In vitro characterization of anti-glucosylceramide rabbit antisera

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Glucosylceramides (GlcCer) are biosynthetic precursors of glycosphingolipids. They are widely distributed in biological systems where they exhibit numerous biological functions. Studies on the localization of glucosylceramides in different tissues have used biochemical methods only since specific antibodies against GlcCer were not previously available. We have characterized two commercially available rabbit antisera which were prepared against GlcCer of plant origin (1-O-(β-D-glucopyranosyl)-N-acyl-4-hydroxysphinganine; GlcCer-3) or human origin (1-O-(β-D-glucopyranosyl)-N-acyl-sphingosine; GlcCer-2) and claimed to be specific for GlcCer. The antisera were also able to detect specifically GlcCer species in crude lipid extracts from human epidermis after separation by thin-layer chromatography. The reagents are sensitive since both antisera reacted at dilutions higher than 1:500 with their homologous antigen in the nanogram range in thin layer immunostaining or dot-blot assays. The antisera are specific for GlcCer although they did not differentiate between GlcCer-2 and GlcCer-3 containing sphingosine or 4-hydroxysphinganine. The antisera also reacted with N-stearoyl-DL-dihydroglucocerebroside indicating that the naturally occurring structural variations in the amino alcohol moiety are not determining the specificity. No crossreactivity was observed with other mono- or diglycosylceramides (galactosylceramides, lactosylceramide), free ceramides or structurally unrelated lipids (cholesterol, sphingomyelin, or phospholipids). Therefore, the glycosylmoiety seems to represent the major antigenic determinant. Finally, the antisera also proved to be useful for the immunohistochemical localization of GlcCer in human epidermis by which earlier biochemical data on the distribution of GlcCer in the various epidermal layers were confirmed.

Key words: glycolipid antibody/thinlayer immunostaining/immunohistochemistry/epidermal lipids

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Introduction

Glycosphingolipids (GSL) are mediators of numerous biological functions such as cell recognition and cell adhesion, cell differentiation and development, or interaction of host cells with pathogens. While many studies have elucidated the role of galactosylceramide, lactosylceramide and more complex GSL, recent reports indicate also a specific role of glucosylceramides (GlcCer) in cellular function (Ichikawa and Hirabayashi, 1998). GlcCer induce neuronal growth (Schwarz and Futerman, 1997) and mitogenesis of many cell types (Datta and Radin, 1988; Shayman *et al.*, 1991; Yao and Yoshino, 1994; Marsh *et al.*, 1995), and have specific functions in epidermal differentiation (Uchida *et al.*, 1990; Sando *et al.*, 1996). Finally, GlcCer are accumulated in Gaucher's disease where the enzyme β-glucocerebrosidase is not or inefficiently functioning (Brady *et al.*, 1965). Most of the knowledge on the biological functions of GlcCer has resulted from biochemical analyses of extracted lipids from various organs or tissues by separation, quantification, and structural analysis. However, the immunohistochemical localization of GlcCer has not been reported before since no specific antibodies were available so far.

We were especially interested in the localization of GlcCer in human epidermis since GlcCer represent the major precursors of the epidermal ceramides which are essential components of the skin permeability barrier (Holleran *et al.*, 1993). The GlcCer content increases from the basal cell layer up to the outer stratum granulosum about 2-fold, whereas in the stratum corneum only traces of GlcCer are detectable (Lampe *et al.*, 1983; Yardley, 1983; Cox and Squier, 1986). From lipid biochemical and morphological investigations, it has been concluded that GlcCer are transported to the stratum corneum via specialized organelles, the lamellar bodies (LB). The LB are enriched in polar lipids like GlcCer, phospholipids and sterols and contain several acid hydrolases (Freinkel and Traczyk, 1985; Grayson *et al.*, 1985). They first appear in the stratum spinosum and accumulate with ongoing differentiation until they fill up to 25% of the cytosol of the granular cells (Elias and Friend, 1975). Upon reaching the interface of the stratum corneum and stratum granulosum, the LB fuse with the plasma membrane of the uppermost granulocytes and extrude their contents into the intercellular space of the stratum corneum. Concomitantly, the lipids are enzymatically processed into an unpolar mixture of ceramides, fatty acids, and cholesterol which are arranged into multiple lamellae. Together with the surrounding corneocytes, the intercellular lipid lamellae constitute the epidermal permeability barrier (Elias and Menon, 1991; Forslind *et al.*, 1997).

From studies with knock out mice it is evident that the conversion of GlcCer into ceramides at the interface of the

stratum corneum and stratum granulosum is of decisive importance for epidermal permeability barrier construction: accumulation of GlcCer within the lower parts of the stratum corneum due to deficiency of the processing enzyme β-glucocerebrosidase (Holleran *et al.*, 1994; Sidransky *et al.*, 1996) or the related sphingolipid activator protein C (Döring *et al.*, 1999a) results in impaired barrier function. Nevertheless, the immunohistochemical proof for the localization of GlcCer in the epidermis is still lacking.

