A Monoclonal Antibody That Induces Neuronal Apoptosis Binds a Metastasis Marker

Li-tao Zhong, Adriana Manzi, Evan Skowronski, Lucia Notterpek, Arvan L. Fluharty, Kym F. Faull, Irene Masada, Shahrooz Rabizadeh, Maria Varsanyi-Nagy, Youlin Ruan, Justin D. Oh, Larry L. Butcher, and Dale E. Bredesen

Interdepartmental Program in Neuroscience [L. Z., S. R.], Mental Retardation Research Center [A. L. F.], and Department of Psychiatry and Biobehavioral Sciences and the Neuropsychiatric Institute [K. F. F.], University of California, Los Angeles, California 90024; Program on Aging, The Burnham Institute, La Jolla, California 92037 [L. Z., S. R.]; Glycobiology Program, Cancer Center, University of California, La Jolla, California 92030 [M. J.]; Lawrence Livermore National Laboratory, Livermore, California 94551 [E. S.]; Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305-5125 [L. N.]; Glyko, Inc., Novato, California 94949 [I. M.]; Vivorx, Inc., Santa Monica, California 90404-2429 [M. V. N.]; Biomedical Sciences Graduate Program, University of California, San Francisco, California 94143 [Y. R.]; Department of Psychology, University of California, Los Angeles, California 90095-1563 [J. D. O., L. L. B.]; and Buck Institute for Age Research, Novato, California 94945 [D. E. B.]

ABSTRACT

The cell surface molecules controlling apoptosis in cortical neurons are largely unknown. A monoclonal antibody was derived that induces cultured neocortical neurons to undergo apoptosis. A Fab fragment of the antibody, however, lacked the ability to induce cell death. The antigen was purified, and characterized by compositional analysis, fast atom bombardment (FAB) mass spectrometry, sequential exoglycosidase treatments, methylation analysis, and 1H-nuclear magnetic resonance spectroscopy, proving to be isoglobotetraosylceramide (IsoGb4). IsoGb4 has been shown previously to be a metastasis marker, antibodies against which block metastases in a mammary adenocarcinoma model (S. A. Carlsen et al., Cancer Res., 53: 2906–2911, 1993). Addition of the purified antigen to cells lacking this glycolipid demonstrated that it is capable of functioning as a portable apoptosis-transducing molecule. Intracellular ceramide levels were increased after the treatment with the apoptosis-inducing antibody, but the membrane sphingomyelin level remained unchanged. Fumonisin B1 inhibited both the ceramide increase and the apoptosis induced via IsoGb4, which indicated that the ceramide synthase pathway is likely to be involved in apoptosis induction by IsoGb4.

INTRODUCTION

Apoptosis is generally accorded an important role in brain development, neoplasia, response to viral infections, and numerous other cellular processes. However, very little is known about cell surface molecules that participate in the induction of apoptosis in neurons. In some nonneural cells, Fas/Apo-1, which is a member of the TNFR superfamily, functions as a proapoptotic receptor (1, 2). Other members of the TNFR superfamily, such as TNFR1 (3) and lymphotixin-β receptor (4), may also transduce apoptotic signals (conversely, TNF has been reported to have an antiapoptotic effect on neurons; 5). Another superfamily member, the low-affinity neurotrophin receptor (p75NTR), has been shown to mediate apoptosis in some developing neurons (6–8), but the vast majority of mature neocortical neurons do not express p75NTR (9).

We report here the results of a study designed to identify proapoptotic receptors and other proapoptotic surface molecules displayed by neurons. The approach taken was to generate MAbs against a neural cell line and then to screen the antibodies for their abilities to induce neural apoptosis. A MAb derived in this way proved to bind to IsoGb4, which has been demonstrated previously to be a marker for highly metastatic cells in a rat mammary adenocarcinoma model (10). Furthermore, antibodies to IsoGb4 have been shown to inhibit metastases in this same model (10). Our results support the notion that, in addition to proteinaceous cell death receptors such as Fas, certain glycolipids such as IsoGb4 may also serve as proapoptotic cell surface molecules, and that such molecules may play roles in cellular processes such as neoplasia and developmental neuronal cell death.

MATERIALS AND METHODS

Cell Culture. CSM25 cells (described in the “Results” section) were grown in DMEM with 10% FBS at 34°C with 5% CO2.

Primary cortical neuron cultures were prepared as described previously by Notterpek and Rome (11).

Production and Screening of MAbs. BALB/c mice (7–8 weeks old) were immunized by i.p. injection of 107 CSM25 cells suspended in 0.5 ml of PBS; the immunization was repeated each week for 6 weeks. Spleen cells from the immunized mice were fused with P3 myeloma cells to produce MAb hybridomas (12). To screen for apoptosis-inducing MAbs, 25 μl of supernatant from each well was transferred to wells of 96-well plates that had CSM25 grown in 100 μl of medium. After 24 h, the wells were examined microscopically for apoptosis. The death of cells was confirmed by staining with propidium iodide or acridine orange (see the “Results” section below). Once supernatant was found to induce apoptosis reliably, the hybridoma cells in the corresponding well were further subcloned until a single clone was isolated. Clones were expanded by growing in serum-free hybridoma medium (Life Technologies, Inc.) plus 5% FBS supplemented with hypoxanthine and thymine.

