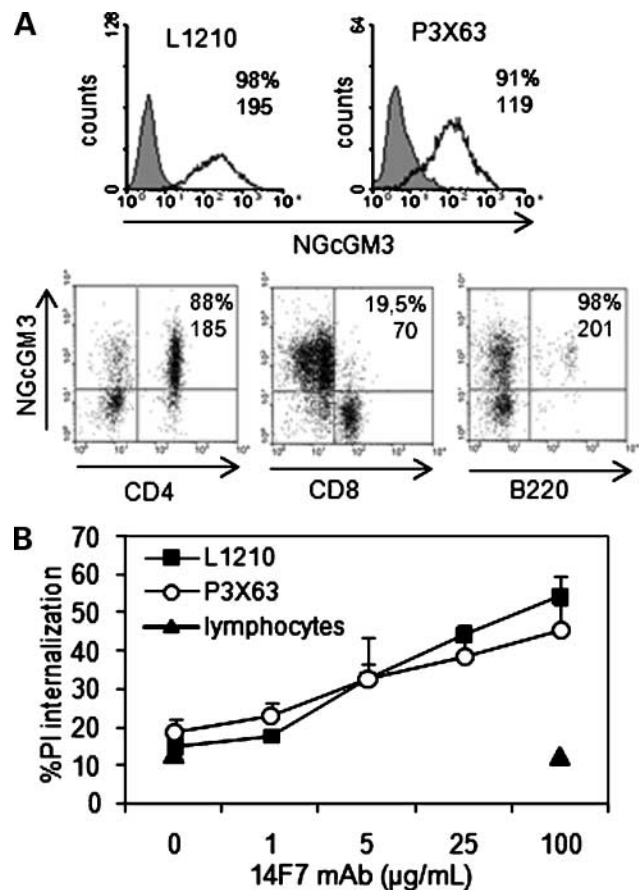


Figure 1. Cell death induced in tumor cell lines by an anti-NGcGM3 mAb. **A**, flow cytometry analysis of NGcGM3 expression on L1210 and P3X63 tumor cell lines and CD4^+ , CD8^+ , and B220^+ subsets of murine lymphocytes. Cell lines were stained with biotin-conjugated isotype-matched control mAb (gray histogram) or biotin-labeled 14F7 mAb (black line) followed by streptavidin-horseradish peroxidase. Each lymphocyte population was evaluated for NGcGM3 expression. Numbers represent percentages and mean fluorescence intensities of NGcGM3-expressing cells. Representative of three independent experiments. **B**, 14F7 mAb-induced cytotoxicity in murine lymphocytes and tumor cell lines. Cells were incubated with different concentrations of 14F7 mAb for 3 h at 37°C. Cell viability was evaluated by PI uptake and cytometry analysis. Points, mean of triplicate measurement; bars, SD.

gangliosides were depleted using D-PDMP, an inhibitor of glucosylceramide synthase. This reagent has been often used to study biological functions in living cells without interfering of cell viability and cell growth (27). The expression of NGcGM3 ganglioside in the plasma membrane of L1210 tumor cells was considerably reduced after treatment with 10 $\mu\text{mol}/\text{L}$ D-PDMP during 3 days (Fig. 2A, inset). 14F7 mAb did not induce cell death in D-PDMP-treated cells, which exhibited a reduced ganglioside expression (Fig. 2A). Exogenous addition of NGcGM3 ganglioside to D-PDMP-treated cells restored the recognition by 14F7 mAb but did not restore its cytotoxic effect. Similar results were obtained with HUT-78 lymphoma cells, which do not express NGcGM3. Incubation of HUT-78 cells with NGcGM3 ganglioside made these cells be recognized by

14F7 mAb, ~99% of positive cells and a mean fluorescence intensity of 115 ± 19 , but these cells did not become sensitive to the cytotoxic effect of 14F7 mAb (data not shown).

Furthermore, the cytotoxic activity of 14F7 mAb was also examined after the preincubation with a 14F7 anti-idiotypic 4G9 mAb. This mAb is able to block the binding of 14F7 mAb to the ganglioside (18). The anti-idiotypic 4G9 mAb impaired 14F7-induced NGcGM3-mediated cell death in a dose-dependent manner (Fig. 2B).

Both Specificity and Affinity of 14F7 mAb Binding Site Are Critical for Cytotoxicity

L1210 cells were treated with intact 14F7 mAb or its Fab and $F(ab')_2$ fragments. Whereas treatment with Fab

fragments failed to induce cell death, $F(ab')_2$ fragments retained the cytotoxic activity of the full-length antibody (Fig. 3A). All these fragments recognized the antigen as well as the whole antibody (Fig. 3B, *inset*). Similarly, the chimeric 14F7 also induced cell death in a dose-dependent manner (Fig. 3B). Interestingly, P3 mAb, an anti-*N*-glycolyl-containing gangliosides murine IgM mAb (28), did not exhibit cytotoxicity in L1210 and P3X63 tumor cells (data not shown). Even chimeric P3 antibody, which shared the same γ -chain isotype than chimeric 14F7, did not show any cytotoxic activity on L1210 cells (Fig. 3B). These antibodies differed also in their antigen recognition capacity; whereas increasing concentrations of chimeric 14F7 antibody inhibited the binding of biotinylated chimeric P3 antibody to NGcGM3-expressing L1210 tumor cells, the opposite situation was not observed (Fig. 3C). Even at 100 $\mu\text{g}/\text{mL}$, the chimeric P3 antibody was not able to inhibit the binding of biotinylated chimeric 14F7 (10 $\mu\text{g}/\text{mL}$) to L1210 tumor cells.

