

Development and characterization of a novel anti-ceramide antibody

Kannan Krishnamurthy, Somsankar Dasgupta, and Erhard Bieberich¹

School of Medicine, Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA 30912

Abstract Ceramide is emerging as a key sphingolipid that regulates a variety of cellular processes. To facilitate the study of ceramide localization and its interaction with cellular proteins, we have developed a novel antibody against ceramide. Our results indicate that the antibody (rabbit IgG) specifically recognizes ceramide in lipid overlay assays and detects ceramide species with different fatty acid chain lengths that include C2, C8, C16, C18, C20, and C24. The new antibody was compared with the commercially available anti-ceramide antibody (mouse IgM) in immunocytochemistry experiments to study the localization of ceramide. Although both antibodies stain the same regions on the cell membrane, the rabbit IgG reveals the distribution of ceramide in compartments that are not well identified with the commercially available antibody. In addition to staining of ceramide in protrusions of the plasma membrane, the rabbit IgG also detects ceramide in the Golgi apparatus. Pharmacological depletion or increase of ceramide levels results in a corresponding change in staining intensity, confirming the specificity of the antibody. These results indicate that the rabbit IgG is a suitable antibody to determine the localization of ceramide and its interaction with proteins by immunocytochemistry.—Krishnamurthy, K., S. Dasgupta, and E. Bieberich. **Development and characterization of a novel anti-ceramide antibody.** *J. Lipid Res.* 2007. 48: 968–975.

Supplementary key words rabbit immunoglobulin G • intracellular ceramide localization • immunocytochemistry

Ceramide and its derivatives are now recognized as key lipid mediators involved in a variety of cell signaling events, such as stress response (1), apoptosis (2–5), and differentiation (6, 7). De novo ceramide synthesis is initiated by the condensation of serine and palmitoyl-CoA in the endoplasmic reticulum, catalyzed by serine palmitoyl transferase (8). Ceramide is then transported to the Golgi, where it acts as a precursor for the synthesis of other sphingolipids (9). Ceramide is also generated from sphingomyelin by the action of various isoforms of sphingomyelinase at the cell membrane and in lysosomes.

Either one or both of these pathways are activated in response to diverse stimuli, leading to ceramide generation and the initiation of ceramide-mediated cell signaling events.

In recent years, it has become apparent that various pools of ceramide and its derivatives can coexist in a cell. Ceramide, generated in response to various stimuli, has been reported to be increased in different organelles, including the mitochondrion (10) and the nucleus (11, 12). Furthermore, being a second messenger, ceramide has been shown to activate a variety of protein kinases [e.g., Kinase Suppressor of Ras (KSR) and protein kinase C ζ] (13–15), protein phosphatases (e.g., PP1 and PP2A) (16, 17), and a protease (cathepsin D) (18). Differential activation of these proteins determines the nature of the signal transduced downstream of ceramide upregulation. To elucidate the exact role of ceramide in various signaling pathways, we need to be able to examine ceramide in its cellular context. Common methods to measure ceramide content in cells, such as TLC using radioactive precursors, the diacylglycerol kinase assay, or even mass spectrometry, reflect global changes in ceramide content in the entire population of cells. These methods, reviewed previously (19), do not give us precise information about the subcellular localization of ceramide or reveal its interaction with cellular proteins.

Ceramide-specific antibodies can be used as a tool to overcome these problems and address other issues, such as the intracellular transport of ceramide and the uptake and trafficking of exogenously added ceramide. There are two commercially available antibodies that have been reported to recognize ceramide. An extensive study has been performed comparing both antibodies (20). This study concluded that the polyclonal anti-ceramide mouse IgM preparation (currently available only from Germany) is specific for ceramide and dihydroceramide, whereas the monoclonal mouse IgM antibody (available in the

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Abbreviations: GlcCer, glucosylceramide; KLH, keyhole limpet hemocyanin; NB-dNJ, *N*-butyl-deoxynojirimycin.

¹To whom correspondence should be addressed.

e-mail: ebieberich@mail.mcgc.edu

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United States) recognizes dihydroceramide, phosphatidylcholine, and sphingomyelin. Although this study focused on identifying the structural determinants of sphingolipids recognized by both of these antibodies, it did not provide experimental evidence for the utility of either antibody for immunocytochemical studies. To resolve this issue, we generated a novel antibody against ceramide raised in rabbit. Our primary objective was to identify an antibody that is specific for ceramide and that can be used for the immunocytochemical detection of ceramide in fixed or living cells. In addition, a novel assay was developed that allows for the analysis of the specificity of anti-ceramide antibodies used for immunocytochemistry.

