Structural determinants of sphingolipid recognition by commercially available anti-ceramide antibodies

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Abstract **The sphingolipid mediator ceramide is involved in cellular processes such as apoptosis, differentiation, responses to cytokines, and stress responses. Experimental evidence suggests that the intracellular location of ceramide may be a key factor in determining its ultimate cellular effects. One approach to ceramide localization is the use of recently developed anti-ceramide antibodies for immunocytochemical studies. Two such commercial preparations are now available; we sought to compare and contrast their specificity for ceramide and/or other cellular lipids. By using lipid overlay assays and a diverse panel of sphingolipids, we were able to delineate the specificity and thus, the utility of these reagents. Our results indicate that one of these anti-ceramide preparations is quite specific for ceramide and dihydroceramide, whereas the other preparation recognizes dihydroceramide, phosphatidylcholine, and sphingomyelin. Furthermore, through the use of chemically modified ceramides in similar assays, we were able to determine some structural determinants of lipid recognition by both of these reagents.**—Cowart, L. A., Z. Szulc, A. Bielawska, and Y. A. Hannun. **Structural determinants of sphingolipid recognition by commercially available anti-ceramide antibodies.** ***J. Lipid Res.*** **2002.** 43: **2042–2048.**

Supplementary key words ceramide • antibody • lipid-antibody interaction • sphingolipid • lipid • lipid chemistry • signal transduction

Ceramide and other sphingolipids are now recognized as lipid mediators of cellular responses to stress and extracellular signals such as TNF, FAS, and chemotherapeutic agents (1–3). These signals induce elevation of cellular ceramide levels, which is important for the execution of cellular processes including differentiation, senescence, and apoptosis (4, 5).

Ceramide can be generated de novo, via serine palmitoyl transferase which generates a sphingoid base, followed by the *N*-linked addition of an acyl chain by ceramide synthase (6). Ceramide goes on to be incorporated into complex sphingolipids including sphingomyelin, and thus can also be generated from SM breakdown by SMase (7).

Much of the recent literature indicates that effects of ceramide are in part dependent on the subcellular localization of its formation. De novo ceramide is generated in the endoplasmic reticulum and Golgi apparatus (6), whereas SMase-derived ceramide is generated in intracellular compartments where SM and SMase are colocalized, particularly in the lysosomes (8), plasma membrane (9, 10), and possibly mitochondria (11). Therefore, determining the precise subcellular localization of ceramide is desirable.

Other issues in the field of ceramide research that would profit from the ability to determine the subcellular localization of ceramide include the intracellular transport of ceramide, which is a highly hydrophobic molecule and does not diffuse freely in the cell (12). Additionally, the uptake and subsequent intracellular targeting of exogenously added ceramides and ceramide derivatives could benefit from tools that would allow ceramide localization.

Potentially aiding in these endeavors has been the development of antibodies directed against ceramide. These antibodies are promising tools for establishing the topology of ceramide generation and/or subcellular localization. Two commercially available antibodies have been described as ceramide-specific. Each has been used experimentally by us and others in attempts to determine locations of ceramide generation in situ. In seeking to evaluate the specificity of these reagents, we conducted lipid overlay assays coupled with structure-function analysis. Both antibodies have potential experimental applications; however, it is important to consider their specificities in experimental design.

METHODS

Materials

Anti-ceramide IgM antibodies were purchased from GlycoTech Produktions und Handels GmbH (MAS 0010, lot number D3610a; Kükels, Germany) and Alexis Deutschland GmbH

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; SMase, sphingomyelinase.

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(804-196-T050, clone MID 15B4; Grünberg, Germany). Anti-mouse IgM-HRP conjugate was purchased from Calbiochem (Darmstadt, Germany).

Selected lipids were prepared as described (13, 14). Other lipids (SM, α-hydroxyceramides, PS, PE, PI, and PG) were purchased from Matreya (Pleasant Gap, PA).