Here we report on commercially available rabbit sera claimed to be specific for GlcCer. We determined their *in vitro* binding properties, their specificity and sensitivity and demonstrate their use for the identification of GlcCer by EIA, on TLC plates by immunostaining, and for the immunohistochemical localization in human epidermis.

Results

Dot-blot assays

From a commercial source we obtained six individual rabbit antisera against glucosylceramide, two of which were obtained after immunization with 1-O-(β-D-glucopyranosyl)-N-acyl-sphingosine (GlcCer-2), and four of which were obtained after immunization with 1-O-(β-D-glucopyranosyl)-N-acyl-4-hydroxy-sphinganine (GlcCer-3). In a first screening, serial dilutions of antiserum were tested with their homologous antigen by dot blot assays. As seen in Figure 1A, all four sera reacted with GlcCer-3, whereby serum number 4 yielded the best reactivity and serum number 2 the lowest. The two antisera against GlcCer-2 had a comparable reactivity with the homologous antigen (Figure 1B). For further characterization, we used a pool of the four antisera against GlcCer-3 and serum number 1 against GlcCer-2; these two reagents will be referred as anti-GlcCer-3 and anti-GlcCer-2 in the following.

Anti-GlcCer-2 and anti-GlcCer-3 were first tested by checkerboard titrations in dot-blot assays with the antigens used for immunization. Serial dilutions of each antiserum were tested with antigen amounts ranging from 8 to 1000 ng (Figure 2). Anti-GlcCer-3 antiserum gave a similar reaction pattern with the homologous GlcCer-3 (Figure 2A) and the heterologous GlcCer-2 (Figure 2C) antigen; however, the latter was one dilution step less sensitive. High amounts of antigen (1 µg) were still detected at a serum dilution of 1:2000 and less than 100 ng of antigen were detected at an antiserum dilution of 1:1000. The antiserum against GlcCer-2 of human origin was less active and could also not differentiate between GlcCer-2 and GlcCer-3 as an antigen (Figure 2B,D).

The specificity of the antisera against GlcCer-3 (Figure 3A) and GlcCer-2 (Figure 3B) was tested by dot-blots using a variety of structurally related or unrelated lipids and glycolipids at a serum dilution of 1:1000 and 1 or 0.1 µg of antigen. Both sera gave a positive reaction with GlcCer-2 (lane 8), GlcCer-3 (lane 7) and N-stearoyl-DL-dihydro-glucocerebroside (lane 4). Whereas galactosylceramides (lanes 1 and 2) did not react, lactosylceramide (lane 11) and sterylglucoside (lane 6) yielded a faint reaction with anti-GlcCer-3 which, however, was only seen with high amounts of antigen (1 µg) and clearly distinguishable from positive reactions which were still observed with 0.1 µg of antigen.
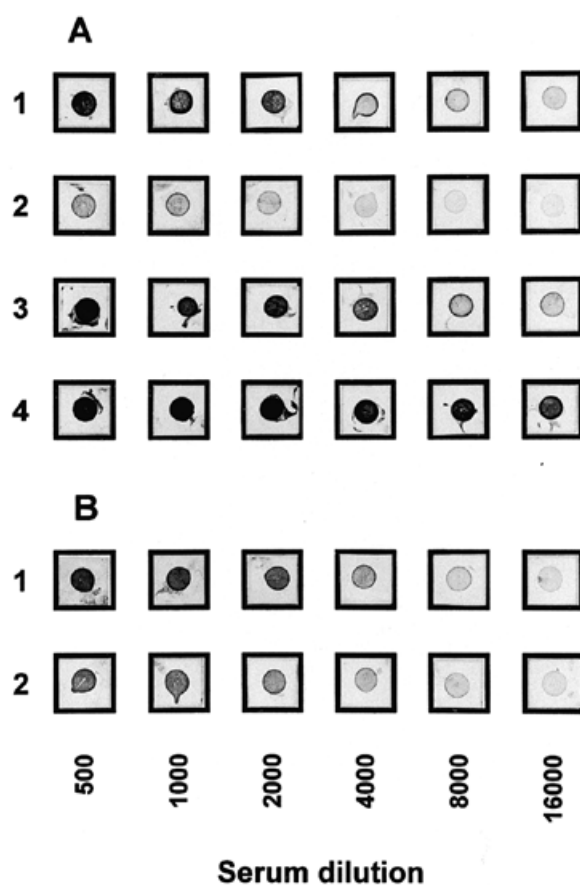
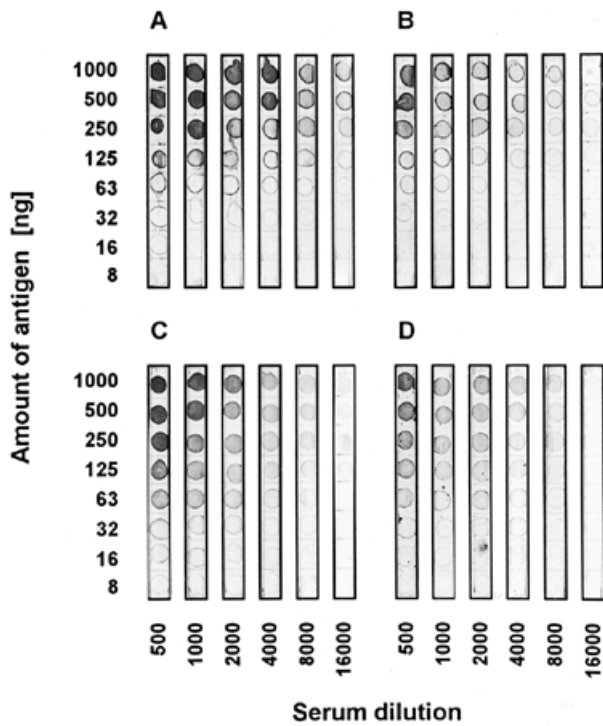**Fig. 1.** Screening of rabbit antisera against GlcCer-2 and GlcCer-3 by dot-blot assays. GlcCer-3 (**A**) or GlcCer-2 (**B**), 1 µg each, were spotted onto nylon membranes and reacted with serial dilutions of antisera from individual rabbits immunized with GlcCer-3 (**A**) or GlcCer-2 (**B**).

