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Mouse anti-ceramide antiserum: a specific tool for the detection of endogenous ceramide

Gabriele Vielhaber[3,4], Lore Brade[3], Buko Lindner[3], Stephan Pfeiffer[1,4], Roger Wepf[4], Ulrich Hintze[4], Klaus-Peter Wittern[4], and Helmut Brade[2,3]

[3]Research Center Borstel, Center for Medicine and Biosciences, Parkallee 22, D-23845 Borstel, Germany, and [4]Analytical Research Department, Beiersdorf AG, Unnastr. 48, D-20245 Hamburg, Germany

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Ceramide is a pivotal molecule in signal transduction and an essential structural component of the epidermal permeability barrier. The epidermis is marked by a high concentration of ceramide and by a unique spectrum of ceramide species: Besides the two ceramide structures commonly found in mammalian tissue, *N*-acylsphingosine and *N*-2-hydroxyacylsphingosine, six additional ceramides differing in the grade of hydroxylation of either the sphingosine base or the fatty acid have been identified in the epidermis. Here we report on the characterization of an IgM-enriched polyclonal mouse serum against ceramide. In dot blot assays with purified epidermal lipids the antiserum bound to a similar extent to *N*-acyl-sphingosine (ceramide 2), *N*-acyl-4-hydroxysphinganine (ceramide 3), and *N*-(2-hydroxyacyl)-sphingosine (ceramide 5), whereas no specific reaction was detected with glycosylceramides, sphingomyelin, free sphingosine, phospholipids, or cholesterol. In contrast, a monoclonal IgM antibody, also claimed to be specific for ceramide, was shown to bind specifically to sphingomyelin and therefore was not further investigated. In thin-layer chromatography immunostaining with purified lipids a strong and highly reproducible reaction of the antiserum with ceramide 2 and ceramide 5 was observed, whereas the reaction with ceramide 1 and ceramide 3 was weaker and more variable. Ceramide 2 and ceramide 5 were detected in the nanomolar range at serum dilutions of up to 1:100 by dot blot and thin-layer immunostaining. In thin-layer chromatography immunostaining of crude lipid extracts from human epidermis, the antiserum also reacted with *N*-(2-hydroxyacyl)-4-hydroxysphinganine (ceramide 6) and *N*-(2-hydroxyacyl)-6-hydroxysphingosine (ceramide 7). Furthermore, the suitability of the antiserum for the detection of endogenous ceramide by immunolight microscopy was demonstrated on cryoprocessed human skin tissue. Double immunofluorescence labeling experiments with the anti-ceramide antiserum and the recently described anti-glucosylceramide antiserum (Brade *et al.*, 2000, *Glycobiology* 10, 629) showed that both lipids are concentrated in separate epidermal sites. Whereas anti-ceramide stained the dermal and basal epidermal cells as well as the corneocytes, anti-glucosylceramide staining was concentrated in the stratum granulosum. In conclusion, the specificity and sensitivity of the reagent will enable studies on the subcellular distribution and biological functions of endogenous ceramide.

Key words: sphingolipids/thin-layer immunostaining/epidermis/immunohistochemistry/glucosylceramide/antibodies

Introduction

Ceramide has been identified as an important effector in signal transduction and regulation of specific cell functions (reviewed in Perry and Hannun, 1998), for example, differentiation and apoptosis. Synthetic ceramide analogs activate protein phosphatases (Dobrowsky *et al.*, 1993; Chalfant *et al.*, 1999); protein kinases such as ceramide-activated protein kinase (Yao *et al.*, 1995), Raf (Huwiler *et al.*, 1996) and PKCζ (Müller *et al.*, 1995); as well as interleukin-converting enzyme-like proteases (Yoshimura *et al.*, 1998; Mizushima *et al.*, 1996). In addition, short-chain ceramide analogs interfere with the function of mitochondria by inducing the release of cytochrome c from the mitochondrial inner membrane (Ghafourifar *et al.*, 1999). On the other hand, endogenously generated ceramide is actually discussed as a downstream mediator of apoptosis rather than being itself an essential second messenger (Hannun, 1996; Watts *et al.*, 1999; Tepper *et al.*, 1999).