EXPERIMENTAL PROCEDURES

Materials

New Zealand White female rabbits were purchased from Myrtle's Rabbitry (Thompson Station, TN). *D-erythro*-C18 ceramide was from Matreya (Pleasant Gap, PA). Hoechst 33258, myriocin, HRP-conjugated anti-rabbit IgG, keyhole limpet hemocyanin (KLH), and SIGMA FAST™ *o*-phenylenediamine dihydrochloride peroxidase substrate tablets were from Sigma-Aldrich (St. Louis, MO). Immulon 1B flat-bottomed 96-well microtiter plates were from Thermo Electron Corp. (Milford, MA). SphingoStrips™, Alexa Fluor® 647-conjugated phalloidin, and Alexa Fluor® 594-conjugated wheat germ agglutinin were obtained from Invitrogen (Carlsbad, CA). Cy3-conjugated donkey anti-rabbit IgG, Cy2-conjugated donkey anti-rabbit IgG, Cy2-conjugated donkey anti-mouse IgM, μ -chain-specific and normal donkey serum were purchased from Jackson ImmunoResearch (West Grove, PA). The polyclonal mouse anti-ceramide IgM MAS0020 was from Glyco-biotech (Kukels, Germany). Bacterial sphingomyelinase was from Calbiochem (San Diego, CA). Blocking-grade dry milk and nitrocellulose membranes were from Bio-Rad (Hercules, CA).

Preparation of ceramide antibody

Immunization of rabbits. C18 ceramide (2 mg) was dissolved in chloroform-methanol (2:1, v/v) and dried under a steady stream of nitrogen. One milliliter of KLH (2 mg/ml) in PBS was added to the dried residue. This was mixed with an equal volume of Freund's Complete Adjuvant to form an emulsion. One milliliter of the emulsion (~1 mg of ceramide) was injected subcutaneously into the flanks of one rabbit. Booster doses were injected at 2, 4, 8, and 10 weeks after the initial injection using Freund's Incomplete Adjuvant. A small volume of blood (2–5 ml) was collected by ear-vein puncture of the rabbit at a definite time interval to determine the titer of the antiserum. When the desired titer was obtained, the rabbit was anesthetized and bled through cardiac puncture and the serum was collected from clotted blood. All procedures involving animals were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals. Furthermore, these procedures were performed in compliance with the guidelines issued by the Committee on Animal Use for Research and Education at the Medical College of Georgia.

Purification of anti-ceramide antibody. We used ELISA to determine the titer of the antiserum. Briefly, Immulon 1B flat-bottomed 96-well microtiter plates were coated with 500 ng of

C18 ceramide in ethanol. After washing off unbound ceramide, the reaction was blocked with 1% BSA in PBS. The wells were then incubated with various dilutions (1:50 to 1:400) of immunized and preimmune serum in 1% BSA/PBS at 37°C for 1 h. The unbound antibody was removed by repeated washing with PBS. HRP-conjugated anti-rabbit IgG secondary antibody (1:2,000) was added to the plate and incubated for 37°C for 1 h. After thorough washing, the wells were incubated with *o*-phenylenediamine dihydrochloride peroxidase substrate for 5–15 min and the reaction was terminated using 3 N H₂SO₄. The absorbance was measured at 492 nm.

To purify the IgG fraction, the total immunoglobulin from serum was precipitated using 45% ammonium sulfate and stirred overnight at 4°C. The pellet was collected by centrifugation, dissolved in a defined volume of PBS, and dialyzed against PBS to remove the ammonium sulfate with several changes of PBS. The solution after dialysis was further clarified by centrifugation, mixed with 0.02% azide, and preserved at –20°C for further purification. To purify the IgG fraction, a portion of the immunoglobulin solution was passed through a protein A column and the eluate (citrate buffer elution) was concentrated with polyethylene glycol. Any potential anti-KLH antibody was removed by passing the IgG fraction through a gel column containing KLH immobilized on agarose. For long-term storage, the IgG fraction was mixed with glycerol (1:1), divided into aliquots, and stored at –20°C.

Lipid overlay assay

Lipid overlay assays were performed using either SphingoStrips™ or by spotting lipids on a nitrocellulose membrane as described previously (20). Briefly, lipids were dissolved in chloroform-methanol (2:1, v/v), spotted on the nitrocellulose membrane, and allowed to dry at room temperature for 30 min. The membrane was blocked with 10% dry milk in PBS. Membranes were incubated with the anti-ceramide antibodies diluted in the blocking buffer at 4°C overnight with gentle shaking. Membranes were washed five times with PBS with vigorous shaking at room temperature and then incubated with HRP-conjugated secondary antibodies for 2–3 h at room temperature. The membranes were washed five times with PBS + 0.2% Tween-20 with vigorous shaking at room temperature. Antibody binding was detected using a chemiluminescence system and exposure to X-ray film.