Lipid overlay assay

Lipid-overlay assays were performed similarly to those described by Brade et al. (15). Briefly, lipids were dissolved in $CHCl_3$ at a stock concentration of 10 mM. For membrane-based assays, lipids were diluted to desired concentrations in $CHCl_3$, spotted onto Hybond-C nitrocellulose membranes (Amersham Pharmacia, Buckinghamshire, England), and allowed to dry for 30 min. Blocking was performed for 30 min at room temperature in 10% nonfat dry milk/PBS. For silica gel TLC plate-based assays, lipids were spotted, plates were dried for 30 min, and then incubated in a blocking solution of PBS/0.05% saponin for 1 h prior to blocking in milk as above. Antibodies were diluted 1:500 in blocking buffer and incubated with blots at room temperature overnight with gentle shaking. Membranes were washed with vigorous shaking at room temperature in PBS with five changes over 30 min. The anti-mouse IgM-horseradish peroxidase conjugate was diluted 1:2,000 in blocking buffer and incubated 2–3 h at room temperature. Washing was repeated exactly as before. Lipid-antibody complex was detected using the enhanced chemiluminescence kit from Amersham Pharmacia (Buckinghamshire, England) and exposure to film.

RESULTS

Two commercially available anti-ceramide antibody preparations were evaluated for ceramide selectivity. Both antibodies are of the IgM isotype. The first available antibody is a monoclonal anti-ceramide mouse IgM, raised against C_{14} ceramide covalently linked to BSA at its *N*-acyl omega terminus (Alexis, Grünberg, Germany; now available from Sigma, St. Louis, MO). The second available antibody is a polyclonal mouse antiserum enriched for IgM (Glycotech, Kükels, Germany); information as to the antigen used to raise this antibody is not available.

Ceramide specificity

In order to verify the specificity of the antibodies for ceramide, we performed lipid overlay assays. This technique has been used extensively as a qualitative screen for antibody-antigen interactions (15, 16). We were concerned with the possibility that the anti-ceramide antibodies may interact with other similar molecules, such as other sphingolipids or glycerophospholipids. Therefore, we tested D-*erythro*-C_{16} ceramide, PI, PE, PS, PG, and PC. We observed a strong recognition of C_{16}-ceramide by the polyclonal antiserum, and a much weaker recognition by the monoclonal antibody. Furthermore, though neither antibody recognized PI, the monoclonal antibody recognized PC very strongly, whereas PC recognition by the polyclonal antiserum was barely detectable (**Fig. 1A**). No detectable recognition of PE, PG, PS, PA, or DAG was observed by either antibody preparation (not shown). From these data, we concluded that among these lipids, the polyclonal antiserum is selective for ceramide, whereas the monoclonal anti-ceramide antibody is actually more reactive with PC than with ceramide.

Because SM is composed of ceramide and a phosphocholine headgroup and the monoclonal antibody recognized PC, whereas the polyclonal antiserum recognized ceramide, we sought to determine if SM would be recognized by either antibody preparation. As shown in Fig. 1A, recognition of PC is much stronger than recognition of SM; however, the lipid-overlay assay demonstrated a clearly detectable recognition of SM by the monoclonal antibody upon overexposure (Fig. 1B). This result is in agreement with previously published findings that also demonstrated that the monoclonal antibody recognizes SM (17). Importantly, even at long exposure times, there was no detection of the polyclonal antibody interaction with SM.

Since ceramide shares many structural features with other sphingolipids, including the sphingoid moiety, we tested the ability for the anti-ceramide antibody preparations to recognize a panel of sphingolipids, including