Besides its importance in signal transduction processes, ceramide displays an essential role as a structural component of the epidermal permeability barrier against transcutaneous water loss, which is located in the stratum corneum. Here, ceramide, together with cholesterol and free fatty acids, forms multiple intercellular lipid lamellae, which are embedded in a rigid matrix of corneocytes (Elias and Menon, 1991; Forslind *et al.*, 1997). Specialized secretory organelles, the lamellar bodies, deliver the barrier lipids to the stratum corneum. They are enriched in a polar lipid mixture of glucosylceramides, phospholipids, and sterols and contain several acid hydrolases (Freinkel and Traczyk, 1985). At the stratum granulosum–stratum corneum interface the lamellar bodies fuse with the plasma membrane of the uppermost granulocyte and secrete their contents into the intercellular space of the stratum corneum. Concomitantly, the lipids are enzymatically hydrolyzed into ceramide, cholesterol, and free fatty acids.

To date, eight epidermal ceramide species are known, varying in the hydroxylation of the sphingosine backbone and of the fatty acid moiety (Figure 1; Robson *et al.*, 1994; Stewart and Downing, 1999). Among them, two ceramide species exist

[1]Present address: Central Microscopy, Biology Center, University of Kiel, Ohlshausenstr. 40, D-24098 Kiel, Germany

[2]To whom correspondence should be addressed

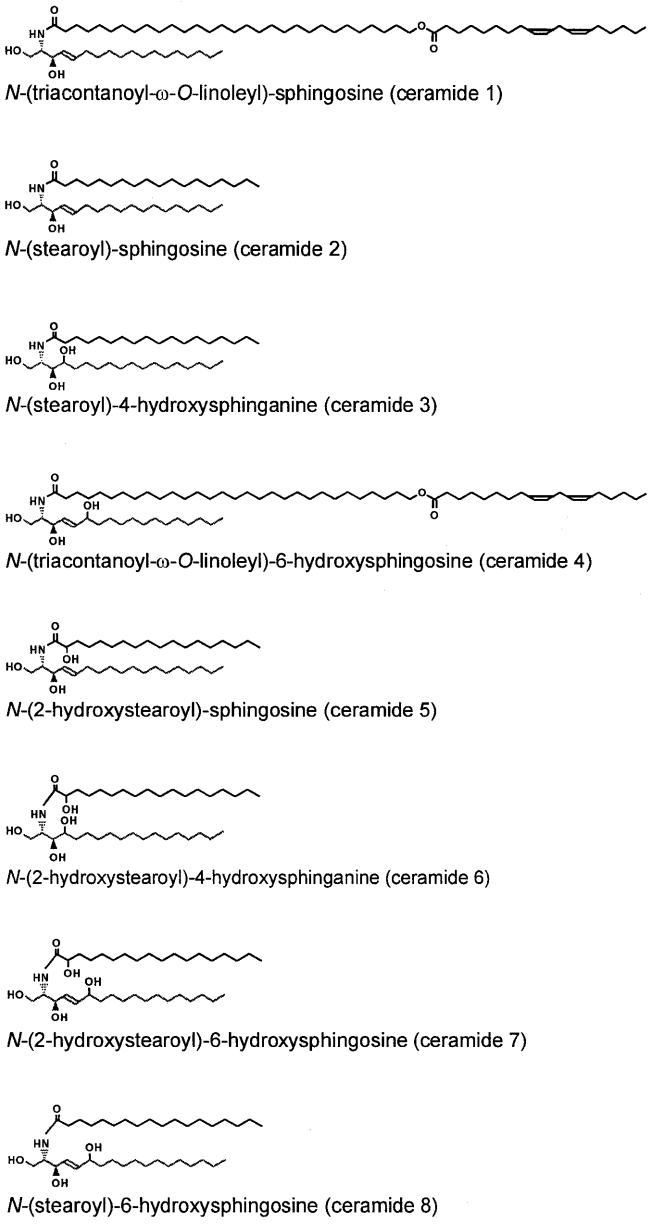

Fig. 1. Chemical structures of ceramide species occurring in human epidermis. Note that the chain length of the amide linked fatty acid and the sphingosine base vary in natural ceramides. Ceramides 1–7 according to Robson *et al.* (1994), ceramide 8 according to Brade, 2000.

that possess an ω-hydroxyacyl moiety (Swartzendruber *et al.*, 1987; Wertz *et al.*, 1987). They are covalently bound to proteins of the cornified envelope, a compact polymer structure composed of protein and lipid that coats the surface of the corneocytes (Marekov and Steinert, 1998). Glucosylceramide and, to a minor extent, sphingomyelin are the major precursors of epidermal barrier ceramide, as demonstrated by studies on mice deficient in the respective acid hydrolases, β-glucocerebrosidase and acid sphingomyelinase (Holleran *et al.*, 1993, 1994; Jensen *et al.*, 1999; Schmuth *et al.*, 2000).