TLC and TLC-immunostaining

Purified glycolipid antigens were separated by TLC and visualized with standard spray reagents (Figure 4A) or by immunostaining with rabbit anti-GlcCer-2 (Figure 4B) or anti-GlcCer-3 (Figure 4C). The data obtained by dot-blot were confirmed and the specificity of the TLC-immunostaining was even clearer than in dot-blots. The antiserum against GlcCer-2 (Figure 4B) clearly visualized N-stearoyl-DL-dihydro-glucocerebroside (lane 4), GlcCer-3 (lane 7) and GlcCer-2 (lane 8) with comparable intensities. Thus, this antiserum did not differentiate between the homologous antigen GlcCer-2 and the heterologous antigens GlcCer-3 and N-stearoyl-DL-dihydroglucocerebroside, respectively. The antiserum against GlcCer-3 (Figure 4C) also reacted with GlcCer-2, GlcCer-3, and N-stearoyl-DL-dihydroglucocerebroside, however, the reactivity with the homologous antigen GlcCer-3 was significantly stronger than the other positive reactions. In addition, steryl-glucoside was slightly positive under these conditions. Although this antiserum is not regarded to be monospecific for the homologous antigen, it can distinguish to a certain extent between GlcCer-2 and GlcCer-3.

To provide evidence that the antisera used in TLC-overlay staining also reacted with crude lipid mixtures, in addition to
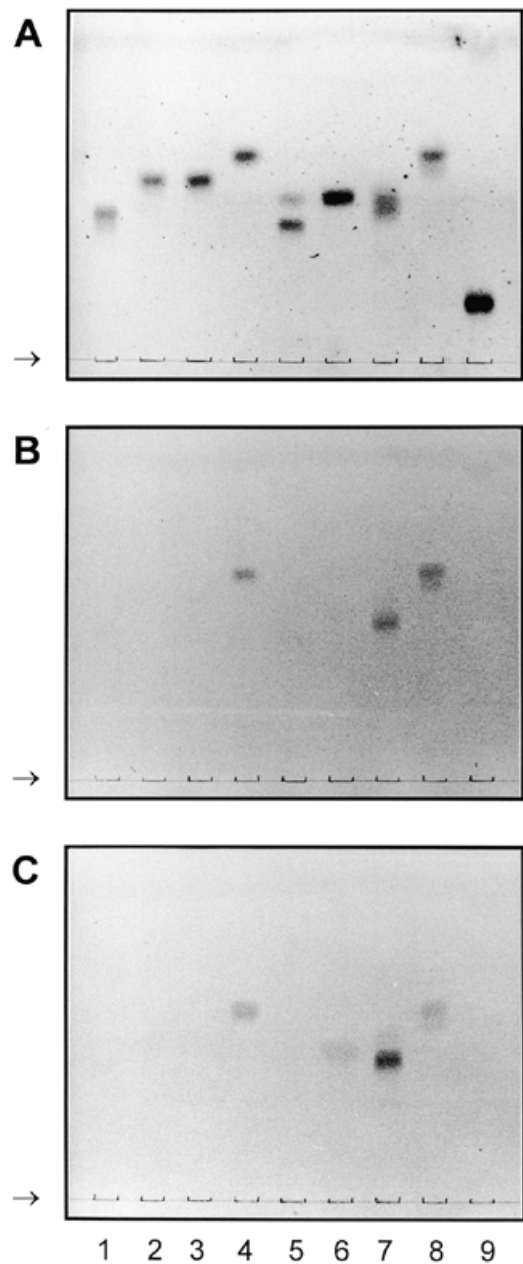
Fig. 2. Checkerboard titrations of anti-GlcCer-2 and anti-GlcCer-3 rabbit antisera in dot-blot assays. Amounts of 8 to 1000 ng of GlcCer-2 (**C** and **D**) or GlcCer-3 (**A** and **B**) were spotted onto nylon membranes and reacted with serial dilutions of rabbit antiserum against GlcCer-2 (**B** and **D**) or GlcCer-3 (**A** and **C**).
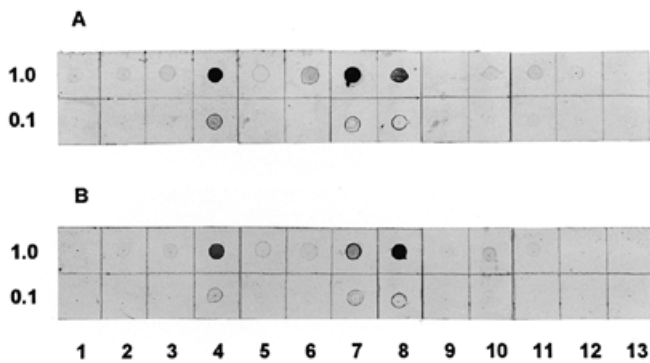