14F7 mAb-Induced Cell Death Involved Loss of Membrane Integrity

Morphologic analysis showed that L1210 tumor cells treated with 14F7 mAb at 37°C exhibited swelling measured by an increase in forward scatter (Fig. 4A). PI incorporation indicated nonviable cells that have lost plasma membrane integrity. Also, 14F7 mAb-treated cells rapidly aggregated in clusters in suspension culture. L1210 tumor cells treated with an isotype-matched control antibody did not show any increase in PI uptake or morphologic changes.

Otherwise, L1210 tumor cells treated with the anti-NGcGM3 ganglioside murine and chimeric antibodies developed large membrane lesions as shown by scanning electron microscopy (Fig. 4B). Most usually, a single membrane lesion has been observed on individual cells, reaching a diameter $\sim 1.2 \pm 0.6 \mu\text{m}$. Cells incubated with a negative control mAb did not show any morphologic change.

14F7 mAb-Induced Cell Death Was Nonapoptotic but Involved Cytoskeleton Molecules

Although this complement-independent cytotoxic effect was usually observed at physiologic conditions (37°C), it was also observed at 4°C and even under serum-free culture conditions or PBS (data not shown). Also, neither protein synthesis nor transcription was required for this cell death mechanism. Treatment with cycloheximide and actinomycin D, inhibitors of *de novo* protein and mRNA synthesis respectively, did not affect the cytotoxic effect of 14F7 mAb (Fig. 5A). Moreover metabolic inhibitors as sodium azide and EDTA did not produce any change in cytotoxicity (data not shown). Evidences of cell viability loss by antibody treatment were obtained even after 5 min of incubation.

To evaluate caspase-dependent apoptotic signaling pathway, caspase activation was analyzed in antibody-treated tumor cell lysates by Western blot assays using antibodies against initiator caspase-8 and caspase-9, cleavage effector caspase-3, and poly(ADP-ribose) polymerase. No cleavages

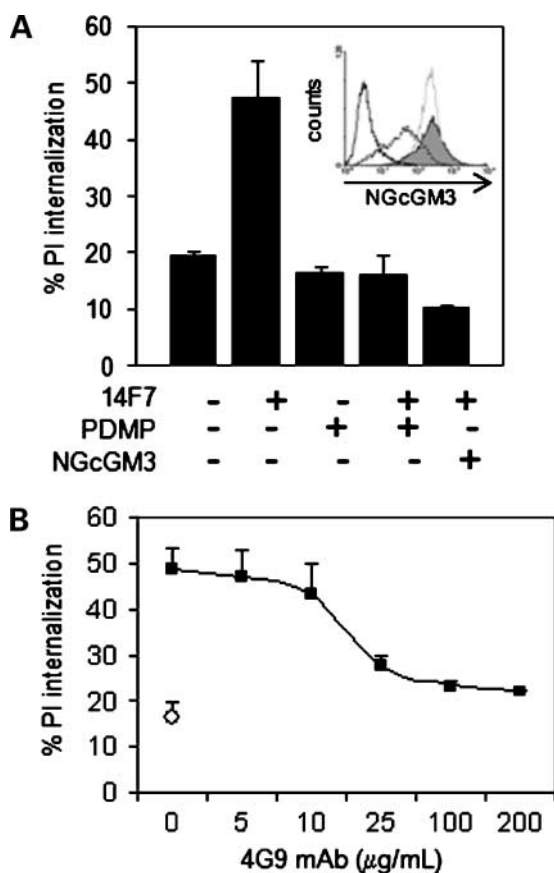
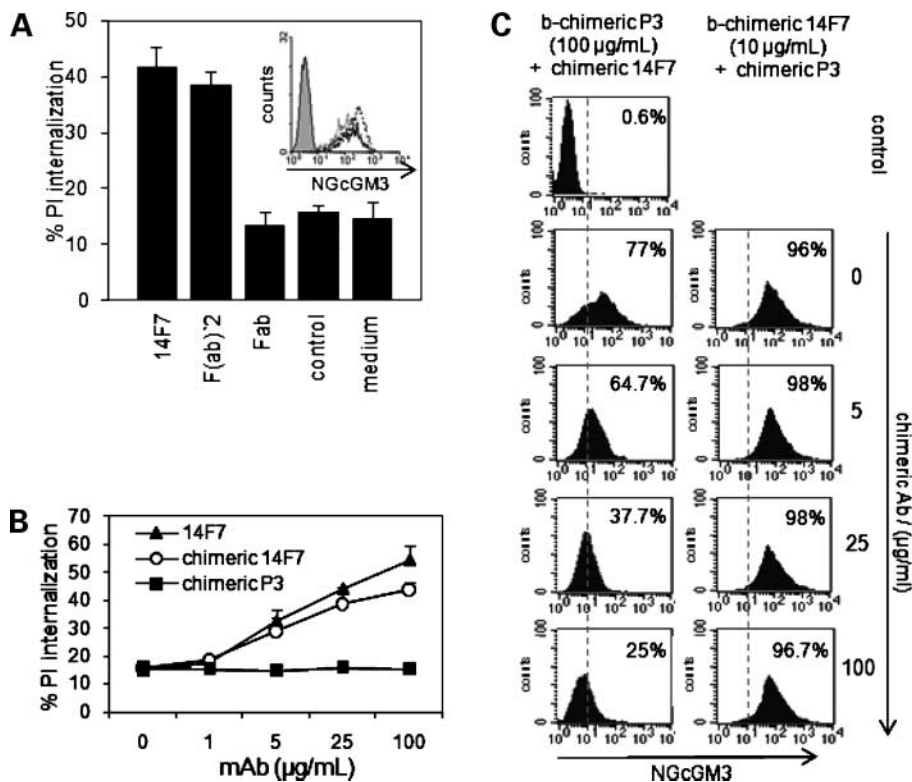