Considering the multiple targets of ceramide action, it is of increasing interest to identify the subcellular localization of endogenous ceramide at the microscopic level. However, a suitable antibody has not been available so far. Here we report on the characterization of two commercially available reagents claimed to be specific for ceramide. One of these is an immunoglobulin (Ig)M-enriched polyclonal mouse serum, the other a monoclonal antibody of the IgM isotype. It will be shown by dot blot assays, thin-layer chromatography (TLC) immunostaining, and immuno-light microscopy that the antiserum specifically reacts with various ceramide species in the nanomolar range, whereas the monoclonal antibody will be shown to be specific for sphingomyelin in dot blot assays.

Results

Specificity of anti-ceramide antibodies in dot blot assays

The specificity of the mouse anti-ceramide antibodies was first tested in dot blot assays using various purified lipids (Figure 2). The antiserum was tested at dilutions of 1:25 and 1:100 (Figure 2A) and the monoclonal antibody at a concentration of 20 μg/ml (Figure 2B), both with 10, 2, and 0.4 nmol of antigen. The antiserum reacted specifically with all ceramides tested except ceramide 1 (Cer-1, Figure 1) at both dilutions and showed no cross-reactivity with glycosylceramides, sphingomyelin, sphingosine, phospholipids, or cholesterol when 2 nmol or less antigen were present (Figure 2A). A faint reaction with Cer-1, cholesterol, and phosphatidylethanolamine was seen only with high amounts (≥ 10 nmol) of antigen and was clearly distinguishable from reactions that were still observed with 0.4 nmol of antigen. As shown in Figure 2B, the monoclonal antibody specifically recognized sphingomyelin but not ceramides or any other lipid included in the test. Because the present study was aimed at the characterization of anti-ceramide antibodies, the monoclonal antibody was not included in the following experiments.

The antiserum binds specifically to ceramide in immunostaining of thin-layer chromatograms

When a mixture of standard lipids in amounts ranging from 80 to 0.63 nmol was separated by TLC and visualized by immunostaining with anti-ceramide serum at a dilution of 1:100 (Figure 3B), as little as 1 nmol of the reference substances ceramide 2 (Cer-2, *N*-acylsphingosine) or ceramide 5 (Cer-5, *N*-(2-hydroxyacyl)-sphingosine) were detected, whereas Cer-1 and ceramide 3 (Cer-3, *N*-acyl-4-hydroxysphinganine) gave a staining at the detection limit. Cholesterol was not specifically detected, but if high amounts (80 nmol) were present, a faint reaction was observed.

Anti-ceramide antiserum reacts with several epidermal ceramide species in TLC immunostaining

To confirm that the antiserum also detects naturally occurring lipids, a crude epidermal lipid extract was separated by preparative TLC, and the ceramide fraction was scraped off, rechromatographed on a TLC plate, and stained with anti-ceramide antiserum. Approximately half of the free cholesterol and free fatty acids was retained in the ceramide fraction, as judged from comparison of the ceramide fraction and the lipids remaining after separation by TLC and visualization with spray reagent. As shown in Figure 4, the antiserum reacted with all standard ceramides (Cer-1, Cer-2, Cer-3, Cer-5) and with epidermal Cer-2, Cer-3,