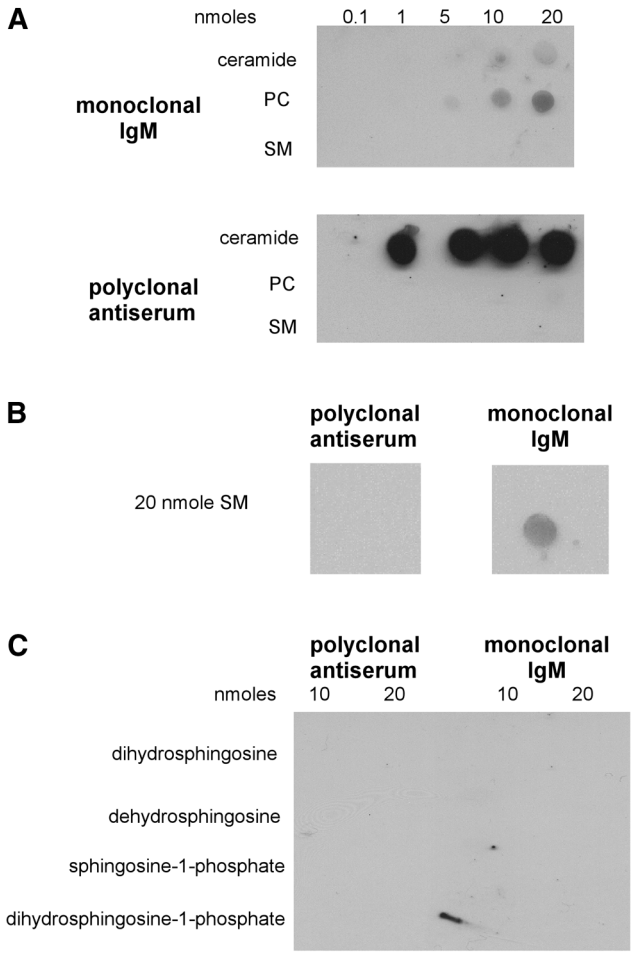

Fig. 1. Anti-ceramide specificity. Lipids were spotted onto nitrocellulose membranes and blotted with anti-ceramide antibody dilutions as described in Methods. A: Indicated amounts of ceramide, sphingomyelin, and phosphatidylcholine. B: Overexposure demonstrated the interaction of the monoclonal antibody with SM. C: There was no detection of interaction of either antibody with dihydrosphingosine, dehydrosphingosine, sphingosine-1-phosphate, or dihydrosphingosine-1-phosphate.

sphingoid bases and sphingoid base phosphates. Neither preparation recognized D-*erythro*-dihydrosphingosine, D-*erythro*-dehydrosphingosine, D-*erythryo*-dihydrosphingosine 1-phosphate, or D-*erythro*-sphingosine-1-phosphate (Fig. 1C). This suggested that *N*-acylation is required for antibody recognition by the antiserum.

N-Acyl chain length selectivity and stereoselectivity of ceramide recognition

In order to identify structural determinants of ceramide recognition by the polyclonal antiserum, as well as to identify ceramide isomers which may possibly be recognized by the monoclonal antibody, we used the same assay to test recognition of several synthetic ceramide analogs (**Fig. 2**), including D-*erythro*-ceramides with various *N*-acyl chain lengths and their stereoisomers. Neither preparation recognized ceramides with *N*-acyl chain lengths of 2, 6, or 8 carbons (not shown). Furthermore, we found that the polyclonal antiserum was unable to recognize C_{10} ceramide, but interacted with C_{14}- and C_{16}-ceramides very strongly (**Fig. 3A**, Fig. 1A). Interestingly, recognition was significantly decreased in the C_{24} ceramide (Fig. 3A), suggesting that only a certain range of carbon chain length is recognizable by the polyclonal antiserum. Furthermore, though the monoclonal antibody only weakly recognized C_{16} ceramide (Fig. 1A), we observed significant recognition of C_{14} and C_{24} (Fig. 3A). These results show a distinct preference of both antibodies for specific *N*-acyl chain lengths.

Due to their increased water solubilities relative to natural ceramides, short-chain ceramides are frequently used in experimental situations. It is interesting to note that these short-chain ceramides were not recognized by the anti-ceramide antiserum. However, experimental evidence suggests that exogenously added ceramides can acquire long chain fatty acids (by deacylation followed by reacylation with endogenous fatty acids) once the lipid enters the cell (18). In this case, the *N*-reacylated exogenous ceramide could possibly be detected.

Natural sphingolipids have D-*erythro* stereochemistry at the C_2-C_3 positions. We tested antibody recognition of the unnatural L-*erythro* and D- and L-*threo* ceramide stereoisomers, and found that the polyclonal antiserum recognized all these stereoisomers approximately equally (Fig. 3B). This may indicate that the C_2-C_3 region of ceramide is not an epitope for the antiserum. Interestingly, the monoclonal anti-ceramide showed significant detection of the D-*threo* ceramide (Fig. 3B). This recognition is not a likely experimental consideration, however, as these are unnatural ceramides whose cellular concentrations are exceedingly low or absent.