Figure 2. NGcGM3 expression was not sufficient to induce cell death by 14F7 mAb. **A**, nontreated L1210 cells, D-PDMP-treated cells, and L1210 cells after exogenous reincorporation of NGcGM3 ganglioside were exposed to 14F7 mAb (25 $\mu\text{g}/\text{mL}$; 3 h) followed by flow cytometry analysis of PI incorporation. *Inset*, expression of NGcGM3 ganglioside in nontreated L1210 cell line (filled histogram), after treatment with D-PDMP (dotted line) and after reincorporation of NGcGM3 ganglioside (gray line), was detected with biotinylated-14F7 mAb and RPE-conjugated streptavidin (black line) by flow cytometry. Representative of three independent experiments. **B**, 14F7-induced cell death impaired by anti-idiotypic 4G9 mAb. 14F7 mAb was incubated previously in the presence of different concentrations of 4G9 mAb at 37°C for 1 h followed by cytotoxicity assay as described in Materials and Methods. White circle, cytotoxicity without mAb treatment. Points or columns, mean of triplicate measurements; bars, SD.

Figure 3. 14F7 mAb variable region was relevant for cytotoxicity. **A**, L1210 cells were incubated with 0.17 $\mu\text{mol/L}$ Fab and F(ab')_2 fragments of 14F7 mAb, compared with the whole antibody, and cell death was analyzed by PI incorporation as described. *Inset*, histogram represents NGcGM3 recognition in L1210 cells by 14F7 mAb (black line), F(ab')_2 (dotted line), Fab (gray line) fragments, and FITC-labeled anti-mouse Fab antibody (solid gray histogram). **B**, cytotoxicity induced in L1210 cells by different concentrations of chimeric variants of 14F7 and P3 mAbs. 4G9 mAb was used as isotype-matched irrelevant mAb. *Points*, mean of triplicate measurements; *bars*, SD. **C**, competitive antibody binding assay between chimeric 14F7 and chimeric P3 mAbs. L1210 cells were incubated with biotinylated chimeric P3 (100 $\mu\text{g/mL}$) or chimeric 14F7 (10 $\mu\text{g/mL}$), and different concentrations (0, 5, 25, and 100 $\mu\text{g/mL}$) of chimeric 14F7 or P3 were added, respectively. Tumor cell staining was analyzed by flow cytometry analysis using RPE-conjugated streptavidin. Numbers represent percentage of antibody-stained positive cells. Representative of three independent experiments.



at any caspase or their substrate were observed in L1210 cell line treated with 14F7 mAb (Fig. 5B). Additionally, the caspase inhibitor zVAD.fmk did not impair 14F7 mAb-induced cell death (data not shown). Furthermore, DNA laddering was evaluated (Fig. 5C). Internucleosomal DNA fragmentation was not observed after treatment with the anti-NGcGM3 ganglioside mAb during 24 h of incubation. A positive control of vinblastine-treated L1210 tumor cells exhibited a chromosomal DNA reduction and laddering.

Some caspase-independent mechanisms have been related with Fas death receptor. We also studied the 14F7 mAb-induced cytotoxic effect in a L1210 variant cell line transfected with murine Fas receptor. Unexpectedly, 14F7 mAb had the same cytotoxic effect in both tumor cells, suggesting that Fas receptor protein is not involved in this mechanism (Fig. 5D). Caspase activation was not observed in Fas-expressing transfected L1210 cells (data not shown), a similar result to the one obtained with parental Fas-negative tumor cells.

To explore the mechanism of cell death induced by this anti-NGcGM3 mAb, we also examined the effect of cytoskeleton organization. First, to assess actin filament integrity in 14F7 mAb-induced cell death, L1210 cells were treated with cytochalasin B, an inhibitor of actin polymerization. This treatment completely inhibited 14F7 mAb-induced cell death (Fig. 6A). We also analyzed the involvement of ezrin, a protein that mediates actin association to the plasma membrane. After incubation with 14F7 mAb, an increase in ezrin phosphorylation was detected

(Fig. 6B). Phosphorylated bands from samples treated with 14F7 mAb were 1.5 more intense than ones treated with the isotype-matched control antibody. These data indicate that cytoskeleton rearrangement is a key event in the 14F7 mAb-induced NGcGM3-mediated cell death mechanism.