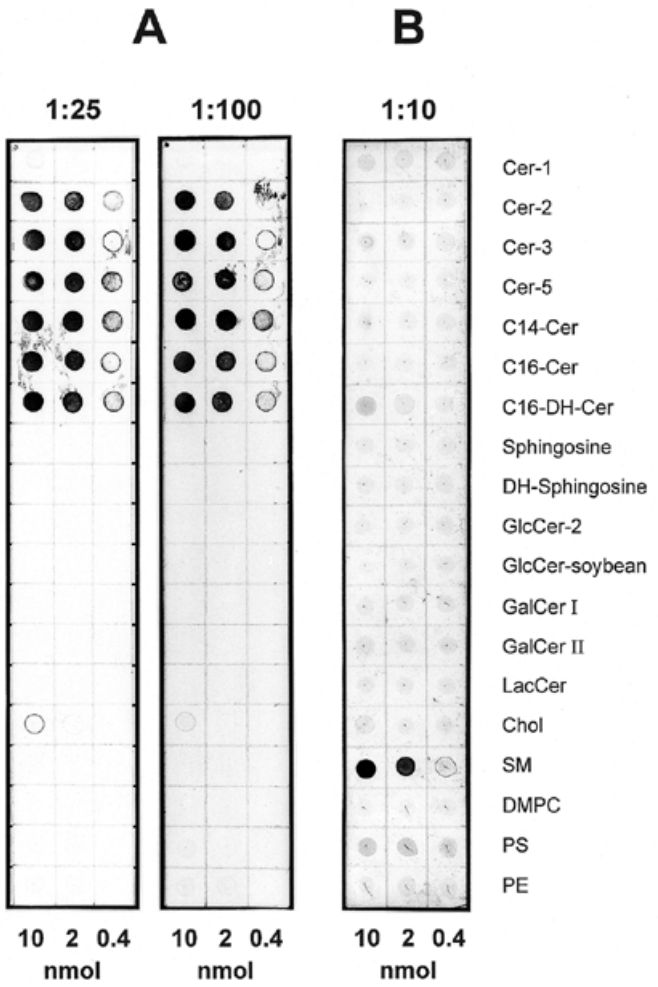

Fig. 2. Specificity of mouse antiserum and mouse monoclonal antibody against ceramide in dot blot assays. Aliquots of 0.4, 2.0, or 10.0 nmol of antigen were spotted onto nylon membranes and reacted with polyclonal mouse anti-ceramide antiserum (**A**) or monoclonal mouse anti-ceramide antibody (**B**) at the indicated dilutions. Cer-1, ceramide-1; Cer-2, ceramide-2; Cer-3, ceramide-3; Cer-5, ceramide 5; C14-Cer, N-myristoyl-D-sphingosine; C16-Cer, N-palmitoyl-D-sphingosine; C16-DH-Cer, N-palmitoyl-dihydro-sphingosine; DH-sphingosine, dihydro-sphingosine; GlcCer-soybean, glucosylceramides from soybean; GlcCer-2, glucosylceramide 2; GalCer I, galactosylceramide I ; GalCer II, galactosylceramide II; LacCer, lactosylceramide; Chol, cholesterol; SM, N-palmitoyl-D-sphingomyelin; DMPC, dimyristoylphosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. For chemical structures see Figure 1.

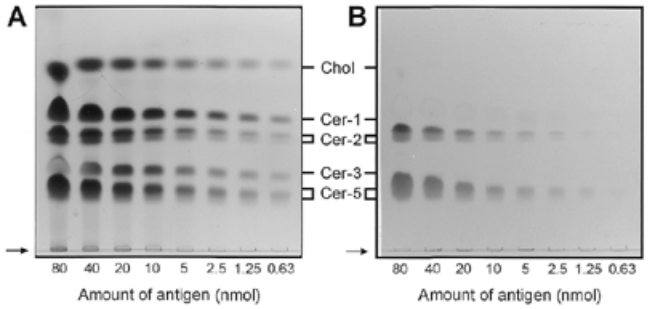

Fig. 3. Sensitivity of anti-ceramide mouse antiserum in immunostaining of TLC plates. Aliquots of purified lipids (80–0.63 nmol) were separated by TLC and visualized with a spray reagent (**A**) or by immunostaining with mouse anti-ceramide (**B**). The antigens were ceramide 1 (Cer-1), ceramide 2 (Cer-2), ceramide 3 (Cer-3), ceramide 5 (Cer-5), and cholesterol (Chol). The arrow indicates the origin. For chemical structures see Figure 1.