Immunocytochemistry and flow cytometry

For immunocytochemistry, F11 cells were grown on coverslips. Cells were incubated with myriocin for 3 days to inhibit *de novo* ceramide biosynthesis and reduce ceramide levels. To determine whether the antibody would cross-react with glycosphingolipids in the membrane, cells were incubated for 4 days with 250 μ M *N*-butyl-deoxynojirimycin (NB-dNJ), a glucosylceramide (GlcCer) synthase inhibitor known to inhibit glycosphingolipid biosynthesis (21, 22). Control and myriocin- or NB-dNJ-treated cells were fixed with 4% *p*-formaldehyde in PBS for 20 min at room temperature. To release endogenous ceramide from sphingomyelin, fixed cells were incubated for 1 h at 37°C with 0.3 units (1 U/ml) of *Staphylococcus aureus* sphingomyelinase. Immunocytochemistry for ceramide was performed without the use of detergent for permeabilization. The immunostaining of fixed cells or embryonic brain sections followed procedures described previously (15) using a blocking solution of 3% ovalbumin and 2% donkey serum in PBS and primary and secondary antibodies diluted in 0.1% ovalbumin in PBS. Epifluorescence microscopy was performed with an Axiophot microscope (Carl Zeiss MicroImaging, Inc.) equipped with a Spot II charge-coupled device camera. Confocal fluorescence microscopy was performed using a Zeiss

LSM510 confocal laser scanning microscope equipped with a two photon argon laser at 488 nm (Cy2), 543 nm (Cy3), or 633 nm (Alexa Fluor® 647).

For flow cytometry analysis, control and myriocin-incubated F11 cells were trypsinized and passed through a 40 μ m mesh. The cells were resuspended in 100 μ l of blocking buffer (3% ovalbumin in PBS) and incubated at room temperature for 15 min. The cells were then stained with the anti-ceramide rabbit IgG antibody diluted in 0.5% ovalbumin in PBS at 4°C for 1 h. After washing with PBS three times, cells were stained with Cy2-conjugated anti-rabbit IgG secondary antibody at 4°C for 1 h. Cells were washed with PBS, and stained cells were analyzed by flow cytometry, measuring the fluorescence emission at 530 nm. The results from three independent labeling experiments were normalized against the control and represented as bar graphs.

RESULTS

The rabbit IgG fraction recognizes ceramide in lipid overlay assays

The antiserum and purified rabbit IgG fraction was tested in lipid ELISA and overlay assays using lipid-coated Immulon 1B 96-well plates, nitrocellulose membranes, and SphingoStrips as described in Experimental Procedures. The 96-well plates were coated with 500 ng of ceramide or other lipid species per well. The staining reaction of the preimmune serum was equivalent to that of the negative control without serum and was <15% of the positive reaction. A positive reaction was found for C18 ceramide, the ceramide species used for immunization of the rabbits (Fig. 1A). The anti-ceramide IgG fraction, however, did not react significantly with sphingomyelin or phosphatidylcholine. To use an assay method with higher sensitivity, 1.0 or 10 nmol of different ceramide species was spotted on nitrocellulose followed by lipid overlay immunostaining. Figure 1B shows that the rabbit IgG recognized C16, C18, C20, and C24 ceramide in a dose-dependent manner. Consistent with the results from the lipid ELISA, the strongest reaction was seen with C18 ceramide. The antibody also recognized C2 ceramide, but only at higher concentrations.

To confirm the specificity of the anti-ceramide antibody, the lipid overlay assay was performed with SphingoStrips, commercially available membranes spotted with 100 pmol of various sphingolipids and related lipids. Figure 1C shows the layout and composition of lipids on the SphingoStrip. The rabbit IgG fraction reacted strongly and specifically with ceramide (Fig. 1D). No reactivity was detected with phosphatidylcholine or sphingomyelin. This specificity was consistent with that of the polyclonal IgM anti-ceramide antibody that has been shown to recognize ceramide in a similar assay (20). As with the anti-ceramide IgM, the rabbit anti-ceramide IgG detected dihydroceramide of appropriate fatty acid chain length (Fig. 1E). Anti-ceramide rabbit IgG also reacted with phytoceramide, although significantly less intensely than with ceramide. Together, these results indicate that the rabbit IgG fraction specifically recognizes ceramide in lipid overlay assays, similar to the polyclonal mouse IgM antibody, and is ca-

pable of detecting ceramide species with different fatty acid chain lengths.