Discussion

It has been described that NAcGM3 is crucial for cell survival (29); however, this ganglioside can also induce apoptosis or modulate death receptors activation (14). Nevertheless, we lack information concerning the possible role of NGcGM3 for cell viability. This *N*-glycosylated ganglioside has a higher expression in tumors compared with normal human tissues (3), and its immunosuppressive effect and relevance for tumor progression has been recently reported (30). Here, we describe for the first time that NGcGM3 mediates an oncosis-like cell death mechanism involving loss of membrane integrity.

First, we found a differential expression of NGcGM3 in murine lymphocyte primary subsets. A higher expression was found in B and CD4^+ T lymphocytes compared with CD8^+ T lymphocytes. This is the first report that shows a different expression of NGcGM3 in lymphocyte populations. NGcGM2 ganglioside is one of the major components of murine lymphocytes followed by NGcGD1a (31). Also, it has been reported that GM1 ganglioside expression is increased in CD4-CD8^+ mature murine thymocytes in comparison with $\text{CD4}^+\text{CD8}^-$ cells (32). As gangliosides

are usually organized into lipid rafts where they modulate several signaling pathways, it is possible to speculate that these NGcGM3 differences could be correlated with different functions of this ganglioside in these murine lymphocyte subsets.

NGcGM3 ganglioside expression at the plasma membrane was a necessary but not a sufficient condition to this antibody-induced cell death. Only tumor cells were sensitive to 14F7 mAb cytotoxic effect, whereas cell death was not observed in normal lymphocytes. However, abrogating NGcGM3 expression or blocking the antibody-epitope impaired 14F7 mAb-induced cell death. Interestingly, exogenous reincorporation of NGcGM3 in PMDP-treated L1210 cells did not recover the cytotoxic effect. However, it has been reported that exogenous addition of gangliosides can disturb membrane organization (33) and even redistribute protein localization. Gangliosides are usually associated with several proteins into glycolipid-enriched microdomains. Anti-GD2 antibodies induced apoptosis, interfering the association of GD2 ganglioside with β_1 -integrin and focal adhesion kinase (16). Differential NGcGM3 ganglioside distribution into lipid rafts between normal and tumor cells could explain differences in 14F7

mAb-induced cytotoxicity. Further studies to determine the influence of lipid raft composition in tumor cell sensitivity to 14F7 mAb are required.

The complement-independent NGcGM3-mediated cell death induced by the 14F7 mAb did not fulfill the operational criteria defining apoptosis. 14F7 mAb-induced cell death was a rapid process characterized by increased cell size and permeability and cell aggregation. This process seemed to be energy independent, did not require *de novo* protein synthesis, and was not affected by metabolic inhibitors. Moreover, no evidences of DNA fragmentation, chromatin modification, or caspase activation were found. However, 14F7 mAb-treated cells showed giant lesions at the plasma membrane, much greater than the pores formed by complement, perforin, or bacterial toxins; which most usually have a diameter ~ 0.1 to $0.3 \mu\text{m}$ (34).

It has been described as a cell death mechanism with similar features to 14F7 mAb-induced cytotoxicity on activated lymphocytes by an anti-MHC-I-like membrane protein, RE2 mAb (35). Moreover, this type of cell death has also been observed for other mAbs with different specificity, such as a type I transmembrane mucin (36), and a *N*-acetyl lactosamine structure (37). Although this type of