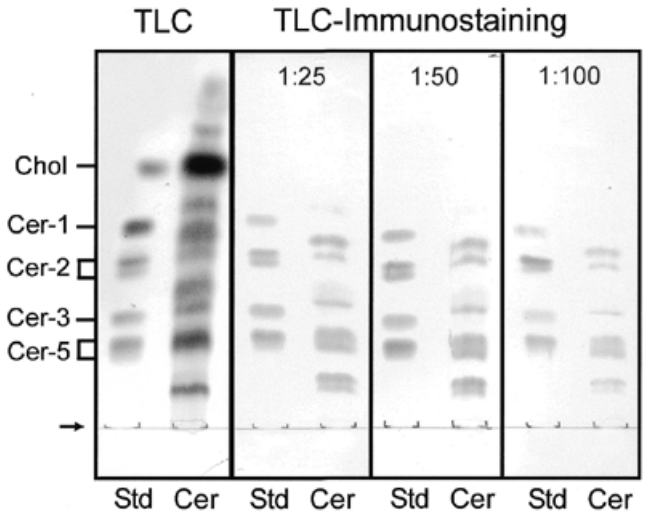

Fig. 4. Reactivity of anti-ceramide mouse antiserum with epidermal lipid extracts in TLC immunostaining. Aliquots of an epidermal lipid extract enriched in ceramides (2.4 mg lipid per lane) (Cer) were separated by TLC and visualized with a spray reagent or by immunostaining with mouse anti-Cer diluted 1:25, 1:50, and 1:100, respectively. For details see *Materials and methods*. The standard lipid mixture (Std) was composed of cholesterol (Chol), ceramide 1 (Cer-1), ceramide 2 (Cer-2), ceramide 3 (Cer-3), and ceramide 5 (Cer-5) (8 nmol lipid each). The arrow indicates the origin. For chemical structures see Figure 1.

Cer-5, Cer-6, and Cer-7, but not with cholesterol and free fatty acids in dilutions from 1:25 to 1:100. At a dilution of 1:25, the antibody also detected epidermal Cer-1, whereas no reaction with Cer-4 was observed. The most intense reaction was observed with Cer-2, Cer-5, and Cer-6. The molecular weight of the epidermal ceramides recognized by the antiserum was confirmed by MALDI-TOF mass spectrometry (data not shown).

The antiserum detects ceramide in human epidermis by immunofluorescence light microscopy

To provide further evidence for the specificity of the anti-ceramide antiserum, we performed a double immunofluorescence staining on cryoprocessed samples of human epidermis (Pfeiffer *et al.*, 2000) with anti-ceramide and anti-glucosylceramide antisera (Brade *et al.*, 2000). As shown in Figure 5, the antisera clearly stained distinct epidermal sites: anti-ceramide fluorescence was concentrated in dermal cells and epidermal cells of the basal, spinous, and horny layers, whereas anti-glucosyl-ceramide stained only the cells of the stratum granulosum and upper stratum spinosum. Basal keratinocytes and dermal fibroblasts showed a prominent fluorescence around the cell nuclei, in the cytoplasm, and at cell boundaries, whereas this staining pattern gradually faded toward the upper epidermal layers. In addition, the boundaries of the uppermost granular cells as well as those of the corneocytes were intensely stained.