The rabbit IgG fraction can be used to visualize ceramide by immunocytochemistry of fixed cells and tissue

To determine whether the rabbit IgG fraction could be used for immunocytochemistry, we stained F11 neuroblastoma cells using both the rabbit IgG fraction and the polyclonal IgM antibody for ceramide. The cells were fixed but not permeabilized before staining, because permeabilization could affect the distribution of lipids in the membrane. Figure 2A (left) shows that at lower dilution of the primary antibody (1:50), both antibodies bound to the same regions on the plasma membrane, indicated by the appearance of yellow pseudocolor in the overlay. At higher dilutions of the primary antibody (1:200), the staining with rabbit IgG was still visible, whereas the signal from the polyclonal IgM was greatly diminished (right), indicating that the rabbit IgG antibody was more sensitive or present at a higher concentration. Figure 2B (left) also shows that when cells were incubated with myriocin (an inhibitor of de novo ceramide biosynthesis) for 3 days, the intensity of the signal was reduced with both antibodies. Incubation with myriocin is known to reduce ceramide levels in cells (23), consistent with diminished staining using the two antibodies. To quantify this effect, control and myriocin-incubated F11 cells were stained with the rabbit IgG fraction and analyzed by flow cytometry, as described in Experimental Procedures. Figure 2B (right) shows that the fluorescence intensity of the myriocin-incubated cells was reduced by ~40% compared with that of control cells. This was consistent with the observation that myriocin incubation of F11 cells for 3 days reduced ceramide levels by ~50%, as determined by high-performance thin-layer chromatography (data not shown).

On the other hand, when cells were incubated with NB-dNJ (an inhibitor of GlcCer synthase), the intensity of the signal was not changed significantly (Fig. 2C). GlcCer synthase is the first enzyme in glycosphingolipid biosynthesis and uses ceramide as a substrate. Inhibiting this enzyme has been shown to reduce glycosphingolipid levels without altering ceramide levels (22). If the antibody were to cross-react with glycosphingolipids, we would expect to see a reduction in the staining intensity. The absence of such a reduction (Fig. 2C) indicated that this antibody was specific for ceramide. This specificity was also confirmed by the absence of a fluorescence signal when using the IgG fraction preadsorbed to ceramide (Fig. 2D). To further test the specificity of the rabbit IgG, we incubated F11 cells with bacterial sphingomyelinase before performing immunocytochemistry. Sphingomyelinase is a phospholipase C-like enzyme that hydrolyzes sphingomyelin to release phosphorylcholine and ceramide. Incubation with bacterial sphingomyelinase has been shown to increase ceramide levels at the plasma membrane (24). Figure 3A shows that sphingomyelinase-incubated cells contained increased ceramide levels, as detected by the rabbit IgG fraction compared with control cells.