Immunohistochemical Staining. To prepare adherent cell lines for immunostaining, cells were grown on chamber slides (Collaborative Research, Inc.) coated with polylysine. For nonadherent cells, cells were deposited on glass

Received 2/19/01; accepted 5/29/01.

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.

1 Supported in part by NIH Grants NS33376 (to D. E. B.) and T32-NS07356 (to Y. R.), and by a grant from the San Diego chapter of the American Parkinson Disease Association. Compositional analyses were performed at the University of California-San Diego Glycobiology Core.

2 Present address: University of California, San Francisco, Department of Physiology, Box 0725, San Francisco, California 94143.

3 Present address: Clinical Pharmacology, NIH, NINDS-ETB, Building 10, Room SC201, 9000 Rockville Pike, Bethesda, MD 20892.

4 To whom requests for reprints should be addressed, at Buck Institute for Age Research, 8001 Redwood Boulevard, Novato, CA 94945-1400. Phone: (415) 209-2090; Fax: (415) 209-2230; E-mail: dbredesen@buckinstitute.org.

11 To whom requests for reprints should be addressed, at Buck Institute for Age Research, 8001 Redwood Boulevard, Novato, CA 94945-1400. Phone: (415) 209-2090; Fax: (415) 209-2230; E-mail: dbredesen@buckinstitute.org.

12 The abbreviations used are: TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; GB3, globotoxosylceramide; GB4, globotetrasylceramide; IsoGb4, isoGb4; BTN, fetal bovine serum; FAB, fast atom bombardment; HPLC, high-performance liquid chromatography; SOD, superoxide dismutase; MAb, monoclonal antibody; NAIM-1, neural apoptosis-inducing monoclonal 1; G3L, glycopterolipid; NMR, nuclear magnetic resonance; TOCSY, total correlation spectroscopy.
slides by centrifugation at 500 rpm for 3 min on a side-spin centrifuge. For staining rat tissues, a 3-month-old Sprague Dawley rat was killed by ether and the organs were quickly removed and immediately immersed in liquid nitrogen. Frozen tissues were then cut on a freezing microtome into 15-μm sections, which were picked up on slides.

For staining, the slides were washed once in PBS and fixed with 1% formalin for 10 min. The fixed cells were washed twice in PBS, incubated in a 5% PBS in PBS blocking solution for 1 h and then were incubated for 3 h with NAIM-1 antibody (0.01 μg/ml) or control mouse IgG3. The slides were washed three times in PBS and further incubated with rabbit antimouse IgG antibody conjugated with alkaline phosphatase (1 ng/ml; Promega) for 1 h and were washed 5 times in PBS color was developed in nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution.

NAIM-1 Antibody Purification. Cultured CSM25 cells were washed in PBS and extracted with chloroform-methanol-water (30:60:8) as previously described (13). The extraction was then loaded on a DEAE-Sephadex A-25 resin column, and the flow-through solvent was collected and reapplied to the column three times. The final flow-through was collected and dried and redissolved in chloroform-methanol-water (2:4:3). The solution was then loaded onto a standard Sep-Pak C18 cartridge (Millipore), which was pre-washed sequentially by the injection of 5 ml each of water, methanol, chloroform, methanol, and water, and the eluate collected and reapplied to the cartridge twice, followed by 5 ml of the same solvent. The cartridge was then washed with 5 ml water and the NAIM-1 antigen eluted with 5 ml of methanol. The eluted sample was evaporated to dryness, resuspended in 0.5 ml of chloroform-methanol (1:1), and injected into an IatroScan column (1.2 × 120 cm; Iatron Laboratories, Tokyo, Japan) which was prewashed with 30 ml of chloroform-methanol-water (48:42:10). The column was eluted with a linear gradient from (53:42:5) to (48:42:10) over 60 min at a flow rate of 0.4 ml per min. Fractions of the eluates were collected each 0.5 min yielding 0.2-ml fractions. The samples were dried by Speed-vac, resuspended at a concentration of ~1 mg/ml in chloroform-methanol (1:1) and applied on a TLC plate at 100 μl per spot (concentrations were estimated by comparing TLC band strengths with those obtained from GB4 and GB3; Matreya, Inc.). The plate was developed with a mixture of 60 ml chloroform, 42.5 ml methanol, and 10 ml water. For TLC purification, the NAIM-1 antigen was visualized by iodine vapor staining and scraped from the plate. The scraped gel was then collected into a microfuge tube and suspended in 1 ml of chloroform-methanol (1:1), vortexed and sonicated and eventually centrifuged at 14,000 rpm in a microcentrifuge for 5 min. The supernatant, containing NAIM-1 antigen, was passed through a C18 Sep-Pak cartridge and the NAIM-1 antigen eluted in methanol.

Ceramide Glycanase Assay. A NAIM-1 antigen sample (10 mg in chloroform-methanol, 1:1) was mixed with sodium cholate (10 mg) in chloroform-methanol (1:1, v/v) and was evaporated to dryness. The residue was dissolved in sodium acetate buffer (0.1 M pH 5.0; 10 ml). Ceramide glycanase (V-Labs; 20 μM/ml, 10 ml) was then added and incubated for 24 h at 37°C (14). After reaction, the samples were dried under nitrogen, resuspended in 3 ml of water, and dialyzed to remove salts. The dialyzed sample was dried down in a Speed-vac and used for further analysis.