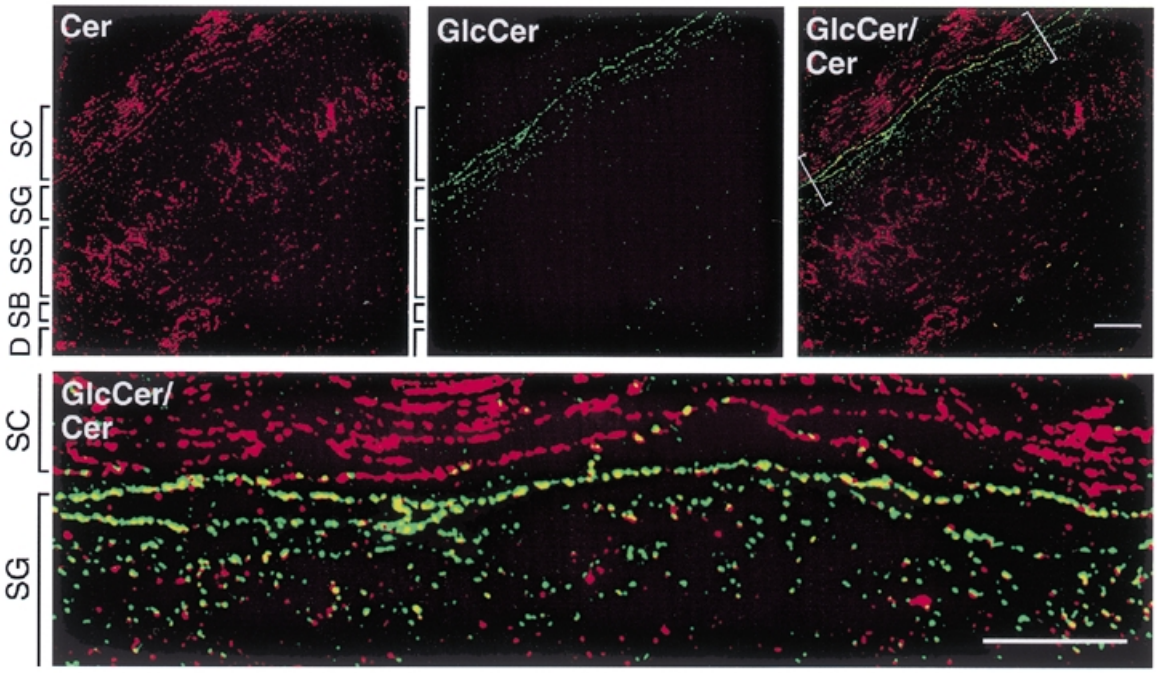

Fig. 5. Ceramide and glucosylceramide are complementary localized in human skin. Double immunofluorescence staining of ceramide and glucosylceramide on semithin sections of high-pressure frozen and freeze-substituted human skin, embedded in Lowicryl HM20. Upper panel: SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; D, dermis. Right picture: The brackets depict the region enlarged in the lower panel; bar, 20 µm. Lower panel: Note the occasional colocalization of ceramide and glucosylceramide in the upper stratum granulosum as reflected by the yellow spots; bar, 10 µm.

As previously reported for GlcCer (Brade *et al.*, 2000), a low dehydration temperature during preparation of the tissue for electron microscopy was of decisive importance for the retention of Cer in the sample, whereas the staining pattern was largely unchanged if chemical fixation was used instead of physical fixation. If the dehydration was performed in a graded ethanol series at temperatures over –50°C, the signal significantly blurred. After dehydration at room temperature, Cer was no longer detectable (data not shown). Thus, Cer and GlcCer immunohistochemistry should only be performed with specimens dehydrated at temperatures below –50°C.

Enzyme-linked immunosorbent assay

The antiserum was also tested by enzyme-linked immunosorgent assay (ELISA). Figure 6 shows binding curves of serially diluted antiserum with graded amounts of Cer-5 ranging from 0.032 nmol/well (open diamonds) to 4 nmol/well (filled circles) as a solid-phase antigen. Over a broad range of antigen concentrations the binding curves showed similar slopes. With very low concentrations of 0.063 and 0.032 nmol antigen per well, the steepness of the curves started to decrease. Also the ranges of confidence values were dependent on the ceramide concentrations applied to the plate. With 4.0 and 2.0 nmol per well the confidence values did not exceed 10%, with 1.0 and 0.5 nmol per well they remained below 15%, whereas with lower antigen concentrations the confidence values increased up to a range of 20%. No specific binding was observed with a control mouse serum, which had not been enriched for anti-ceramide antibody.

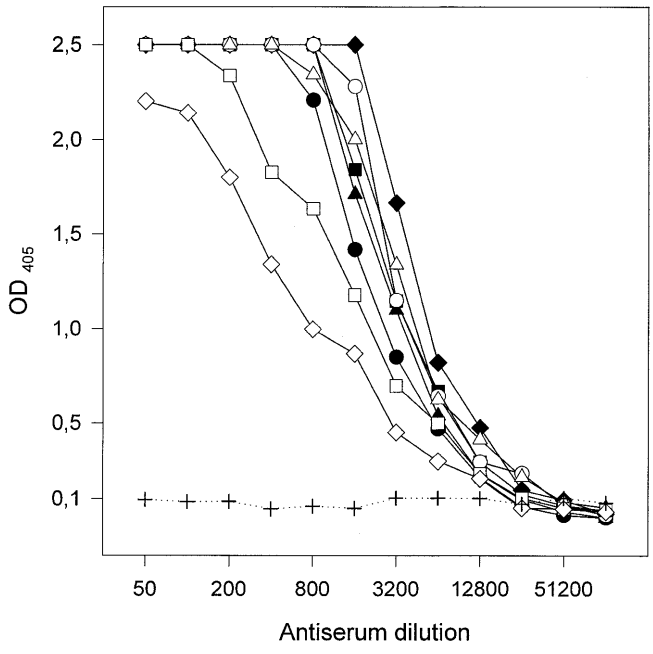

Fig. 6. Binding characteristics of anti-ceramide mouse antiserum with Cer-5 in ELISA. Microtiter plates were coated with 4 (filled circle), 2 (filled triangle), 1 (filled square), 0.5 (filled diamond), 0.25 (open circle), 0.125 (open triangle), 0.063 (open square), or 0.032 (open diamond) nmol/well and reacted with serial twofold dilutions of mouse anti-Cer. The dotted line (cross) represents the reactivity of the serum without enrichment for anti-ceramide antibodies with the highest amount of antigen (4 nmol/well). Values are means of quadruplicates with confidence values not exceeding 20%.

Discussion

There is a broad interest in specific antibodies against ceramides for the immunochemical detection of these lipids in tissues and cells for those studying the biosynthesis, localization, and function of ceramides in complex biological systems.