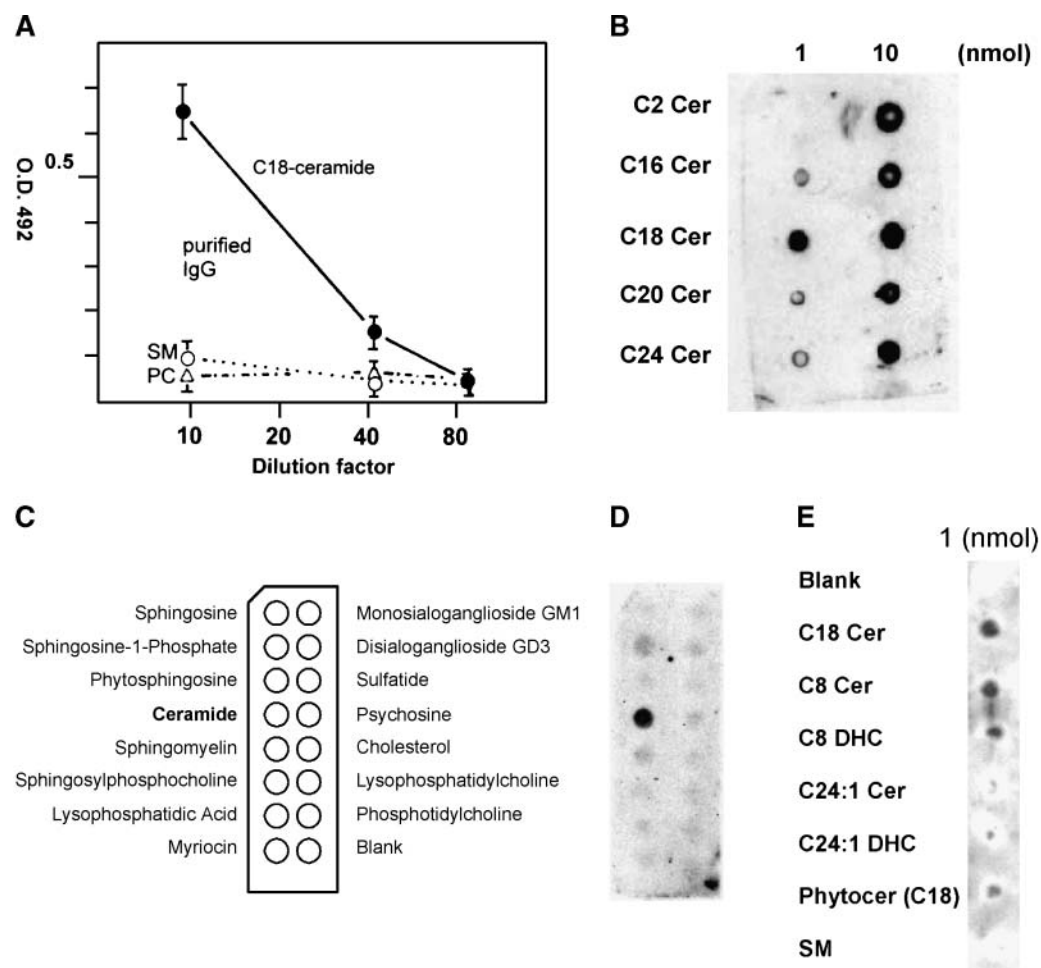


Fig. 1. Anti-ceramide rabbit IgG specifically recognizes ceramide in lipid overlay assays. **A:** Lipid ELISA using 500 ng of ceramide or other lipid species coated on the surface of wells on a 96-well Immulon 1B plate. Means are shown after subtraction of the absorption obtained with preimmune serum, and bars represent SEM ($n = 3$). Note that sphingomyelin (SM) and phosphatidylcholine (PC) did not show significant antibody binding. O.D., optical density. **B:** Ceramides containing fatty acids of different chain lengths were spotted on nitrocellulose membranes and probed with the rabbit IgG, which recognizes most ceramide (Cer) species in a dose-dependent manner. Note that the antibody shows the strongest reaction for C18 ceramide, which was used for the initial immunization. **C:** Layout of SphingoStrips used to test the specificity of anti-ceramide antibody. Each spot contains 100 pmol of the indicated lipid. **D:** SphingoStrips were probed with the newly developed rabbit IgG as described in Experimental Procedures. Note that the antibody shows a specific reaction with the spot that corresponds to ceramide. **E:** Comparison of ceramide and dihydroceramide (DHC) binding of the anti-ceramide rabbit IgG. Note that C8 dihydroceramide is bound as well as C8 ceramide. The antibody also shows some binding to phytoceramide [*N*-stearoyl phytosphingosine (Phytocer)].

The distribution of ceramide on the plasma membrane and within cells was tested in a series of high-resolution fluorescence analyses using F11 cells and the anti-ceramide rabbit IgG fraction. Figure 3B shows that ceramide was enriched in protrusions of the cell membrane and in a perinuclear region that was identified as the Golgi apparatus using costaining with fluorescence-labeled wheat germ agglutinin (Fig. 3C). Remarkably, the anti-ceramide rabbit IgG was also able to detect ceramide in the plasma membrane of neurons of the developing cortical plate and intermediate zone (Fig. 4). The mouse IgM stained ceramide in a similar tissue complex, although mainly in the nuclear region of cortical plate cells.

Although the presence of ceramide in neuronal nuclei is not a priori excluded, it appears to be more likely that the rabbit IgG detected the main cellular distribution site of ceramide (plasma membrane). Together, these results indicated that the rabbit IgG fraction specifically recognized ceramide in fixed cells and tissues and therefore is suitable for the immunocytochemical detection of ceramide.

DISCUSSION

Ceramide has been shown to regulate important processes in cell biology, such as proliferation, apoptosis, and