Exoglycosidase Digestion. The exoglycosidase digestion assay followed previously published procedures (15). NAIM-1 antigen samples were dried and then resuspended in 50 ml of 0.05 M sodium citrate buffer (pH 4.5), containing 1.0 mg/ml sodium taurodeoxycholate and 50 μM of α-galactosidase, or 50 μM of β-galactosidase, or 100 μM of β-N-acetylhexosaminidase, or combinations of these enzymes. The samples were incubated at 37°C for 18 h. After digestion, the samples were processed as in the procedures after ceramide glycanase treatment.

Methylation Analysis. The permethylation of the GSL samples (NAIM-1 antigen and GB4) followed the procedure described previously (16). The sample (~50 μg) was dried in a glass vial under vacuum over phosphorus pentoxide (P2O5) and then dissolved in dry DMSO (100 μl) with the aid of a stirring bar. A slurry of NaOH in dry DMSO (200 μl) was prepared by grinding in a mortar 7 NaOH pellets with 3 ml of DMSO) was added and mixed. Methyl iodide (ICl3; 200 μl) was added and the vial was capped and placed on a stirring plate for 30 min. The reaction was stopped by slowly adding 500 μl of water. The permethylated product was recovered by extraction with chloroform and washing the organic phase with water until neutral. After drying the chloroform layer, the permethylated product was submitted to acetolysis/ hydrolysis with 300 μl of 0.5 n sulfuric acid in 90% acetic acid for 6 h at 80°C.

After cooling to room temperature, the solution was neutralized by adding 1 M sodium hydroxide, and evaporated with the aid of 0.5 ml of ethanol. After overnight drying under vacuum over P2O5, partially methylated sugars were dissolved in 10 mM ammonium hydroxide (0.5 ml) and reduced with sodium borodeuteride by overnight incubation at room temperature. Excess borodeuteride was destroyed by dropwise addition of glacial acetic acid, and the sample was dried in a Speed-vac. Methyl borates were eliminated by repeated evaporation with 0.5-ml aliquots of acidic methanol. This procedure was repeated five times, until the crystals were clear. After overnight drying under vacuum over P2O5, the sample was acetylated by heating with 0.5 ml of acetic anhydride at 100°C for 3 h. Partially methylated/partially acetylated monosaccharide alditoils were recovered in the organic layers by partition in chloroform-water (0.5 ml; 1:1). The organic layer was dried, dissolved in 10 ml of chloroform, and analyzed by gas liquid chromatography-electrospray ionization mass spectrometry using a Hewlett-Packard 5890 gas chromatograph with a 5971 MS unit. The sample was injected into a DB-5 fused silica capillary column. The run was started at 50°C, this temperature was kept for 2 min, and then a gradient from 50°C to 150°C at 20°C/min (5 min), and from 150°C to 250°C at 4°C/min (25.0 min) was used for elution. The run was monitored by electron impact mass spectrometry, and the peaks assigned on the basis of their fragmentation pattern and retention times as compared with those of known standards (17, 18).

NMR. The sample (30 μg) was dissolved in 100 μl of deuterated chloroform-methanol (CCl3D:MeOD; 1:1, v/v) to exchange hydroxyl protons for deuterons, and dried down in a Speed-vac. This process was repeated four times. Finally, the sample was dissolved in 40 μl of hexadeuterated-DMSO (DMSO-d6) containing 4% deuterium oxide (D2O) and transferred to a Nano-cel column. One-dimensional (Fig. 3), and two-dimensional TOCSY 1H-NMR spectra were obtained in a Varian Unity Plus 500 MHz spectrometer fitted with a Nano-NMR (Varian Applications Lab., Palo Alto, CA). The Nano-NMR probe spins samples rapidly (~2 kHz) at the magic angle to remove the magnetic susceptibility contributions to the 1H-NMR line widths. One-dimensional spectra were obtained using presaturation of the hydrogen oxygen deuterium peak. TOCSY two-dimensional spectra were obtained using a 10-kHz MELEV spin lock of either 70 or 120 ms duration. Resonances were referenced to the DMSO multiplet at 2.49 ppm.

Mass Spectra (FAB). Mass spectra of NAIM-1 antigen fractions were obtained by negative ion mode FAB mass spectrometry (19). The NAIM-1 antigen sample, dissolved in chloroform-methanol (1:1), was applied (2 μl) on the surface of the static probe tip, which had been smeared with 1 μl of a tetramethylurea/triethylamine matrix. The sample was scanned [VG ZAB-SE; VG Analytical, Manchester, United Kingdom; 8 kV accelerating potential, cesium bombardment at 22 kV and 2 mA, instrument mass resolution (M/DM, 10% valley of 1000)] from m/z 2100 to 500. An average spectrum was obtained from six to eight independent scans using a multichannel analyzer. The same material after peracetylation with trifluoroacetic acid-glacial acetic acid, 1:1, at room temperature for 24 h was analyzed in the positive ion FAB using meta-nitrobenzyl alcohol as matrix. The data were collected by scanning from m/z 2600 to 700 with an instrument resolution of about 1500 (M/DM, 10% valley).