Fig. 3. Specificity of anti-GlcCer-2 and anti-GlcCer-3 rabbit antisera in dot-blot assays. Aliquots of 1 or 0.1 µg of antigen were spotted onto nylon membranes and reacted with rabbit antiserum (diluted 1:1000) against GlcCer-3 (**A**) or GlcCer-2 (**B**). The antigens were: galactosylceramide I (1), galactosylceramide II (2), N-stearoyl-DL-dihydrogalactocerebroside (3), N-stearoyl-DL-dihydroglucocerebroside (4), sterylgalactoside (5), sterylglucoside (6), GlcCer-3 (7), GlcCer-2 (8), ceramide-2 (9), ceramide-3 (10), lactosylceramide (11), cholesterol (12) and dimyristoylphosphatidylcholine (13).

Fig. 4. Specificity of anti-GlcCer-2 and anti-GlcCer-3 rabbit antisera in immunostaining of TLC plates. Purified lipid antigens were separated by TLC and visualized with a spray reagent (**A**) or by immunostaining with rabbit anti-GlcCer-2 (**B**) or anti-GlcCer-3 (**C**). For details see *Materials and methods*. The antigens (2 µg each) were galactosylceramide I (1), galactosylceramide II (2), N-stearoyl-DL-dihydrogalactocerebroside (3), N-stearoyl-DL-dihydroglucocerebroside (4), sterylgalactoside (5), sterylglucoside (6), GlcCer-3 (7), GlcCer-2 (8), and lactosylceramide (9). The arrow indicates the origin.

isolated and characterized lipids, we used lipid extracts from human epidermis which were separated by TLC and visualized with spray reagents or by immunostaining with anti-GlcCer-3. As shown in Figure 5, anti-GlcCer-3 clearly identified several bands within the R_f-range of epidermal cerebrosides (bracket in lane 2). It is noted that other lipids such as ceramide, cholesterol, or various phospholipids did not yield a positive reaction.

Since TLC-immunostaining and immunohistochemistry of glycolipids in general are methods of low sensitivity, we determined the sensitivity of anti-GlcCer-3 under TLC immunostaining conditions. The homologous antigen was serially diluted, separated by TLC and visualized by immunostaining as described before. As little as 60 ng of GlcCer-3 were still detected (Figure 6).
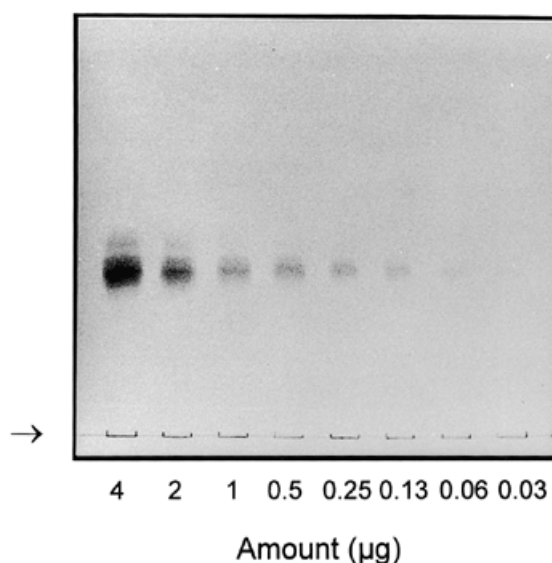
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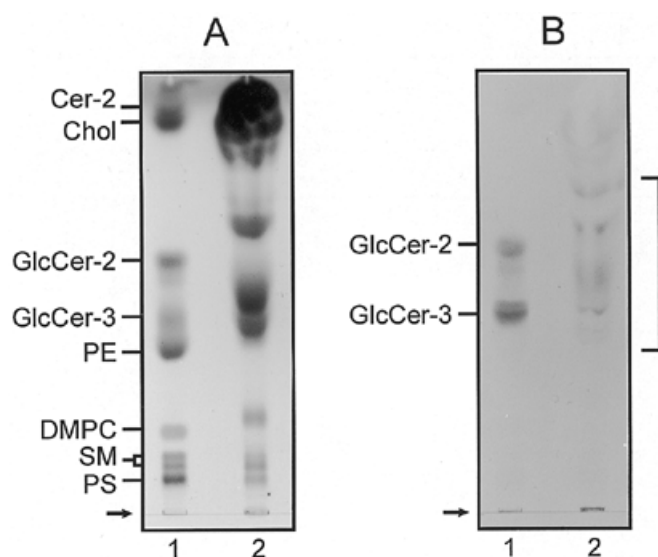
Fig. 5. Reactivity of anti-GlcCer-3 rabbit antiserum with epidermal lipids in TLC immunostaining. Aliquots of an epidermal lipid extract (335 μg each) (lane 2) were separated by TLC and visualized with a spray reagent (**A**) or by immunostaining with rabbit anti-GlcCer-3 (**B**). For details see *Materials and methods*. The standard lipid mixture (lane 1) was composed of cholesterol (Chol), Ceramide-2 (Cer-2), GlcCer-2, GlcCer-3, sphingomyelin (SM), phosphatidylethanolamine (PE), dimyristoylphosphatidylcholine (DMPC) and phosphatidylserine (PS) (1.6 nmol lipid each). The arrow indicates the origin.