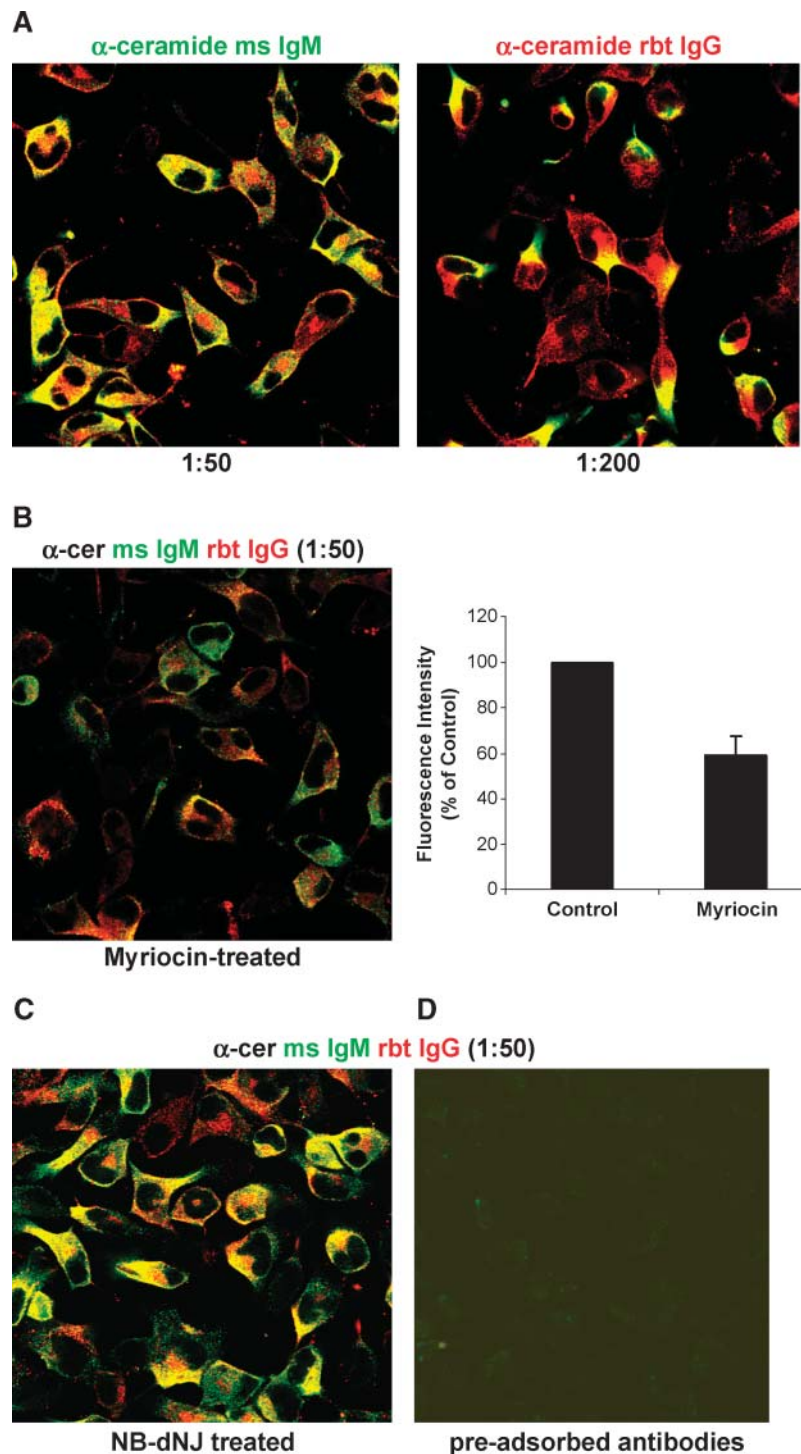


Fig. 2. The rabbit IgG antibody specifically recognizes ceramide in immunocytochemistry. **A:** Immunocytochemistry and confocal laser scanning microscopy of F11 neuroblastoma cells using rabbit (rbt) IgG anti-ceramide antibody (Cy3; red) and polyclonal mouse (ms) IgM antibody (Cy2; green) at different dilutions of the primary antibodies. Note that at higher dilution, the rabbit IgG shows a robust signal, whereas the signal from the mouse IgM is diminished. **B:** Reduction of ceramide levels in F11 cells using the serine palmitoyl transferase inhibitor myriocin reduces the antibody signal from both antibodies compared with the controls in **A**, indicating the specificity of the antibodies toward ceramide (left). The reduction in staining intensity with the rabbit IgG was quantified by flow cytometry. Note that the staining intensity of myriocin-incubated cells was reduced by ~40% compared with the control (right). Error bars represent mean \pm SD from three independent experiments. α -cer, anti-ceramide. **C:** Incubation of F11 cells with the glucosylceramide synthase inhibitor *N*-butyl-deoxynojirimycin (NB-dNJ) causes no significant change in the staining intensity with both antibodies, once again indicating the specificity of the antibodies toward ceramide. **D:** Mouse and rabbit anti-ceramide antibodies were preadsorbed to ceramide. Competition with ceramide in suspension abolished the fluorescence signal.