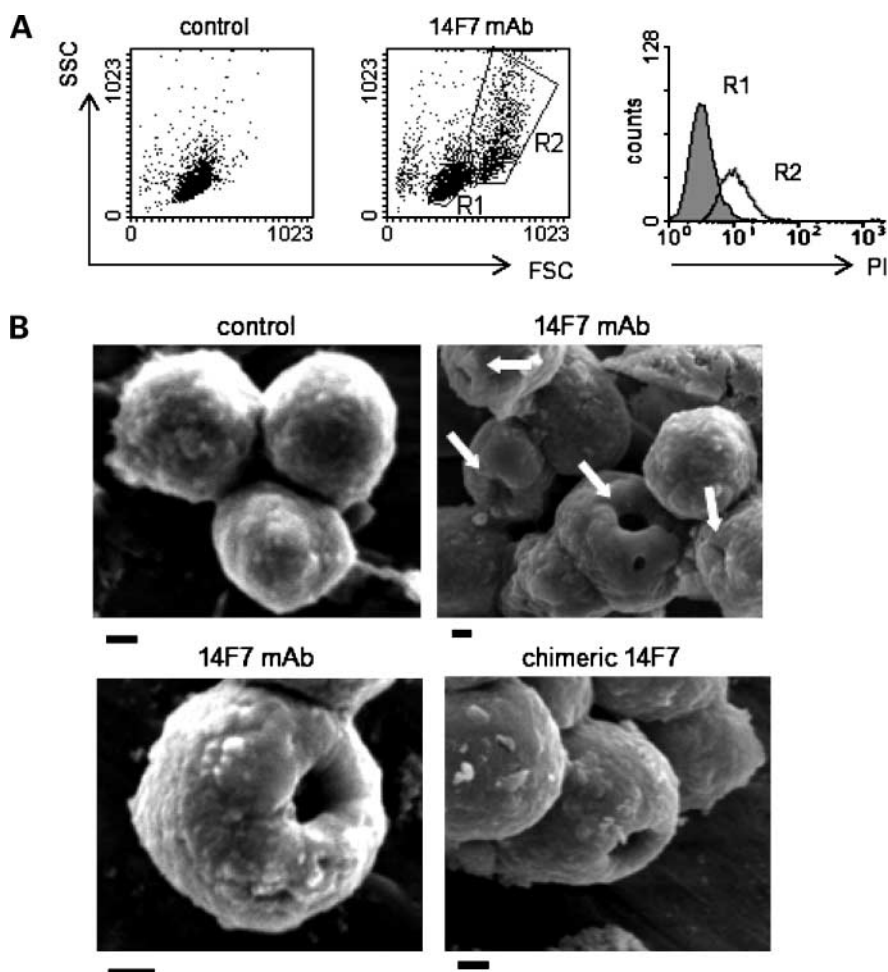
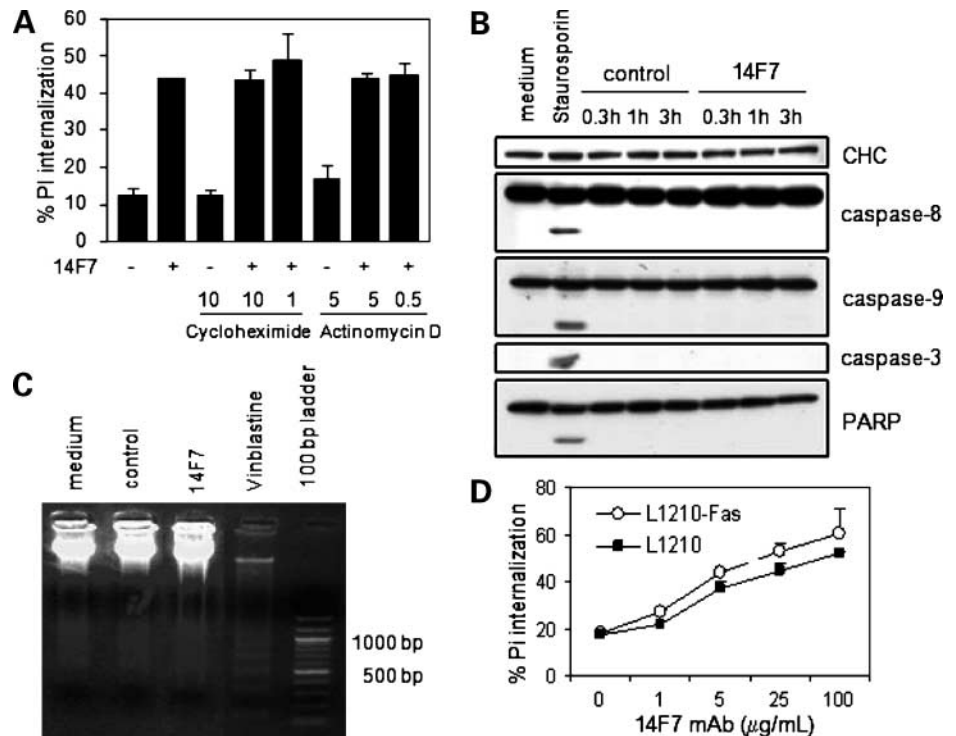


Figure 4. 14F7 mAb-induced cell death involved morphologic changes. **A**, morphologic changes in L1210 cells after treatment with 14F7 mAb and an isotype-matched control mAb (iorC5; $25 \mu\text{g}/\text{mL}$) for 3 h at 37°C . Forward scatter and side scatter measurements in treated cells are shown. PI incorporation by 14F7 mAb-treated cells is also shown. **B**, L1210 cells were incubated with 14F7 mAb, an isotype-matched control mAb (4G9) or chimeric 14F7 for 3 h at 37°C , and then analyzed by scanning electron microscopy as described in Materials and Methods.

Figure 5. NGcGM3-mediated cell death was nonapoptotic. **A**, cycloheximide and actinomycin D were added at different concentrations (numbers represent drug concentration at $\mu\text{g/mL}$) during 24 h before 14F7 mAb treatment (25 $\mu\text{g/mL}$). Cell death was estimated by PI internalization. **B**, L1210 cells were treated with 14F7 mAb for different times, and the activation of caspase-8, caspase-9, and caspase-3, and poly(ADP-ribose) polymerase (PARP) was determined by Western blotting with the appropriated antibodies. **C**, for internucleosomal DNA fragmentation analysis, L1210 cells were treated with cell culture medium, 14F7 mAb, an isotype-matched control mAb (iorC5), and vinblastine during 24 h of incubation followed by nuclear DNA extraction as described in Materials and Methods. Representative of three independent experiments. **D**, 14F7 mAb-induced cell death was evaluated by PI uptake at different antibody concentrations in parental L1210 cells (Fas-negative) and transfected Fas-positive L1210 cells. *Points*, mean of triplicate measurements; *bars*, SD.



mechanism was first described on lymphocytic cells, it seems that it is not restricted to hematopoietic tissues; as recently it was reported a mAb that induces this mechanism in colorectal tumor cells by a *N*-linked carbohydrate antigen (38). Some authors have correlated these mechanisms with oncosis, described as a nonapoptotic cell death accompanied by cellular swelling and increased membrane permeability (39). Although the molecular pathway leading to oncosis is unknown, it has been reported as a consequence of ionic pumps failures at the plasma membrane, decreased levels of cellular ATP, and membrane disturbing (39, 40). However, this is the first report of an oncosis-like cell death mediated by NGcGM3 ganglioside.