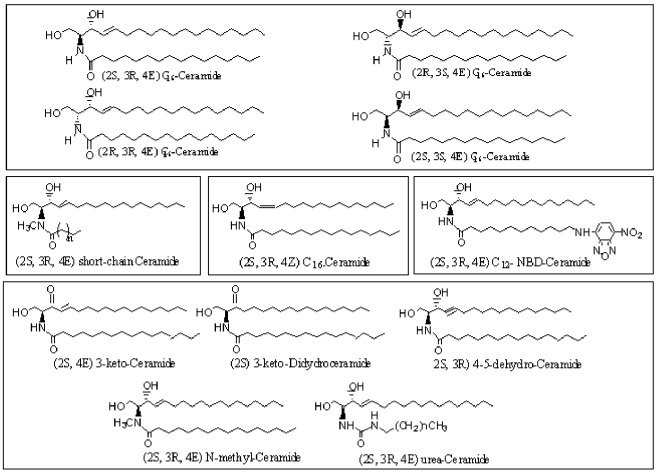

Fig. 2. Structures of synthetic ceramide analogs used in this study.

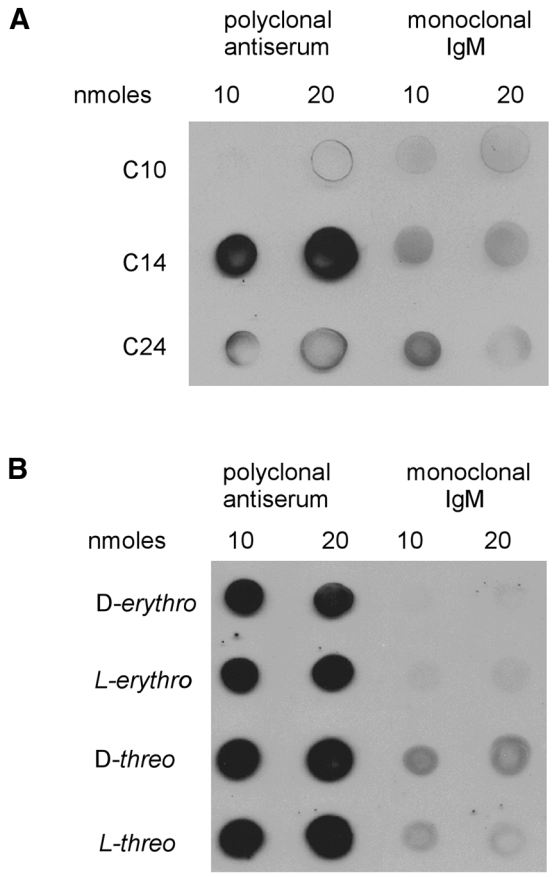

Fig. 3. Chain length and stereochemical specificity of antibody recognition of ceramides. Lipids were spotted onto nitrocellulose membranes and blotted with anti-ceramide antibody dilutions as described in Methods. A: *N*-acyl chain length specificity. All compounds were the D-*erythro* configuration. B: Stereospecificity. All compounds contained *N*-acyl groups of 16 carbons (C16).

Modified ceramides

In an effort to understand structural requirements for recognition by the antibody preparations, we used several synthetic ceramide analogs and ceramides with chemical modifications that are not naturally occurring (Fig. 2). Interestingly, C_{16}-*N*-methylceramide was recognized by both antibody preparations (**Fig. 4**); however, D-*erythro*-C_{16}-urea ceramide was not recognized by either preparation (Fig. 4). Furthermore, the experimentally useful ceramide derivative NBD ceramide was not recognized by either the polyclonal antiserum or the monoclonal antibody (Fig. 4).