Incorporation of IsoGb4/NAIM-1 Antigen into Cells. HeLa or Ramos cells were grown in wells of 12-well tissue culture plates at 37°C, 5% CO2 in DMEM containing 10% FBS. For incorporation experiments, cells were washed three times with serum-free DMEM, and then maintained in DMEM containing 1% FBS. At the beginning of the experiment, each well contained 10,000 HeLa cells or 50,000 Ramos cells. HPLC purified NAIM-1 antigen was suspended in 50 μl of methanol-chloroform (1:1) at a concentration of 5 mg/ml in a 1.5-ml microcentrifuge tube. A 500-μl sample of boiling distilled water was added rapidly to the tube, and the tube was heated in a boiling water bath for 10 min. Finally, the sample was dissolved in 40 μl of water at a concentration of 1 mg/ml and incubated in 5% CO2 at 37°C. After 72 h, HeLa cells were removed from the plates by trypsinization for 10 min and were washed three times in DMEM with 10% FBS. Ramos cells were washed once in DMEM and then treated in the same manner as were HeLa cells. One purpose of trypsin treatment was to remove any NAIM-1 antigen that was nonspecifically associated with cell surface proteins. The cells were then subjected to either the apoptosis-inducing assay or immunostaining.

Downloaded from cancersres.aacrjournals.org on April 10, 2017. © 2001 American Association for Cancer Research.
Transfection with Bcl-2, p35, and Cu-Zn SOD Expression Constructs. For Bcl-2 transfection, an 850-bp EcoRI-HindIII fragment containing the open reading frame of the human bcl-2 cDNA was obtained from Professor Michael Cleary at Stanford University (Stanford, CA). It was inserted into the pBabe-puro retroviral expression vector supplied by Hartmut Land (Imperial Cancer Research Fund, London, United Kingdom). This plasmid and pBabe-puro alone as control were transfected into the packaging cell line GP+86 (20), and stable transformants were selected in 5 μg/ml puromycin (21). Cell lines with the highest viral titer (~10^7/ml) were used to prepare viral stocks. After being infected with the recombinant retroviruses, stable CSM25 transformants were selected in 10 μg/ml puromycin and pooled (22). Transfection of p35 and Cu-Zn SOD was achieved by the same method as described by Rabizadeh et al. (23, 24).

Ceramide and Sphingomyelin Hydrolysis Assays. Ceramide was measured by the diacylglycerol kinase assay as described previously (25). Cells were extracted with chloroform:methanol:1N HCl (100:100:1). The organic phase extracts were dried under N₂ and subjected to mild alkaline hydrolysis (0.1 N methanoic KOH for 1 h at 37°C). Samples were reextracted, and the organic phase was dried under N₂. Ceramide in each sample was resuspended in a 100-μl reaction mixture containing 150 μg of cardiolipin, 280 μM diethylenetriaminepentaacetic acid, 51 mM octyl-β-D-glucopyranoside, 50 mM NaCl, 51 mM imidazole, 1 mM EDTA, 1.5 mM MgCl₂, 2 mM DTT, 0.7% glycerol, 70 μM β-mercaptoethanol, 1 mM ATP, 10 μCi of [γ-32P]ATP (3000 Ci/mmol), and 35 μg/ml diacylglycerol kinase (pH 6.5). After 30 min at room temperature, the reaction was stopped by extraction of lipids with 1 ml of chloroform:methanol:1N HCl (100:100:1), 170 μl of buffered saline solution [135 mM NaCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, and 10 mM HEPES (pH 7.2)], and 30 μl of 100 mM EDTA. The lower organic phase was dried under N₂. Ceramide 1-phosphate was resolved by TLC on silica-gel-60 plates using a solvent system of chloroform:methanol:1N HCl (100:100:1), 170 μl of buffered saline solution [135 mM NaCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, and 10 mM HEPES (pH 7.2)], and 30 μl of 100 mM EDTA. The lower organic phase was dried under N₂. Ceramide 1-phosphate was resolved by TLC on silica-gel-60 plates using a solvent system of chloroform:methanol:acetic acid (65:15:5) and detected by autoradiography, and incorporated 32P was quantified by liquid scintillation counting of the corresponding TLC spots. Ceramide 1-phosphate was verified as a homogenous sample, which is an IgG3, was added at a concentration of 10^6 μg/ml, and incubated for 48 h. Propidium iodide (10 μg/ml) was then added and photographs were taken 30 min later. a and c are from the same field of neurons treated with control IgG3, b and d are from the same field of neurons treated with NAIM-1. Some nuclei in d are fragmented, and others homogeneously stained, both characteristic of apoptosis. a and b, phase contrast; c and d, fluorescence; e, photomicrograph of NAIM-1 immunostaining of rat cortical section, demonstrating the expression of NAIM-1 antigen by neocortical neurons (neurons of the hippocampus did not demonstrate similar staining). The section was prepared from a frozen 3-month-old Sprague Dawley rat brain, fixed in 3% formalin for 10 min, and incubated with NAIM-1 (1 μg/ml). Horseradish peroxidase-conjugated secondary antibody was used, followed by development with 3,3′-diaminobenzidine. All, ×200.