Fig. 6. Sensitivity of anti-GlcCer-3 rabbit antiserum with the homologous antigen in TLC immunostaining. Aliquots of GlcCer-3 (4 μg to 30 ng) were separated by TLC and stained with rabbit anti-GlcCer-3.

EIA

Antisera were also tested by EIA. Figure 7 shows the binding of anti-GlcCer-3 antiserum with graded amounts of GlcCer-3 ranging from 3.2 ng/well (open squares) to 400 ng/well (solid circles) as a solid-phase antigen. With decreasing amounts of antigen the binding curves were shifted to higher antiserum concentrations. With less than 10 ng/well (open triangles and open squares), no more specific binding was observed compared to the reactivity of a preimmune serum (dotted line in Figure 7). Confidence values in the range of 20% were relatively high and no better values were obtained using other protocols varying the antigen-coating process or the blocking conditions (data not shown).

Immunohistochemistry

Since the localization of glycosphingolipids in tissues is certainly one of the most attractive applications of these new reagents, immunofluorescence microscopy studies were performed on semithin sections of cryoprocessed human skin biopsies from three different individuals (each n = 4). As seen in Figure 8A, a spotted staining pattern was observed in the upper layer of the stratum spinosum and the whole area of the stratum granulosum with the anti-GlcCer-3 antiserum. Whereas the staining was randomly distributed in the stratum spinosum and lower stratum granulosum, it concentrated at the keratinocyte cell membranes or the intercellular space in the uppermost two layers of the stratum spinosum. The corresponding preimmune serum did not show any signal (Figure 8B). Staining with the anti-GlcCer-2 antiserum resulted in the same pattern as observed with the anti-GlcCer-3 antiserum except that a lower dilution (1:30) had to be used (data not shown).

Discussion

In this study we investigated commercial antisera against 1-O-(β-D-glucopyranosyl)-N-acyl-sphingosine (GlcCer-2) and 1-O-(β-D-glucopyranosyl)-N-acyl-4-hydroxysphinganine (GlcCer-3) antisera which were worldwide not available so far. The antisera described here are specific; they do neither react with any other GSL than GlcCer on the one hand nor with free ceramide on the other. Thus, the terminal β-glucopyranose seems to be an essential characteristic of the epitope. Structural variations within the ceramide moiety, such as the type and chain length of the N-linked acyl residue or the presence of sphingosine, dihydrosphingosine, or 4-hydroxysphinganine seem to be of minor relevance for the epitope specificity. The antisera are not only specific but also sensitive allowing dilutions of up to 1:1000 for TLC overlay immunostaining and of up to 1:100 in immunohistochemistry. In addition, the GlcCer-3 antiserum reacts specifically with the cerebroside fraction of a crude natural lipid extract from human epidermis. The immunohistochemical results confirm this view. Since the antisera do neither react with other cerebrosides, in particular not with lactosyl- or galactosylceramide, or with ceramide, they will be extremely helpful tools to study the biosynthesis and biodegradation of GSL in general. For those who are experienced in the field of glycolipid immunochemistry, it is well known that antibodies against glycolipid antigens most often exhibit low affinities on the one hand and often cause false-positive reactions due to nonspecific interaction ("stickiness") on the other. Therefore, the experimental conditions have to be determined for each individual test system whereby special attention has to be paid to the blocking solutions and to the concentrations of antigen or antibody.