Other ceramide analogs were investigated for recognition by the antibody preparations. Interestingly, the monoclonal

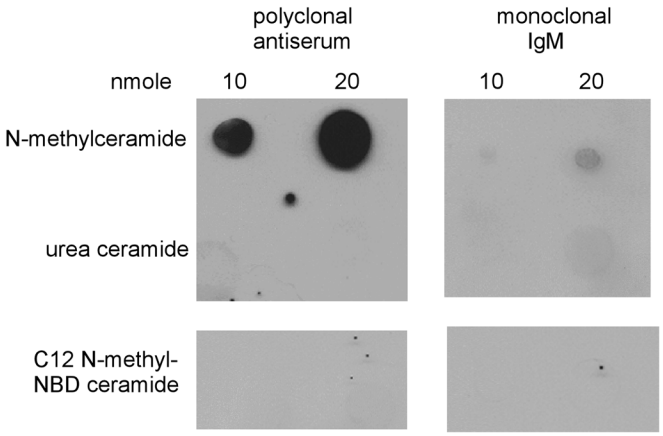

Fig. 4. Recognition of modified ceramides by antibody preparations. Lipids were spotted onto nitrocellulose membranes and blotted with anti-ceramide antibody dilutions as described in Methods.

antibody, which failed to recognize D-*erythro*-C_{16} ceramide, showed a strong recognition of 3-keto-dihydroceramide, 3-keto-ceramide, D-*erythro*-dihydroceramide, an intermediate in ceramide biosynthesis, and the unnatural dehydroceramide, which has a triple bond at the 4,5 position (**Fig. 5**). Furthermore, the monoclonal antibody recognized *cis*-ceramide, which is an unnatural ceramide analog that has a *cis* conformation around the 4,5-double bond, as opposed to natural ceramides that have a *trans* conformation around this bond (Fig. 5). Additionally, the polyclonal antiserum recognized 3-keto-ceramide and 3-keto-dihidroceramide, in addition to dihydroceramide and 4,5-*cis*-ceramide (Fig. 5).

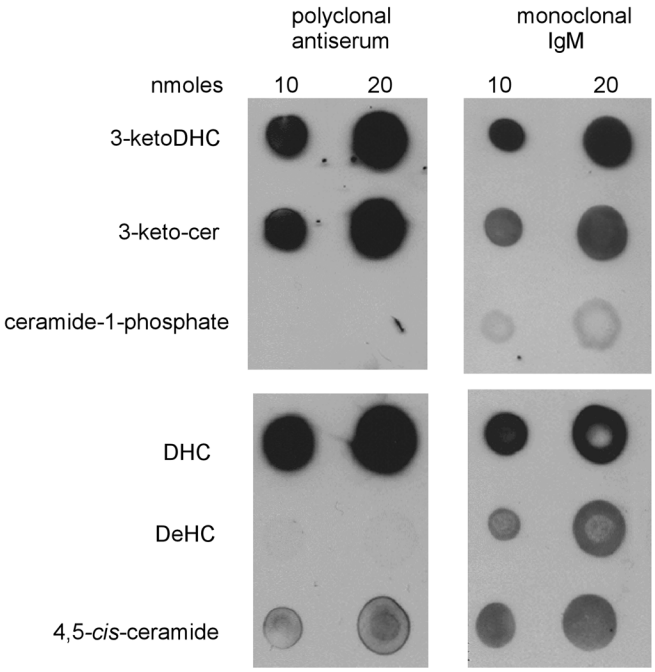

Fig. 5. Recognition of unnatural ceramide derivations by antibody preparations. Lipids were spotted onto nitrocellulose membranes and blotted with anti-ceramide antibody dilutions as described in Methods.

In contrast to the monoclonal antibody, the polyclonal did not recognize dehydroceramide (Fig. 5).

We tested the ability of both antibodies to recognize hydroxylated ceramides: phytoceramide (4-hydroxy-dihydroceramide) species that are the predominant yeast ceramides as well as α-hydroxy-ceramides that are components of galactosphingolipids. Neither antibody preparation recognized phytoceramides, indicating that these reagents are not useful for most yeast-based experiments (not shown). Interestingly, both antibodies recognized a mixture of purified natural α-hydroxy-ceramides (**Fig. 6**). Furthermore, we synthesized a racemic mixture of D-*erythro*-α-hydroxy-C_{16} ceramide which resolved under our chromatographic conditions into two stereoisomers, termed A and B, for the less and more polar isomers, respectively (TLC: SiO_2, solvent system: chloroform-methanol, 95:5, v/v; isomer A: R_f = 0.26, isomer B: R_f = 0.17). Though D- or L-stereochemistries have not been assigned to these two isomers, stereospecificity was observed in the interaction of the monoclonal antibody with these compounds, in that only isomer A was recognized (Fig. 6). The recognition of both the natural purified compound and synthetic isomer A by the monoclonal antibody may suggest that isomer A possesses the natural D-configuration of α-hydroxylated ceramides. On the other hand, the polyclonal anitserum reacted with both isomers, indicating a lack of stereoselectivity for α-hydroxylation (Fig. 6).