RESULTS

A Neural Apoptosis-inducing MAb (NAIM-1). To identify cell surface molecules controlling neuronal cell death, subclones of the temperature-sensitive neural cell line, CSM 14.1 (26, 27), were produced and screened for their propensities to undergo apoptosis after serum withdrawal. Of 43 subclones, the most apoptosis-prone was designated CSM25, and this subclone was used to produce MAbs (12). Thirteen thousand MAbs were screened for apoptosis induction in CSM25. Only one MAb was found to induce apoptosis in CSM25, and this was designated NAIM-1. Immunohistochemical studies demonstrated binding of NAIM-1 to neurons of the adult rat neocortex, but not to astrocytes or oligodendroglia (Fig. 1). Studies in primary cultures demonstrated concordance between expression of the antigen and apoptosis induction by NAIM-1; neurons (Fig. 1) and microglia were induced to undergo apoptosis by NAIM-1, whereas astrocytes and oligodendroglia were not. Induction of apoptosis by NAIM-1 was inhibited by the expression of bcl-2, p35, and sod1, confirming that NAIM-1 does not simply lead to cell death nonspecifically (Fig. 2), but rather functions via an apoptotic pathway. Furthermore, Annexin-V staining and labeling with a biotinylated caspase inhibitor (28) also demonstrated that NAIM-1 induces apoptosis (data not shown). The Fab fragment of the NAIM-1 antibody did not induce apoptosis in CSM25 cells (data not shown), indicating that cross-
Fig. 3. Characterization of the NAIM-1 antigen. a, orcinol staining of NAIM-1 antigen and other glycolipids on TLC plate, demonstrating that the NAIM-1 antigen is a glycolipid. The TLC was run with 60:42.5:10 chloroform:methanol:water. Lane 1, 5 μg of neutral GSLs, including (from top to bottom on the TLC plate) cerebroside, lactosyl ceramide, ceramide trihexoside, and globoside (Gb4); Lane 2, 2 μg of Gb4; Lane 3, 2 μg of purified NAIM-1 antigen; Lane 4, 2 μg of Gb4. b, cleavage of the NAIM-1 antigen by ceramide glycanase. NAIM-1 antigen samples (6 μg for each treatment) were enzymatically digested and then were extracted with chloroform and methanol. After centrifugation, the supernatants were lyophilized, resuspended in water, and dialyzed. The samples were then dried down again and dissolved in chloroform/methanol (1:1) and loaded onto the TLC plate. The plate was then immunostained using NAIM-1 as primary antibody. Lane 1, NAIM-1 antigen treated with ceramide glycanase (20 mU/ml); Lane 2, NAIM-1 antigen treated with ceramide glycanase reaction buffer only; Lane 3, NAIM-1 antigen treated with phospholipase C (type I; 10 units/ml; Sigma Chemical Co., St. Louis, MO); Lane 4, NAIM-1 antigen treated with proteinase K (100 μg/ml; Sigma Chemical Co.). c, FAB mass spectrometry, negative ion mode. The signal at m/z 1336.0 was indistinguishable from what would be expected from deprotonated NAIM-1 antigen containing Δ₆₉-tetra(c)anoic acid as the fatty acid constituent (calculated 1335.9 for the (M-H)⁻ ion). The signal at 1336.0 was obtained from one of the two peaks of HPLC-purified NAIM-1 antigen, with the other peak giving m/z 1225.8, compatible with a 16:0 fatty acyl constituent. The spectrum that included the 1336.0 peak also showed low-intensity fragment ions resulting from the consecutive losses of 203.1 and 162.1 daltons from the (M-H)⁻ consistent with consecutive losses of N-acetyl-hexosamine (calculated loss of 203.1 daltons) and a hexose moiety (calculated loss of 162.1 daltons). The spectrum also revealed a weak signal at m/z 1333.9 consistent with the presence of a small amount of Δ₆₉-tetra(c)anoic acid constituent. Confirming the interpretation of these data, positive ion FAB of the peracetylated HPLC-purified material revealed a clean spectrum containing only two significant ions: a strong signal at m/z 1823.9, which was in close agreement with what would be expected for the (MH-H₂O)⁺ cation derived from NAIM-1 antigen containing Δ₆₉-tetra(c)anoic acid (calculated m/z = 1824.0), and an intense ion at m/z = 784.0, which was unexplained. Negative ion FAB of other preparations of NAIM-1 antigen again revealed signals demonstrating that the predominant fatty acyl components are 24:1 and 16:0, with minor components of 24:0 and 24:2. Negative ion FAB spectra were obtained from chloroform/methanol (1:1) sample solutions by applying 1 μl to the static probe tip surface that had been smeared with tetramethylurea/triethylamine (1:1). Data were collected (VG ZAB-SE; 8 kV accelerating potential, cesium bombardment at 22 kV and 2 Å, instrument mass resolution (M/ΔM, 10% valley) of about 1000) by scanning from m/z 2100 to 500; and the average spectrum, collected from six to eight independent scans using a multichannel analyzer, was smoothed, centroided, and mass measured, using for calibration the signals from a separate introduction of cesium iodide. The pattern and relative intensities of the (M-H)⁻, (M-203.1)⁻ and (M-(203.1+162.1)⁻ ions from the NAIM-1 antigen matched what was obtained from Gb3 and Gb4 standards (Matreya, Inc.) when analyzed under the same conditions. d and e, 1H-NMR analysis of the purified NAIM-1 antigen. Exchangeable protons were converted to deuteriums by repeated dissolution/evaporation in CDCl₃/MeOD (2:1, v/v). The sample (~40 μg) was dissolved in 40 μl of DMSO-d₆/D₂O 96:4 (v/v) and placed in a Nano cell for analysis in a Varian Unity Plus 500 MHz NMR spectrometer (Varian Applications Laboratories, Palo Alto, CA) using a Nano-NMR probe at 30°C. This probe spins rapidly (~2 kHz) at the magic angle removing magnetic susceptibility contributions to the ¹H-NMR line widths. This produces high resolution spectra with small sample volumes (~40 μl), thus lowering solvent contaminants and background (45). Chemical shifts are relative to tetramethylsilane but were actually referenced to the Me₂SO multiplet at 2.49 ppm.
linking of the NAIM-1 antigen is required to initiate the cell death process.