The manifold applications of these reagents are evident and cannot be discussed here in all aspects but the role of GlcCer-2
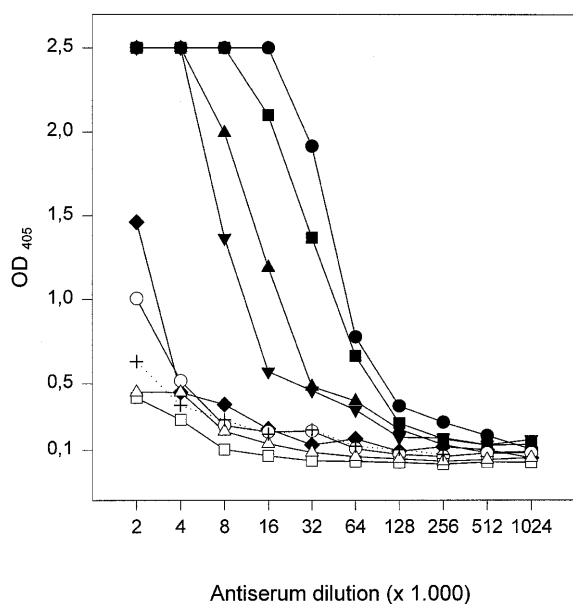
Fig. 7. Binding characteristics of anti-GlcCer-3 rabbit antiserum with its homologous antigen in EIA. Microtiter plates were coated with 400 (solid circles), 200 (solid squares), 100 (solid triangles), 50 (solid inverted triangles), 25 (solid diamonds), 12.5 (open circles), 6.3 (open triangles), or 3.2 (open squares) ng/well and reacted with serial two-fold dilutions of rabbit anti-GlcCer-3. The dotted line (cross) represents the reactivity of the corresponding preimmune serum with the highest amount of antigen (400 ng/well). Values are means of quadruplicates with confidence values not exceeding 20%.

and GlcCer-3 in the epidermis of mammalians for maintaining the barrier function of the skin is one well established example. Therefore, we proved the high quality and the specificity of these reagents by immunohistochemistry of human skin biopsies. Moreover, it was possible for the first time to visualize the distribution of a direct precursor of an epidermal barrier lipid in human epidermis. The observed immunofluorescence staining pattern for anti-GlcCer with concentration of GlcCer in the granular and upper spinous layers corresponds well to the known lipid analytical data of GlcCer distribution in human epidermis (Lampe *et al.*, 1983; Yardley, 1983). However, the lack of signal in the lower epidermis points out that the concentration of GlcCer has to exceed a critical threshold to cause a signal in immunohistochemistry. This threshold is reached by the increasing accumulation of GlcCer in LB with ongoing epidermal differentiation as seen from our light microscopical data. In addition, preliminary immuno-electron microscopy experiments support the general assumption that GlcCer are transported via LB to the stratum corneum.

The samples used in this study have been prepared by a protocol developed for transmission electron microscopy with the aim of an optimal lipid and protein antigen preservation (Pfeiffer *et al.*, forthcoming). However, we detected a similar antigenic staining pattern in chemically fixed skin biopsies which had been dehydrated stepwise by a graded ethanol series with simultaneous lowering of the temperature from 273 K to 223 K (data not shown). If the dehydration was performed continuously at 273 K, the staining blurred and a remarkable background was obtained. These findings suggest that one should be careful of completely dehydrating the tissue with

ethanol at 273 K or higher temperatures for anti-GlcCer immunohistochemistry.

Since it was not the primary aim of the present study to focus on the function of GlcCer in epidermis, we did not determine the identity of all epidermal cerebroside species reacting with the GlcCer-3 antiserum. At least six different glucosylceramide species of porcine epidermis (Wertz and Downing, 1983) and of human keratinocyte cell cultures (Hamakana *et al.*, 1993), respectively, have been structurally characterized. Among these ω-hydroxylated GlcCer are of special interest, two of which have been also identified in human epidermis (Hamakana *et al.*, 1989). They are discussed as precursors of corresponding ceramides which are covalently bound to protein components of the cornified cell envelope (Swartzendruber *et al.*, 1987; Wertz *et al.*, 1989). The latter represents a rigid polymer structure that is composed of protein and lipid and coats the surface of the corneocytes (Nemes and Steinert, 1999). Recent work provided evidence that at least a portion of ω-hydroxylated GlcCer is first covalently bound to the cornified cell envelope (Döring *et al.*, 1999a,b), before they are catabolized to protein-bound ceramides. Therefore, a reactivity with ω-hydroxylated glucosylceramides would be helpful to elucidate the ultrastructural relations of lipids and proteins in the cornified envelope. We are currently investigating this possibility.

Anti-glycolipid antibodies are potential tools for the diagnosis and therapy of diseases, e.g., cancer (Alfonso and Zeuthen, 1996; Hakomori and Zhang, 1997) and neurological disorders (Fredman and Lekman, 1997). The anti-GlcCer antibodies described herein may be a useful tool for the rapid follow-up of enzyme replacement therapy of patients with Gaucher disease type 3 (Gornati *et al.*, 1998) by EIA instead of glycolipid analysis. Moreover, it will help in the diagnosis of multidrug-resistant cancer cells which are characterized by high cellular levels of GlcCer (Lavie *et al.*, 1996, 1997).