Recently, two reagents with claimed specificity for ceramide became commercially available: a monoclonal mouse IgM antibody and an IgM-enriched polyclonal mouse serum. However, already in the first set of experiments (dot blot assays) the monoclonal antibody turned out to be specific not for ceramide but for sphingomyelin. Because our study was aimed at the characterization of anti-ceramide reagents, the monoclonal antibody was omitted from further experiments.

In all methods applied, the antiserum specifically reacted with purified and natural Cer-2 and Cer-5. Comparing the results of the dot blot assays (Figure 2A) and the TLC immunostaining experiments (Figures 3 and 4), the strong reaction with purified Cer-2 and Cer-5 was highly reproducible in all experiments and with both methods, whereas the weaker reactions of anti-Cer with purified Cer-1 and Cer-3 exhibited some variability, which was more pronounced in TLC immunostaining than in dot blot assays. This suggests that the adherence of the ceramides on the silica gel TLC support was more critical than on the nylon membrane used for the dot blot assay. Because overlay immunostaining of TLC plates is known to vary in intensity from one experiment to another, the different staining shown in Figures 3 and 4 was not surprising. Since *N*-(ω-hydroxyacyl)-6-hydroxysphingosine (ceramide 4), liberated from protein-bound ceramides of the cornified envelope, was well detected with the anti-ceramide serum (Vielhaber *et al*., unpublished data), the long acyl chain and the ω-hydroxy group cannot be the only reason for the negative outcome of the experiment shown in Figure 3. However, the complete unreactiveness of Cer-1 in dot blot (Figure 2) was not expected. It seems that Cer-1 adopts a different conformation on nylon membranes than after chromatography on TLC plates. Further experiments using chemically synthesized analogues of ceramide may help answer this question.

In the ELISA system, a higher variability in the weaker reactions was also seen. With decreasing antigen concentrations (1 nmol per well and less) the confidence value increased. On the other hand, ELISA is highly sensitive, allowing the detection of ceramides in the low nanomolar range and has the advantage of being a quantitative assay. Based on these results, an ELISA inhibition test can be envisaged in which liposome-incorporated lipids could be tested and compared quantitatively for their inhibitory capacity.

In addition, the antiserum recognized all epidermal ceramides (Figure 4). However, the reaction with epidermal Cer-1 was weak, and the reactivity against epidermal Cer-4 could not be judged from these experiments due to the low amounts of these lipids present in the epidermal extract. Remarkably, there was a strong immune reaction with epidermal Cer-6 taking into account the low amounts of Cer-6 present in the epidermal lipid extract, whereas the reaction with epidermal Cer-7 was only moderate. Thus, the proximity of the additional hydroxyl group to the hydrophilic "head" of the ceramide molecule seems to decisively support the antigenic recognition.

In some experiments the antiserum also reacted to some extent with cholesterol. However, because the reaction with cholesterol was only observed at concentrations that were 100 times higher than with ceramide (Figure 3B) and because cholesterol and ceramide share no structural similarity, this faint reaction is most likely due to nonspecific binding of IgM antibodies to hydrophobic compounds.

The specificity for ceramide was further confirmed by the results of immuno-light microscopy of cryoprocessed human skin biopsies (Figure 5), which showed a separate staining pattern of anti-ceramide and anti-glucosylceramide in the epidermis with only poor colocalization of the named lipids in the uppermost stratum granulosum. Furthermore, the concentration of ceramide staining at the corneocyte cell boundaries correlated well with the general assumption that ceramides are localized intercellularly in the stratum corneum and in part covalently bound to the protein matrix of the corneocytes. The intense ceramide fluorescence in the cells of the lower epidermis and of the dermis underlines the high sensitivity of the ceramide antiserum, because the ceramide content per cell is about 30 times lower in the lower epidermis than in the stratum corneum (Yardley, 1983). The fact that no vesicular staining pattern in the stratum granulosum was obtained with anti-ceramide suggests that, in contrast to glucosylceramide (Wertz *et al.*, 1984; Freinkel and Traczyk, 1985; Elias and Menon, 1991; Brade *et al.*, 2000), ceramide is not localized within the lamellar bodies. Immuno-gold electron microscopical investigations have resolved the subcellular distribution of ceramide in the cells of the lower epidermis and will be published elsewhere.

Conclusions

With the aid of a polyclonal antiserum specific for ceramide it is now possible to visualize the distribution of endogenous ceramide in subcellular compartments of human tissue. This reagent will help to further elucidate the structural diversity and biological function of distinct ceramide species.