It is not clear if these cell death mechanisms already described have some common molecular events, but it seems that cytoskeleton molecules are most usually involved. Several reports of complement-independent and caspase-independent cytotoxicity showed that actin aggregation was a key event in such phenomena (35, 38, 41, 42). In our case, the fact that ezrin phosphorylation occurred after treatment with 14F7 mAb suggests a strong connection between NGcGM3 and actin filaments. ERM proteins may modulate intermediate signaling events that are essential for cytoskeleton changes, cell death, and permeability increase (43).

Otherwise, it seems that any antibody recognizing NGcGM3 could not induce a complement-independent cell death. Human sera contain naturally high levels of anti-*N*-glycolyl gangliosides antibodies even of IgG isotype (6, 44), which only induce complement-mediated mechanism in leukemia cells (7). Our results suggest that this NGcGM3-mediated cell death seems to be a particular

property of 14F7 variable region. In fact, cross-linking of carbohydrate epitopes but not Fc region could be involved in cell death, because 14F7-Fab fragment did not induce cytotoxicity, whereas chimeric 14F7 antibody and F(ab')_2 fragments retained the capacity to induce it. Moreover, we have described P3 mAb, which recognizes *N*-glycolyl sialic acid-containing gangliosides and sulfatides (28), but did not show any cytotoxic effect on tumor cells even when we compared both chimeric antibodies having the same human Fc constant region. It has been suggested that positive charges at H-CDR3 segment of VH4-34 derived mAbs correlate with oncosis-like cell death mechanisms elicited by these antibodies (45). Interestingly, it was found a motif RXRR in the H-CDR3 of 14F7 mAb (23). Modeling studies together with the crystal structure of 14F7 mAb supported the importance of R98 and R100a in the recognition of NGcGM3 oligosaccharide by this antibody (46). It is possible that these arginines could also be relevant for cytotoxic activity. However, P3 mAb has a similar sequence in the H-CDR3 (RXXR), but this mAb was not able to induce direct cell death (47).

The induction of cell death could be dependent of the specific epitope recognized by these mAb on the NGcGM3 molecule. P3 mAb is restricted to the *N*-glycolyl sialic acid residue (48), whereas the epitope recognized by 14F7 mAb involved the disaccharide *N*-glycolyl sialic acid-galactose (46). Also, P3 mAb was encoded by a germ-line VH region (47), whereas 14F7 variable regions were heavily mutated (23), arguing an affinity maturation process. In fact, chimeric 14F7 antibody seemed to have a higher affinity for NGcGM3 than chimeric P3 antibody.

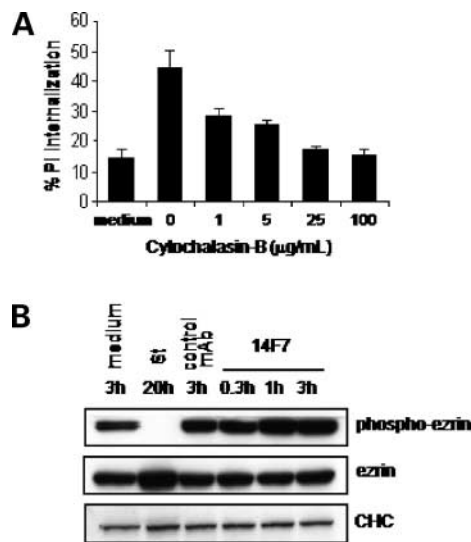


Figure 6. Cytoskeleton activation was required for cytotoxicity. **A**, L1210 cells treated with different concentrations of cytochalasin B were incubated with 14F7 mAb (25 μg/mL, 3 h, 37°C) or an isotype-matched control mAb (iorC5). Cell death was analyzed by PI uptake. **B**, ezrin phosphorylation was analyzed by Western blot using specific antibodies. L1210 cells were treated with 14F7 mAb, an isotype-matched control mAb (iorC5), or staurosporin. Representative of three independent experiments.