The Purification and Identification of NAIM-1 Antigen. The target antigen was found to partition into the chloroform:methanol phase of CSM25 cell extracts, suggesting a lipid component (Fig. 3). Orcinol staining was positive, further suggesting that the antigen is a glycolipid; lack of staining with resorcinol excluded identification as a ganglioside, and lack of binding to a DEAE column confirmed the neutral character of the glycolipid. High-performance TLC followed by immuno-overlay with NAIM-1 was used to facilitate the molecular further. After treatment with endoglycosyl ceramidase and rechromatography, no band was detected, indicating that the antigen is a GSL with a reducing sugar in the ceramide-proximal position (Fig. 3). Comparison with GSL standards chromatographed in parallel showed that the NAIM-1 antigen has a mobility similar to that of GB4 (Fig. 3). Both GB4 and the NAIM-1 antigen produced doublets on HPTLC, indicating ceramide fatty acyl chain length heterogeneity.

Monosaccharide compositional analysis by high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) after acid hydrolysis (2 m trifluoroacetic acid, 4 h, 100°C) indicated the presence of GalNAc, Gal, and Glc in the molar ratio 1:2:1 (data not shown. GalNAc is converted to GalNH₂ by acid hydrolysis). Sequential exoglycosidase treatments followed by gel electrophoresis of the fluorescently tagged oligosaccharides revealed the following sequence and anomericity: GalNAc-β-Gal-α-Gal-β-Glc-β-Cer. Furthermore, treatment with B-N-Ac-hexosaminidase destroyed immunoreactivity, suggesting a requirement for the terminal N-acetylgalactosamine (data not shown). FAB mass spectrometry, in the negative ion mode, was performed on each band of the GSL doublet (Fig. 3). The upper (high mobility) band gave a spectrum compatible with a 24-carbon fatty acid containing one double bond (C24:1), with minor components of C24:0 and C24:2, whereas the lower band produced a spectrum compatible with C16:0. Both spectra were consistent with the presence of sphingosine as the long chain base.

The NAIM-1 antigen was further purified by HPLC on an Iatrobeads column and submitted to methylation analysis and ¹H-NMR spectroscopy. Methylation analysis (Table 1) confirmed the presence of terminal GalNAc and of substitution of the Glc unit at OH-4. The two internal Gal residues were found to be substituted at OH-3, indicating that the NAIM-1 antigen is GalNAc-β1,3-Gal-α1,3-Gal-β1,4-Glc-β1,1-Cer, which is IsoGb4. One-dimensional and two-dimensional ¹H-NMR spectroscopy (Fig. 3), using a Nano-NMR probe, confirmed the identity of the monosaccharide units, their anomericity and molar ratio, and the positions of substitution of the internal Gal units. As further confirmation, a sample of IsoGb4 was obtained as a generous gift from Prof. S. Carlsen, and shown by TLC and immunoblot analysis to be indistinguishable from the NAIM-1 antigen (see below).

Table 1  Methylation analysis of the purified NAIM-1 antigen

<table>
<thead>
<tr>
<th>Compound</th>
<th>GalNAc</th>
<th>Gal</th>
<th>Glc</th>
</tr>
</thead>
<tbody>
<tr>
<td>N,3,4,6-tetramethyl</td>
<td>2,4,6-trimethyl</td>
<td>2,3,6-trimethyl</td>
<td>3,6-trimethyl</td>
</tr>
<tr>
<td>Deduced glycosidic linkages</td>
<td>Terminal</td>
<td>3–</td>
<td>4–</td>
</tr>
<tr>
<td>Gal</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>NAIM-1 antigen</td>
<td>1.0</td>
<td>1.9</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* See "Materials and Methods."