Silica-based lipid overlay assay

A caveat to analysis of lipid-directed antibody specificity is that the different techniques used to evaluate selectivity often yield variable results. This is likely due to different

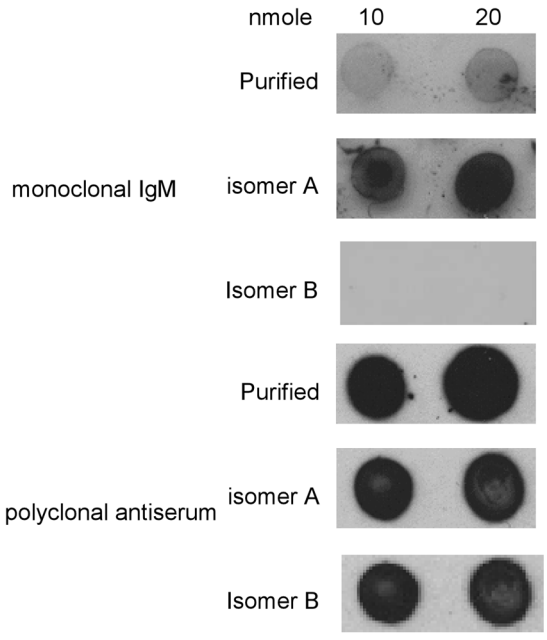

Fig. 6. Recognition of α-hydroxy-ceramides by antibody preparations. Purified natural α-hydroxy-ceramide and synthetic enantiomers of D-*erythro*-α-hydroxy-ceramide were spotted onto membranes and blots were performed as described in Methods.

degrees of epitope exposure provided in each method. For example, in immunocytochemical applications, epitopes may be hidden in cell membranes; in lipid overlay assays, as used here, portions of the lipid molecule important for lipid-antibody interaction may be otherwise occupied in association to the membrane. It is likely that the more hydrophobic portions of the molecule interact with the nitrocellulose membrane, leaving the hydrophilic areas exposed and available for antibody interaction. To test whether exposing the hydrophobic areas would change antibody recognition, we used silica-gel coated aluminum TLC plates as a substrate for the assay. As we observed using nitrocellulose membranes as substrate for the assay, ceramide was recognized by the polyclonal antiserum, but not by the monoclonal antibody (**Fig. 7**). Interestingly, though the monoclonal antibody recognized PC and SM when bound to nitrocellulose, those species were not recognized by the antibody in the silica-based lipid overlay assay (Fig. 7). This could be due to the overall decrease in signal intensity when the assay is performed using silica-coated TLC plates as opposed to nitrocellulose membranes (Figs. 7 and 1, respectively), or, on the other hand, the epitope(s) recognized by the monoclonal antibody may be obscured by the interaction of these lipids with the silica substrate. To address this issue, we repeated the TLC based lipid overlay assay using polyisobutylmethacrylate to coat the TLC plates, thus improving the hydrophobic interactions between plate and lipid, and thereby making available more of the hydrophilic portions of the molecules for interaction with the antibodies. Interestingly, though there was still significant recognition of ceramide by the antibody, the SM and PC remained unrecognized (not shown). This suggests that the lack of recognition of PC and SM by the antibody in the TLC plate assay is due to generally lower signal strength, and does not result from increased availablity of the hydrophobic areas of the molecules for antibody interactions.

DISCUSSION

As a result of the rapid expansion of information about roles of ceramide in cell function, new issues have arisen in the field of ceramide research, including the importance of localizing ceramide formed as a result of various extracellular stimuli, and also issues of ceramide transport. Antibodies specific for ceramide would be valuable tools for the experimental pursuit of these issues. Therefore, we undertook these studies with the purpose of establishing specificity of two such reagents that have become commercially available: a monoclonal anti-ceramide antibody and a polyclonal anti-ceramide antiserum.