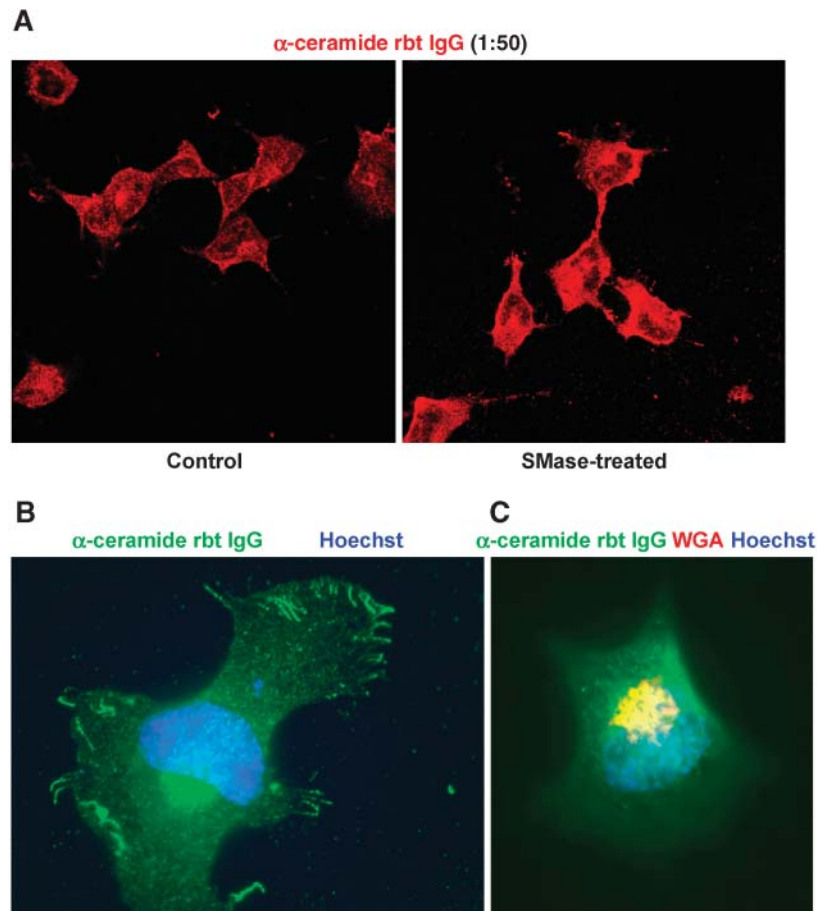


Fig. 3. The novel anti-ceramide antibody specifically recognizes ceramide at the cell membrane and the Golgi. **A:** Immunocytochemistry and confocal laser scanning microscopy of F11 neuroblastoma cells using rabbit (rbt) IgG anti-ceramide antibody (Cy3; red). Incubation of cells with bacterial sphingomyelinase (SMase) for 1 h resulted in increased staining intensity at the plasma membrane compared with the control. **B:** Immunocytochemistry and epifluorescence microscopy of F11 neuroblastoma cells shows that the rabbit IgG anti-ceramide antibody (Cy2; green) stains ceramide in filipodia-like protrusions of the cell membrane (sphingopodia) and shows strong staining at the perinuclear region. **C:** Costaining of cells with wheat germ agglutinin (WGA; red) reveals that the perinuclear region stained by the new anti-ceramide antibody is the Golgi apparatus.

differentiation. Ceramide has also been suggested to be specifically distributed within the cell and structurally organized in lipid microdomains (25). A specifically increased distribution has been described in the endoplasmic reticulum, plasma membrane, Golgi, perinuclear membrane, and mitochondria. Ceramide has also been isolated in a detergent-insoluble membrane fraction that has been suggested to be equivalent to lipid microdomains or rafts. It is tempting to speculate that the structural organization of ceramide rafts in particular membrane areas or subcellular compartments triggers specific cell signaling pathways that regulate biological processes such as apoptosis, proliferation, and differentiation. To date, increased ceramide in particular compartments or microdomains has only been detected by subcellular fractionation, a method susceptible to cross-contamination. Fractionation methods are also problematic because they treat the whole population of cells as homogenous, which is not the case in most cell cultures. Furthermore, the isolation of lipid

rafts using detergent solubilization is artificial in that any intercalation of detergents can change the distribution of lipids in the membrane. Currently, there is little information on the existence of ceramide-containing lipid rafts or membrane domains in living cells using direct visualization methods.

There are two commercially available antibodies against ceramide: monoclonal mouse IgM clone 15B4 and polyclonal mouse IgM MAS0020. The latter has been reported to be more specific for ceramide in overlay assays (20). Both antibodies have been used for immunocytochemistry, flow cytometry, and in vivo ceramide depletion, apparently with no indication for nonspecific reactions with other lipids. However, the results with these two antibodies were dissatisfying in terms of detecting intracellular compartments that were reported to contain ceramide, in particular the Golgi apparatus. Being of the IgM class, they are large antibodies, making penetration of the plasma membrane to detect intracellular ceramide