The Portable Nature of the NAIM-1 Antigen. It is well known that exogenously added GSL may incorporate into cell membranes, either by integrating into the external leaflet of the lipid bilayer or by forming micelles that associate with cell surface proteins. Lipid-lipid interactions between GSL and membrane lipids permit penetration into the lipid phase with eventual disposition of the GSL in a native state. However, associations with membrane proteins via the ceramide moiety may also occur, as well as transport by lipid-binding proteins such as the saposins (29). Thus, the NAIM-1 antigen should be capable of integrating into the cell membrane. This feature would

![Image](https://example.com/image.png)

Fig. 4. Induction of apoptosis in Ramos cells by NAIM-1 (10 µg/ml) after the incorporation of IsoGb4. a–d, photomicrographs of Ramos cells (>200); a, lack of immunostaining of Ramos cells with NAIM-1 antibody (bright field), b, immunostaining of Ramos cells with NAIM-1 antibody following the addition of IsoGb4 purified from C25 cells (bright field), c, lack of apoptosis induction by NAIM-1 in the absence of IsoGb4 addition (phase contrast), d, apoptosis induction by NAIM-1 after IsoGb4 addition (phase contrast). e, quantitation of viability after addition of GB4, IsoGb4, or neither, followed by addition of NAIM-1 antibody. IsoGb4 (2 µg/ml) purified from C25 and, separately, obtained from Prof. S. Carlsen, was added to Ramos cells grown at 37°C in 5% CO₂ in DMEM with 1% FBS and incubated for 24 h. The cells were then washed once in DMEM, treated with trypsin (2.5 mg/ml) to remove protein-bound IsoGb4, and then washed three more times in DMEM. For a and b, the cells were transferred to a glass slide using a cytospin centrifuge and then fixed in 5% formalin for 10 min, and immunostaining done as described in the legend to Fig. 1. For c and d, cells were plated in 24-well plates at a density of 5 × 10⁶ cells per well; then NAIM-1 (10 µg/ml) was added, and the photos taken after 24 h.
hypothetically allow it to function as a portable apoptosis-mediating receptor.

Mangeney et al. (30) have shown that binding of the B-chain of verotoxin to GB3 of Burkitt’s lymphoma cells is sufficient to induce apoptosis. This suggests that the signal transduction components necessary to transduce an apoptotic signal originating with GSL binding are present in Burkitt’s lymphoma cells that are sensitive to the binding chain of verotoxin. Therefore, purified IsoGb4 was added to Burkitt’s lymphoma cells [the Ramos line, one of those shown to be sensitive to verotoxin (30)]. Immunocytochemistry demonstrated trypsin-resistant association of IsoGb4 with Ramos cells, which were rendered sensitive to apoptosis induction by NAIM-1 (Fig. 4). Neither NAIM-1 nor IsoGb4 alone was sufficient to induce apoptosis in Ramos cells (Fig. 4). Furthermore, HeLa cells were resistant to IsoGb4-mediated apoptosis, suggesting that the signal transduction components necessary for the response to NAIM-1 are not completely functional in HeLa cells.

The portable nature of the NAIM-1 antigen was further demonstrated in cell-to-cell migration studies (Fig. 5). Ramos cells acquired the NAIM-1 antigen/IsoGb4 after 24-h incubation with HS-2 cells (31), a mouse hippocampal cell line expressing IsoGb4, and concurrently acquired sensitivity to NAIM-1 (Fig. 5B). In control studies, after a 24-h incubation with IsoGb4-nonexpressing HeLa cells, Ramos cells remained IsoGb4 negative (Fig. 5A).

The Signal Transduction Pathway of NAIM-1-induced Apoptosis. Because ceramide functions as a second messenger in several apoptotic signal transduction pathways (32–35) and because IsoGb4 contains a ceramide moiety, the role of ceramide in the NAIM-1/IsoGb4 apoptotic pathway was investigated. The intracellular concentration of ceramide increased in CSM25 cells after treatment with NAIM-1 antibody (Fig. 6). Generation of ceramide was detectable by 1 h and reached a plateau at 8 h. This increase in ceramide concentration preceded the appearance of the apoptotic morphological changes.

It has been shown that ceramide, generated in the apoptosis pathways induced by TNF-α, FAS, and ionizing radiation, results from hydrolysis of membrane sphingomyelin by a sphingomyelinase (32–35). In those cases, activation of sphingomyelinase and ceramide generation occurs very rapidly (within seconds to minutes after exposure to these agents). However, the earliest time of elevation of ceramide in CSM25 cells after NAIM-1 antibody treatment was 1 h (Fig. 6). Furthermore, during the time that intracellular ceramide levels were increasing (hour 1–4), no decrease in cellular sphingomyelin content was detected (Fig. 7). These findings made it unlikely that a sphingomyelinase featured in NAIM-1/IsoGb4-induced apoptosis.

A second mechanism for ceramide generation has been reported by Bose et al. (36), in which ceramide synthase leads to an increase in ceramide synthesis. Fusonin B1, a natural product of the fungus Fusarium moniliforme, specifically and potently inhibits ceramide synthase (36). We investigated the possibility of ceramide synthase involvement in NAIM-1-induced apoptosis, and found that 30 min of preincubation of CSM25 cells with 10 μM fusonin B1 reduced NAIM-1-induced apoptosis by ~75% (Fig. 8A) and markedly decreased the NAIM-1-associated ceramide elevation (Fig. 8B). Fusonin B1 alone, however, did not affect the CSM25 cells: 100 μM fusonin incubation for 24 h did not change the cell morphology, growth rate, or viability (data not shown). As confirmation of the existence of a ceramide synthase pathway in CSM25 cells, daunorubicin, which induces apoptosis and ceramide elevation through ceramide synthase (36), was added to the cells. Treatment with 1 μM

![Image](cancerres.aacrjournals.org)
daunorubicin for 24 h led to apoptosis in >50% of CSM25 cells, and this effect was also blocked by fumonisin B1 (data not shown).