Sera from patients immunized with NGcGM3/VSSP vaccine or an anti-idiotypic mAb ("antigen mimicry") elicited complement-dependent cell death (10, 49). Work is ongoing to know if these human antibodies could also mediate an oncosis-like phenomenon. In any case, therapeutic schedules of these vaccines should be improved to generate 14F7-like anti-NGcGM3 antibodies. These antibodies could be potentially useful against tumors that generate resistance to complement cascade or apoptotic pathways. Our findings confirm that NGcGM3 ganglioside is a promising candidate for cancer therapy. Clinical trials are now ongoing to examine preliminary efficacy in cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

- Hakomori SI. Inaugural article: the glycosynapse. *Proc Natl Acad Sci U S A* 2002;99:225–32.
- Hakomori S. Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Res* 1996;56:5309–18.
- Malykh YN, Schauer R, Shaw L. *N*-glycolylneuraminic acid in human tumors. *Biochimie* 2001;83:623–34.
- Irie A, Koyama S, Kozutsumi Y, et al. The molecular basis for the absence of *N*-glycolylneuraminic acid in humans. *Biol Chem* 1998;273:15866–71.
- Marquina G, Waki H, Fernández LE, et al. Gangliosides expressed in human breast cancer. *Cancer Res* 1996;56:5165–71.
- Nishimaki T, Kano K, Milgrom F. Hanganutziu-Diecher antigen and antibody in pathologic sera and tissues. *J Immunol* 1979;122:2314–8.
- Nguyen DH, Tangvoranuntakul P, Varki A. Effects of natural human

antibodies against a nonhuman sialic acid that metabolically incorporates into activated and malignant immune cells. *J Immunol* 2005;175:228–36.

8. Bardor M, Nguyen DH, Diaz S, Varki A. Mechanism of uptake and incorporation of the non-human sialic acid *N*-glycolylneuraminic acid into human cells. *J Biol Chem* 2005;280:4228–37.

9. Yin J, Hashimoto A, Izawa M, et al. Hypoxic culture induces expression of sialin, a sialic acid transporter, and cancer-associated gangliosides containing non-human sialic acid on human cancer cells. *Cancer Res* 2006;66:2937–45.

10. Carr A, Rodríguez E, Arango MC, et al. Immunotherapy of advanced breast cancer with a heterophilic ganglioside (NeuGcGM3) cancer vaccine. *J Clin Oncol* 2003;21:1015–21.

11. Carr A, Mullet A, Mazorra Z, et al. A mouse IgG1 monoclonal antibody specific for *N*-glycolyl GM3 ganglioside recognized breast and melanoma tumors. *Hybridoma* 2000;19:241–7.

12. Oliva JP, Valdés Z, Casacó A, et al. Clinical evidences of GM3 (NeuGc) ganglioside expression in human breast cancer using the 14F7 monoclonal antibody labelled with ^{99m}Tc. *Breast Cancer Res Treat* 2006;96:115–21.

13. Carr A, Mesa C, Arango MC, Vazquez AM, Fernández LE. *In vivo* and *in vitro* anti-tumor effect of 14F7 monoclonal antibody. *Hybrid Hybridomics* 2002;21:463–8.

14. Bektas M, Spiegel S. Glycosphingolipids and cell death. *Glycoconj J* 2004;20:39–47.

15. Retter MW, Johnson JC, Peckham DW, et al. Characterization of a proapoptotic antiganglioside GM2 monoclonal antibody and evaluation of its therapeutic effect on melanoma and small cell lung carcinoma xenografts. *Cancer Res* 2005;65:6425–34.

16. Aixinjueluo W, Furukawa K, Zhang Q, et al. Mechanisms for the apoptosis of small cell lung cancer cells induced by anti-GD2 monoclonal antibodies: roles of anoikis. *J Biol Chem* 2005;280:29828–36.

17. Stults CLM, Sweeley CC, Matcher BA. Glycosphingolipids: structure, biological source, and properties. *Methods Enzymol* 1989;179:167–74.

18. Rodríguez M, Llanes L, Pérez A, Pérez R, Vázquez AM. Generation and characterization of an anti-idiotypic monoclonal antibody related to GM3(NeuGc) ganglioside. *Hybrid Hybridomics* 2003;22:307–14.

19. Vazquez AM, Tormo B, Velandia A, et al. Characterization of the colorectal antigen IOR-C2. *Hybridoma* 1992;11:245–51.

20. Lopez-Requena A, Mateo de Acosta C, Perez A, et al. Chimeric anti-*N*-glycolyl-ganglioside and its anti-idiotypic mAbs: immunodominance of their variable regions. *Hybrid Hybridomics* 2003;22:235–43.

21. Coligan JE, Krvisbeck AM, Margulies DH, Shevach EM, Strober W. Purification and fragmentation of antibodies. *Curr Protoc Immunol* 1995;1:285.

22. Roque-Navarro L, Mateo C, Lombardero J, et al. Humanization of predicted T-cell epitopes reduces the immunogenicity of chimeric antibodies: new evidence supporting a simple method. *Hybrid Hybridomics* 2003;24:245–57.

23. Rodríguez M, Roque-Navarro L, López-Requena A, et al. Insights into the immunogenetic basis of two ganglioside-associated idiotypic networks. *Immunobiology* 2007;212:57–70.

24. Coloma MJ, Hastings A, Wims LA, Morrison SL. Novel vectors for the expression of antibody molecules using variable regions generated by polymerase chain reaction. *J Immunol Methods* 1992;159:89–104.

25. Perchellet EM, Sperfslage BJ, Wang Y, et al. Among substituted 9,10-dihydro-9,10-(1,2)benzenoanthracene-1,4,5,8-tetraones, the lead antitumor triptycene bisquinone TT24 blocks nucleoside transport, induces apoptotic DNA fragmentation and decreases the viability of L1210 leukemic cells in the nanomolar range of daunorubicin *in vitro*. *Anticancer Drugs* 2002;13:567–81.