Materials and methods

Lipids

Cer-2 (Ceramide type III composed mainly of *N*-stearoyl-sphingosine) and Cer-5 (Ceramide type IV containing Cer-2 and *N*-(2-hydroxystearoyl)-sphingosine), *N*-palmitoyl-DL-dihydro-sphingosine, D-sphingosine, DL-dihydrosphingosine, GlcCer-2, galactosylceramide type I and II, lactosylceramide, cholesterol, *N*-palmitoyl-D-sphingomyelin, phosphatidylserine, phosphatidyl-ethanolamine, and dimyristoylphosphatidylcholine were purchased from Sigma. *N*-palmitoyl-D-sphingosine and *N*-myristoyl-D-sphingosine were from Toronto Research Chemicals (Ontario, Canada). Cer-3 (*N*-acyl-4-hydroxysphinganine, also known as phytoceramid) was obtained from Cosmoferm (Delft, The Netherlands). Cer-1 [*N*-(triacontanoyl-ω-*O*-linoleoyl)-sphingosine] was chemically synthesized by R.R. Schmidt (University of Konstanz); its chemical structure was confirmed by MALDI-TOF (data not shown). Soybean GlcCer was isolated as described previously (Brade *et al.*, 2000). All substances were dissolved in chloroform/methanol (3:1, v/v). For chemical structures see also Figure 1.

Antibodies

The mouse antiserum against ceramide and the rabbit antiserum against GlcCer were purchased from GlycoTech Produktions- und Handelsgesellschaft mbH (Kuekels, Germany). The monoclonal antibody against Cer was purchased from Alexis Deutschland GmbH (Grünberg, Germany).

Preparation of natural lipid extracts

Epidermal lipid extracts from human breast skin were prepared as described (Doering *et al.*, 1999). For further purification of the epidermal ceramide fraction, 40 mg of epidermal lipids were separated twice on a preparative TLC plate (2 mm silica gel) in $CHCl_3$/MeOH/CH_3COOH (190:9:1; v/v/v). The ceramide fraction was scraped off, eluted from the silica gel with chloroform/methanol (2:1; v/v) by shaking overnight, and the organic phase sucked off and dried under nitrogen.

Dot blot

The dot blot procedure was exactly performed as described (Brade *et al.*, 2000) with amounts of antigen and antiserum dilutions as indicated in Figure 2.

TLC and TLC-immunostaining

Ceramides were separated twice on silica gel 60 TLC plates with aluminum support (Merck) with a solvent system of $CHCl_3$/MeOH/CH_3COOH (190:9:1 v/v/v) and visualized by spraying with 10% $CuSO_4$ and 8% H_3PO_4 in water and heating at 180°C (Imokawa *et al.*, 1991). For TLC-immunostaining the plates were first incubated with 0.1% saponin in washing buffer (50 mM Tris–HCl, pH 7.4, 200 mM NaCl) for 30 min, then blocked with 5% nonfat dry milk in washing buffer for 1 h at room temperature and subsequently incubated with mouse serum against ceramide at the indicated dilution overnight at room temperature. After five washings (5 min each) in washing buffer, the plates were incubated with peroxidase-conjugated goat anti-mouse IgM (heavy and light chain specific, Dianova), diluted 1:1500 in blocking solution for 2 h at room temperature, washed 4 times, and a fifth time in substrate buffer (0.1 M sodium citrate buffer, pH 4.5). Bound antibody was then detected by incubation with substrate solution (10 ml), which was freshly prepared and composed of 8.33 ml substrate buffer, 1.67 ml of 4-chloro-1-naphthol (3 mg/ml in MeOH), and hydrogen peroxide (3.3 µl of a 30% solution).

ELISA

ELISA was performed as described elsewhere (Brade *et al.*, 2000).

Immunohistochemistry

For immunohistochemistry, high-pressure frozen and freeze-substituted human skin biopsies embedded in HM20 were used (Pfeiffer *et al.*, 2000). Immunofluorescence staining was performed according to Brade *et al.* (2000), but with 0.5% bovine serum albumin/0.2% fish gelatin in phosphate-buffered saline as washing buffer. The primary and secondary antibodies were applied simultaneously (dilutions: anti-Cer 1:2; anti-GlcCer 1:50, Rhodamine Red-X-conjugated goat anti-(mouse IgM) 1:200; Alexa488-conjugated goat anti-(rabbit IgG) 1:800). The images were acquired with a CLSM Leica TCS SP.

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Abbreviations

Cer, ceramide; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TLC, thin-layer chromatography.

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