**DISCUSSION**

These results demonstrate that IsoGb4 is a cell surface molecule capable of mediating apoptosis in rat neocortical neurons and in the rat neural cell lines CSM14.1 and CSM25. Induction of apoptosis by GSLs may represent a novel control of apoptosis in at least some cells of the central nervous system. It is noteworthy that an antibody to IsoGb4 inhibits pulmonary metastases from the cell line R3230AC (10); although the mechanism of inhibition of metastasis by anti-IsoGb4 antibodies is as yet unknown, induction of apoptosis represents one possibility.

Although the mechanism of apoptosis initiation by IsoGb4 is unknown, induction of apoptosis in Burkitt’s lymphoma cells by verotoxin binding to Gb3 suggests a common mechanism for GSL involvement in the two cell types. Furthermore, the finding that verotoxin binds to Gb3 suggests that there may be an endogenous ligand for neutral GSLs such as IsoGb4. Although the glycans of IsoGb4 and Gb3 are similar, they are not identical, whereas the ceramide portion of the two molecules may be identical (depending on the fatty acid chain length). The results of our studies indicate that ceramide synthase is responsible for the generation of ceramide in NAIM-1/IsoGb4-induced apoptosis. However, other possible mechanisms, such as the release of ceramide by a cellular endoglycosyl ceramidase, as suggested by Hakomori and Igarashi (37), cannot be excluded at this point. Recent reports of the requirement for the GD3 ganglioside in Fas-induced apoptosis by De Maria et al. (38, 39) support the notion that GSLs may be important control points in the apoptotic program; however, one clear contrast between the results of De Maria et al. and the current results is that De Maria et al. found that sphingomyelin hydrolysis was the mechanism of ceramide generation in GD3-dependent apoptosis, whereas we found that ceramide synthase was required for ceramide generation in NAIM-1/IsoGb4-mediated apoptosis.

The finding that IsoGb4 may function as a portable death receptor raises the previously unexplored issue of mobile death receptors and their possible physiological role(s). One potential role would be the coating of invading malignant cells as a local response to neoplastic invasion. Alternatively, because GSLs may be shed from the cell surface, invading malignant cells might conceivably shed apoptosis-mediating GSLs either to destroy surrounding normal cells or to decoys to prevent their own destruction. Whether or not there proves to be a physiological role for any death receptor mobility, the ability to reconstitute the apoptosis program in Ramos cells should aid in the identification of downstream processing, using labeled IsoGb4 and the NAIM-1 MAb. This finding might also conceivably be used in the treatment of solid tumors in which the signaling components of the pathway are intact.

The identification of neutral GSLs of the globoside series as representatives of a novel class of apoptosis receptors in neocortical neurons and Burkitt’s lymphoma cells may offer insight into several previously unexplained findings in diseases featuring cell death dysregulation: first, circulating antibodies against Gal-β1,3-GalNAc, an epitope displayed by both GSLs and glycoproteins, are associated with motor neuron cell death (40); such antibodies could conceivably act in an analogous fashion to NAIM-1 to induce motor neuron apoptosis. Second, mutations in β-hexosaminidase A, although usually associated with Tay-Sachs disease, may also lead to motor neuron degeneration (41, 42); lack of β-hexosaminidase A leads to a marked increase in GSLs with terminal β-linked N-acetylgalactosamines, which would increase potential substrates for cellular endoglycosyl ceramidases. Third, GM1 has been used as a therapeutic agent in Parkinson’s disease and other conditions featuring cell death (43); this could conceivably function as a decoy receptor for an endogenous GSL-binding ligand [although GM1 also displays a Trk-dependent antiapoptotic activity (44)].

GSLs may prove to play a role in diseases featuring cell death dysregulation, and abnormalities in their control may involve antibody binding to GSLs, genetic abnormalities in synthesis or degradation, or binding by microbial toxins.

**ACKNOWLEDGMENTS**

We thank Sven Carlsen (Department of Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada) for the kind gift of IsoGb4; Robert Yu (Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA) for antibody to Gal-Gb3; Kenneth Conklin for assistance with mass spectral analyses; Christine Stortz (UCSD) for methylated galactose standards; Diane Chugani, Leonard Conklin for assistance with mass spectral analyses; Christine Stortz (UCSD) for methylated galactose standards; Diane Chugani, Leonard Rome, and Michelle Durand (UCLA) for the CSM 14.1 cells; John Reed (Burnham Institute, La Jolla, CA) for RAMOS cells; Harold Weiner (UCLA) for mentoring of L. Z.; Paul Keifer for running the NMR spectra; Diana Matrano for technical assistance; Denise Huajardo, Crystal Herndon, and Elana Cooper for administrative assistance; and Hudson Freeze, Charles Glabe, Ronald Schnaar, Richard Kolesnick, Robert Yu, Douglas Green, and Minoru Fukuda for helpful discussions.
REFERENCES


Downloaded from cancercases.aacrjournals.org on April 10, 2017. © 2001 American Association for Cancer Research.