Our primary concern was the identification of potentially misleading cross-reactivity of these reagents with lipids other than ceramide. A related and important issue was the identification of structural determinants of ceramide required for recognition by each of these antibodies. To address these issues, we tested both endogenously occurring compounds and unnatural ceramide analogs in lipid-overlay assays using purified or synthetic lipids spotted onto nitrocellulose membranes or silica-coated TLC plates.

Table 1 summarizes our findings pertaining to the recognition by these antibodies of naturally occurring lipids. Specificity among naturally occurring lipids is most important because of the relevance of antibody recognition of these compounds to experimental situations where only naturally occurring lipids are present. The major finding pertaining to the utility of these antibodies for ceramide localization is that the monoclonal IgM recognizes phosphatidylcholine, dihydroceramide, and to a lesser extent, sphingomyelin. This indicates that it may not be a useful reagent for localizing ceramide formation in situ. The polyclonal antiserum, however, may be more useful, as it is specific for ceramide and dihydroceramide. Therefore, it may be a desirable reagent for applications where ceramide specificity is required and cross-reactivity with dihydroceramide is of little concern (e.g., when dihydroceramide concentrations are known to be very low).

The specificities of these reagents may allow them to be

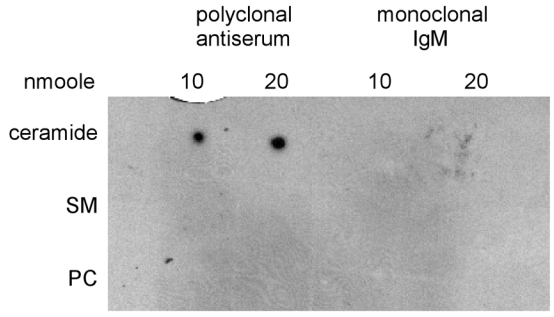

Fig. 7. Recognition of silica-bound lipids by anti-ceramide antibody preparations. Blots were performed on silica-coated TLC plates as described in Methods.

TABLE 1. Recognition of naturally occurring lipids by two anti-ceramide antibody preparations

	Monoclonal IgM	Polyclonal Antiserum
N-acyl chain length		
C_{16}-ceramide	+	++++
C_{24}-ceramide	++	+
C24:1-ceramide	+	++++
Other sphingolipids		
Sphingomyelin	+	−
Dihydroceramide	++++	++++
Dihydrosphingosine	−	−
Sphingosine-1-phosphate	−	−
Dihydrosphingosine-1-phosphate	−	−
Ceramide-1-phosphate	+	−
Other Lipids		
Phosphatidylcholine	+++	−
Phosphatidylinositol	−	−
Phosphatidylserine	−	−
Phosphatidylethanolamine	−	−
Diacylglycerol	−	−

+, Barely detectable; ++, moderately detected; ++++, maximum detection; −, no detection.

used in conjunction to distinguish ceramide from dihydroceramide. The polyclonal antibody can be used first to establish the presence of dihydroceramide and/or ceramide. If distinguishing dihydroceramide from ceramide is desirable, the monoclonal may be subsequently used; if it gives similar results to those obtained using the polyclonal antibody, the compound is likely to be dihydroceramide, which is recognized by both reagents. On the other hand, if the monoclonal antibody fails to recognize the compound recognized by the polyclonal antibody, the compound is likely to be ceramide, which is recognized by the polyclonal antibody only.

As mentioned above, one goal of these studies was to identify the structural determinants of recognition by the antibodies. In order to identify such determinants, we used a panel of synthetic ceramide analogs (Fig. 2). Results of lipid overlay assays are summarized in **Table 2**. Based on data presented here, we conclude that structural determinants of recognition by the polyclonal antiserum include *a*) the presence of a sphingoid moiety, *b*) sphingoid *N*-acylation, *c*) length of the *N*-acyl chain greater than 12-14 carbons, *d*) the absence of a headgroup at C_1, and *e*) either a single or double bond and a *trans* configuration at C4-C5. Furthermore, though *N*-methylation did not perturb antibody interaction, a urea linkage at this position disrupted recognition (Fig. 4). In contrast, identifying the structural determinants for recognition by the monoclonal antibody is less straightforward. In general, the results obtained using the monoclonal antibody did not allow us to draw broad conclusions as to binding determinants. Based on the panel of lipids we tested for interaction with the monoclonal antibody, we conclude that the only steadfast requirements for recognition are *a*) the presence of two alkyl chains (as monoalkyl compounds such as sphingoid bases were not recognized), and *b*) if a sphingoid moiety is present, the presence of the amide linkage from this nitrogen. As sphingolipids with head groups (SM, Fig. 1; ceramide-1-phosphate, Fig. 5) and the glycerolipid PC (Fig. 1), which lacks a sphingoid moiety, were also recognized by the monoclonal antibody, it is more difficult to draw conclusions as to which regions are required for antibody interaction.