Materials and methods

Purification of GlcCer-3 and sterylglucoside

A commercial soybean lecithin fraction (Spectral Services GmbH, Cologne, Germany) was used as the starting material for the isolation of cerebrosides by silica gel column and thin-layer chromatography. Soybean lecithin (7 g) was dissolved in $CHCl_3$/acetone (85:15, vol/vol, 40 ml), applied to a silica gel 60 column (bed volume 60 ml in the same solvent), and eluted by increasing concentration of acetone in $CHCl_3$. A cerebroside fraction eluted at 30 to 33% (vol/vol) acetone in $CHCl_3$. This fraction was subjected to preparative TLC in acetone/toluene/water (91:30:8, vol/vol/vol) yielding a homogenous preparation upon visualization by spraying with anilinonaphthalen-sulfonate and inspection under UV light. The fraction was extracted from the silica gel scrapings by phase partitioning between $CHCl_3$/methanol (2:1, vol/vol, 10 ml) and aqueous NaCl (77 mM, 2.5 ml). The dried sample was acetylated overnight in pyridine (0.5 ml), acetic anhydride (0.2 ml) and dimethylaminopyridine (4 mg). The acetylated product was recovered by phase partitioning between diethylether and aqueous KH_2PO_4 (50 mM) and subjected to preparative TLC in petroleum ether/diethylether (1:2, vol/vol). The acetylated compound was extracted from silica gel scrapings by phase partitioning (diethylether/water) and subjected to deacetylation
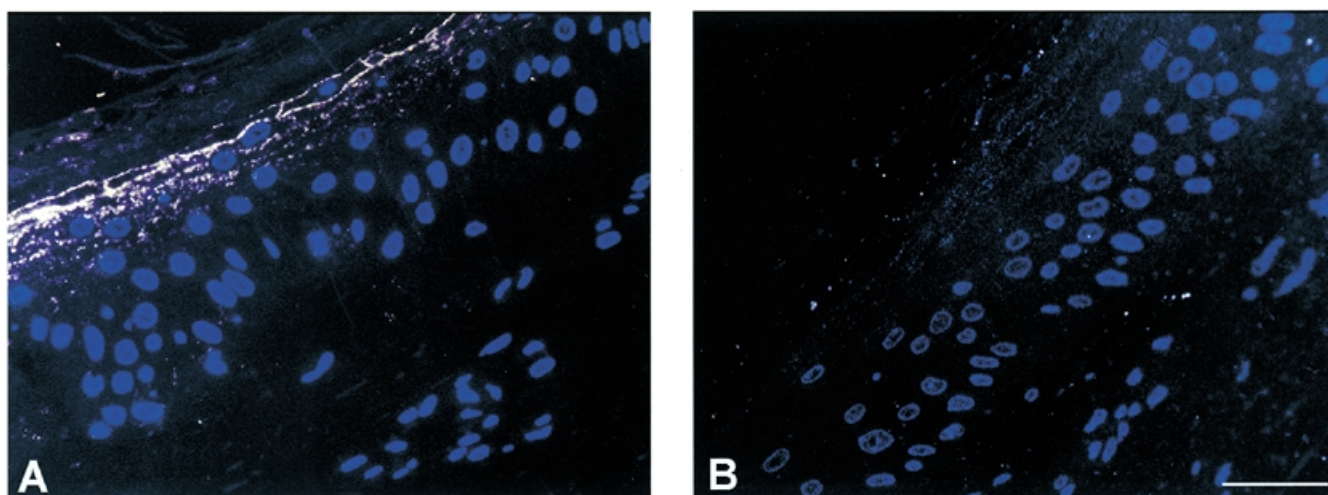
Fig. 8. Localization of GlcCer in human skin. Immunofluorescence staining (yellow spots) on sections of cryoprocessed human skin with a rabbit-anti-GlcCer-3 antiserum (**A**) or preimmune serum (**B**). For better visualization of living epidermal cells, the nuclei were counterstained for with 4,6 diamidino-2-phenylindole (blue color). Scale bar, 20 μm.

with sodium methoxide (0.1 M) in methanol for 15 min at room temperature. The cerebroside was recovered by phase partitioning between $CHCl_3$ and methanol (2:1, 4 ml) and aqueous NaCl (77 mM, 1 ml). The cerebroside was subjected to a final preparative TLC purification in acetone/toluene/water as above. In a typical experiment the yield was 5 mg of pure compound; purity was ascertained by MALDI-MS.

Steryl β-D-glucopyranoside was obtained from the same lecithin fraction. After exhaustive elution with 18% acetone in chloroform (for removal of galactosyldiacylglycerol), steryl β-D-glucopyranoside was eluted with 25% acetone in chloroform. A final purification by preparative TLC in chloroform/methanol (85:15, vol/vol) and recovery by phase partitioning yielded pure steryl glucoside.