26. Muthing J, Steuer H, Peter-Katalinic J, et al. Expression of gangliosides GM3 (NeuAc) and GM3 (NeuGc) in myelomas and hybridomas of mouse, rat, and human origin. *J Biochem* 1994;116:64–73.

27. Lee L, Abe A, Shayman JA. Improved inhibitors of glycosylceramide synthase. *J Biol Chem* 1999;274:14662–9.

28. Vázquez AM, Alfonso M, Lanne B, et al. Generation of a murine monoclonal antibody specific for *N*-glycolylneuraminic acid-containing gangliosides that also recognizes sulfated glycolipids. *Hybridoma* 1995;4:551–6.

29. Noguchi M, Kabayama K, Uemura S, et al. Endogenously produced ganglioside GM3 endows etoposide and doxorubicin resistance by up-regulating Bcl-2 expression in 3LL Lewis lung carcinoma cells. *Glycobiology* 2006;16:641–50.
30. De Leon J, Fernandez A, Mesa C, Clavel M, Fernandez LE. Role of tumour-associated *N*-glycosylated variant of GM3 ganglioside in cancer progression: effect over CD4 expression on T cells. *Cancer Immunol Immunother* 2006;55:443–50.
31. Nakamura K, Hashimoto Y, Yamakawa T, Suzuki A. Genetic polymorphism of ganglioside expression in mouse organs. *J Biochem (Tokyo)* 1988;103:201–8.
32. De Mello Coelho V, Nguyen D, Giri B, Bunbury A, Schaffer E, Taub DD. Quantitative differences in lipid raft components between murine CD4⁺ and CD8⁺ T cells. *BMC Immunol* 2004;5:1–8.
33. Simons M, Friedrichson T, Schulz JB, Pitto M, Masserini M, Kurzchalia TV. Exogenous administration of gangliosides displaces GPI-anchored proteins from lipid microdomains in living cells. *Mol Biol Cell* 1999;10:3187–96.
34. Geny B, Popoff MR. Bacterial protein toxins and lipids: pore formation or toxin entry into cells. *Biol Cell* 2006;98:667–78.
35. Matsuoka S, Asano Y, Sano K, et al. A novel type of cell death of lymphocytes induced by a monoclonal antibody without participation of complement. *J Exp Med* 1995;181:2007–15.
36. Zhang C, Yuhui X, Gu J, Schlossman SF. A cell surface receptor defined by a mAb mediates a unique type of cell death similar to oncosis. *Proc Natl Acad Sci U S A* 1998;95:6290–5.
37. Bhat NM, Bieber NM, Stevenson FK, Teng NNH. Rapid cytotoxicity of human B lymphocytes induced by VH4-34 (VH4.21) gene-encoded monoclonal antibodies. *Clin Exp Immunol* 1996;105:183–90.
38. Loo D, Pryer N, Young P, et al. The glycotope-specific RAV12 monoclonal antibody induces oncosis *in vitro* and has antitumor activity against gastrointestinal adenocarcinoma tumor xenografts *in vivo*. *Mol Cancer Ther* 2007;6:856–65.
39. Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* 1995;146:3–15.
40. Kroemer G, El-Deiry WS, Golstein P, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ* 2005;12:1463–7.
41. Mateo V, Brown EJ, Biron G, et al. Mechanisms of CD47-induced caspase-independent cell death in normal and leukemic cells: link between phosphatidylserine exposure and cytoskeleton organization. *Blood* 2002;100:2882–90.
42. Mone AP, Cheney C, Banks AL, et al. Alemtuzumab induces caspase-independent cell death in human chronic lymphocytic leukemia cells through a lipid raft-dependent mechanism. *Leukemia* 2006;20:272–9.
43. Bretscher A, Edwards K, Fehon RG. ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol* 2002;3:586–99.
44. Martin MJ, Muotri A, Gage F, Varki A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med* 2005;11:228–32.
45. Bhat NM, Bieber NM, Hsu FJ, et al. Rapid cytotoxicity of human B lymphocytes induced by VH4-34 (VH4.21) gene-encoded monoclonal antibodies. II. *Clin Exp Immunol* 1997;108:151–9.
46. Krengel U, Olson LL, Martinez C, et al. Structure and molecular interactions of a unique anti-tumor antibody specific for *N*-glycosyl GM3. *J Biol Chem* 2004;279:5597–603.
47. Pérez A, Lombardero J, Mateo C, et al. Immunogenetic analysis of variable regions encoding AB1 and g-type AB2 antibodies from the NeuGc-containing ganglioside family. *Hybridoma* 2001;20:211–21.
48. Moreno E, Lanne B, Vazquez AM, et al. Delineation of the epitope recognized by an antibody specific for *N*-glycosylneuraminic acid-containing gangliosides. *Glycobiology* 1998;8:695–705.
49. Diaz A, Alfonso M, Alonso R, et al. Immune responses in breast cancer patients immunized with an anti-idiotypic antibody mimicking NeuGc-containing gangliosides. *Clin Immunol* 2003;107:80–9.