TABLE 2. Recognition of synthetic ceramide analogs by two anti-ceramide antibody preparations

Ceramide	Monoclonal IgM	Polyclonal Antiserum
Chiral stereoisomers		
D-*erythro*-C_{16}-ceramide	+	++++
L-*erythro*-C_{16}-ceramide	+	++++
D-*threo*-C_{16}-ceramide	++	++++
L-*threo*-C_{16}-ceramide	++	++++
Short-chain ceramides		
D-*erythro*-C_2-ceramide	−	−
D-*erythro*-C_6-ceramide	−	−
D-*erythro*-C_{10}-ceramide	++	+
cis-Stereoisomer		
D-*erythro*-C_{16}-*cis*-ceramide	+++	++
ω-Ceramides		
D-*erythro*-C_{12}-NBD-ceramide	−	−
Modified D-*erythro*-C_{16}-ceramides		
3-keto-ceramide	++++	++++
3-keto-dihydroceramide	+++	++++
4-5-dehydro-ceramide	++	−
N-methyl-ceramide	++++	++++
urea-ceramide	−	−

+, Barely detectable; ++, moderately detected; ++++, maximum detection; −, no detection.

The reason for the recognition by the monclonal antibody of dehydro-, dihydro, *cis*-, *N*-methyl-, and 3-keto ceramide preferentially to the endogenous D-*erythro*-C16 ceramide remains unclear (Figs. 5, 4, and 1, respectively); however, as dihydroceramide is present in biological samples, the monoclonal antibody may prove useful for localization of dihydrocermide. As mentioned above, however, the recognition of PC and SM by this preparation (Fig. 1) may be problematic in cases where lipid composition of samples is undetermined.

The experimental application of these antibodies that is likely to be of most interest to investigators is in immunocytochemical studies. Because of the complexity of cell systems with respect to the nature of both cellular lipid pools and the topological arrangement of phospho- and sphingolipids within them, it is difficult to use this approach for the fundamental characterization of the antibodies. Rather, by characterizing the antibodies' interactions with purified lipids, we have aimed to provide information about the antibodies that will both facilitate the appropriate design of experimental controls by investigators using these reagents in cell settings and make them aware of the fundamental properties of recognition by these reagents. Indeed, as evidenced by the differences in lipid recognition by the polyclonal antiserum between experiments conducted using nitrocellulose membranes versus silica gel TLC plates, the chemical environment of the recognized lipid obviously plays an important role in determining its degree of recognition by the antibody.

It is important to mention that the monoclonal antibody is intended to be used in enzyme-linked immunosorbent assays, as its specificity was originally established using this application (package insert). ELISA is a more sensitive technique than these lipid-overlay assays and is also quantitative. Indeed, in our laboratory different results were observed depending on the method used to evaluate the antibodies (i.e., lipid overlay assay vs. immunocytochemistry). Similarly, a previous study demonstrated differential recognition of lipids by the antibodies depending on the method used (16). As we have not evaluated the monoclonal antibody using ELISA, we cannot attest to its specificity in this application; however, in both the silica-based and nitrocellulose-based assays, the monoclonal antibody failed to detect D-*erythro* ceramides. The widest application of these antibodies may be in immunocytochemistry, and appropriate controls should be performed for each antibody in each specific application, regardless of which one is chosen for study.

In conclusion, the anti-ceramide polyclonal antiserum is significantly more ceramide-specific than the monoclonal anti-ceramide antibody; when interpreting data or designing experiments, these respective specificities should be taken into careful consideration.

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