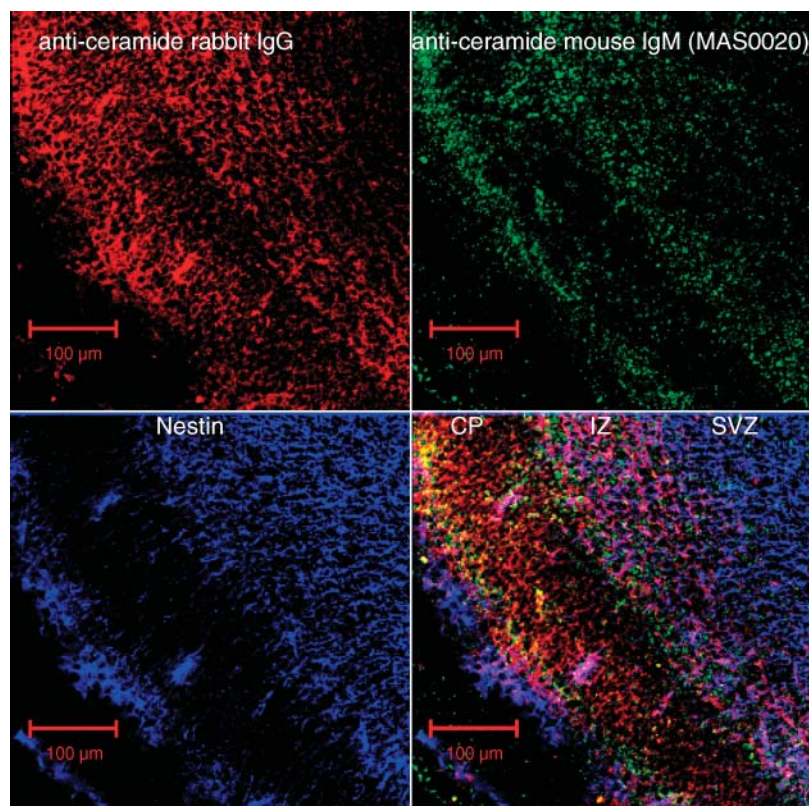



Fig. 4. The rabbit anti-ceramide IgG detects ceramide in fixed cryosections of embryonic mouse brain. Coronal cryosections of embryonic mouse brain (gestational day E14.5) were fixed with 4% *p*-formaldehyde, and ceramide was detected using the mouse anti-ceramide IgM (MAS0020; Cy5; pseudocolored green) or rabbit anti-ceramide IgG (Cy2; pseudocolored red) at a dilution of 1:50 for confocal laser scanning immunofluorescence microscopy. Note that the two antibodies give a strong fluorescence signal for cells in the developing cortical plate (CP) and the intermediate zone (IZ), whereas the mouse IgM stains predominantly the nuclear region. SVZ, subventricular zone.

difficult. To generate additional antibodies with better cell penetration, we immunized rabbits against ceramide.

A standard protocol was developed to test the suitability of a ceramide antibody for immunocytochemistry. The first round of screening used currently known detection methods such as lipid ELISA and lipid overlay assays to quantify the sensitivity and determine the specificity of the ceramide antibody *in vitro*. The second round comprised new screening assays not routinely used but required to demonstrate the suitability of the antibody for immunocytochemistry. The first assay was the ceramide depletion assay, which involved incubating cells with the *de novo* biosynthesis inhibitor myriocin or another inhibitor for glycosphingolipid biosynthesis (NB-dNJ). Cells incubated with myriocin are expected to show less ceramide signal, whereas cells incubated with NB-dNJ will show no change or more ceramide. If the ceramide signal is not reduced by myriocin incubation or reduced by incubation with NB-dNJ, then the antibody is not specific for ceramide. Both the MAS0020 and our new antibody passed this test. The second assay was the ceramide release assay, which involved incubating cells with bacterial sphingomyelinase. This assay is expected to enhance the signal for ceramide. If the signal is not enhanced or even reduced, then the antibody is not spe-

cific for ceramide. The mouse polyclonal and rabbit polyclonal anti-ceramide antibodies passed this test. These assays clearly showed that we generated a novel antibody against ceramide that can be used for immunocytochemistry.

The rabbit antibody detected ceramide at subcellular sites that are known to contain ceramide but were not recognized by MAS0020. This suggested that, at least in some cases, the anti-ceramide rabbit IgG may be better suited for immunocytochemistry than the polyclonal IgM. Of particular interest is the observation that it shows a signal in the Golgi apparatus that may quite naturally emerge as a result of endoplasmic reticulum-to-Golgi transport of ceramide by the ceramide transport protein. The Golgi is also a compartment known to synthesize glycosphingolipids from ceramide (9). Hence, it is expected that a ceramide antibody shows a Golgi resident signal using immunocytochemistry. The reason for our polyclonal rabbit antibody being able to detect a Golgi signal while MAS0020 shows no or only minimal fluorescence in the Golgi may originate in the distinct specificities of the two antibodies for ceramide in membrane domains. It is rather unlikely that an anti-ceramide antibody detects single molecules of ceramide. It is more likely that these antibodies bind to larger complexes that are organized in distinct microdomains. Of

particular interest are protrusions of the plasma membrane that strongly stain for ceramide. We have termed these protrusions “sphingopodia” because they may be functionally involved in shaping the plasma membrane or initiating lamellipodia- or filipodia-dependent processes such as cell migration. The new anti-ceramide antibody provides us with a tool to visualize ceramide generated in response to various stimuli and to identify proteins that interact with ceramide. 

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