Other glycolipids

GlcCer-2, N-stearoyl-DL-dihydro-glucocerebroside, N-stearoyl-DL-dihydro-galactocerebroside, galactosylceramide type I and II, lactosylceramide, cholesterol, phosphatidylethanolamine, phosphatidylserine, dimyristoylphosphatidylcholine and ceramide-2 (Ceramide Typ III) were purchased from Sigma. Ceramide-3 was obtained from Cosmoferm (Delft, The Netherlands). All substances were dissolved in chloroform/methanol (3:1, vol/vol).

Antisera

Rabbit antisera against purified GlcCer-2 and GlcCer-3 were purchased from GlycoTech Produktions und Handelsgesellschaft mbH, Kuekels, Germany. Samples of preimmune sera were also provided.

Preparation of natural lipid extracts

Epidermal lipid extracts from human breast skin were prepared as described by Döring *et al.* (1999a).

TLC and TLC-immunostaining

Glycolipids were separated on silica gel 60 TLC plates with aluminum support (Merck) with a solvent system of $CHCl_3$/

MeOH/25% aqueous NH_4OH (65:35:5, vol/vol/vol) and visualized by spraying with α-naphthol in H_2SO_4 (Figure 4) or 10% $CuSO_4$ and 8% H_3PO_4 in water (Figure 5) and heating at 180°C (Imokawa *et al.*, 1991). For TLC-immunostaining the plates were incubated with blocking buffer (1% polyvinylpyrrolidone and 0.1% nonfat dry milk in 50 mM Tris–HCl pH 7.4, 200 mM NaCl) for 1 h at room temperature and then incubated with rabbit antisera against GlcCer-3 or GlcCer-2 at a dilution of 1:1000 and 1:500, respectively, overnight at room temperature. After 5 washings (5 min each) in washing buffer (50 mM Tris–HCl, pH 7.4, 200 mM NaCl), the plates were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig) G (heavy and light chain specific, Dianova), diluted 1:1000 in blocking buffer for 2 h at room temperature, washed four times as before, 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).

Dot-blot

Antigens were dissolved (1 mg/ml) in $CHCl_3$/MeOH (3:1, vol/vol) and aliquots (1 μl) were dotted onto uncharged nylon membrane (Qiagen 60010) and air dried. All following steps were done at room temperature. After blocking in blotting buffer (50 mM Tris–HCl, 0.2 M NaCl, pH 7.4, supplemented with 10% nonfat dry milk) for 1 h, serial dilutions of rabbit antisera in the same buffer were added, incubated over night and washed 5 times (5 min each) in blotting buffer. Alkaline phosphatase-conjugated goat anti-rabbit IgG (heavy and light chain specific, Dianova) was added (diluted 1:1,000 in blotting buffer) and incubation was continued for another 2 h. After washing as before, 5-bromo-4-chloro-3-indoylphosphate and p-toluidine p-nitroblue tetrazolinum chloride (Bio-Rad) were added as a substrate according to the supplier's instruction. The reaction was stopped after 15 min by the addition of distilled water.

Enzyme-immunoassay (EIA)

PolySorp microtiter plates (U-bottom, Nunc) were coated with glycolipid antigens in methanol and dried under a hood. Unless stated otherwise, 50 μl volumes were used. Plates were blocked with 200 μl of PBS supplemented with 1% bovine serum albumin (PBS-BSA) for 1 h at room temperature followed by removal of the blocking solution by gentle trashing on paper towels. Appropriate antiserum dilutions in PBS-BSA were added and incubated at 4°C overnight. After three washings in PBS-BSA, peroxidase-conjugated goat anti-rabbit IgG (heavy and light chain specific, Dianova) was added (diluted 1:1000) and incubation was continued for 2 h at room temperature. After four washings in PBS (10 mM), the plates incubated with freshly prepared substrate solution, which was composed of azino-di-3-ethylbenzthiazolinsulfonic acid (1 mg) dissolved in substrate buffer (0.1 M sodium citrate, pH 4.5, 1 ml), with sonication in an ultrasound water bath for 3 min followed by the addition of hydrogen peroxide (25 μl of a 0.1% solution). After 30 min at 37°C on a rocking platform, the reaction was stopped by the addition 2% aqueous oxalic acid and the plates were read by a microplate reader (Dynatech MR 700) at 405 nm. All tests were done in quadruplicates.

Tissue preparation and immunofluorescence

Normal human skin biopsies obtained from the forearms of three different individuals were used in this study. The biopsies were high-pressure frozen and freeze-substituted with temperature steps from 183 K to 223 K with acetone saturated with uranyl actetate. Subsequently, the specimens were embedded in HM20 at 223 K. A detailed description of the procedure and its use for immunoelectron microscopy will be published separately (Pfeiffer *et al.*, forthcoming). The following steps were performed at room temperature and all dilutions were prepared in washing buffer (PBS/0.1% Tween 20). Semithin sections (200 nm) were placed on glass cover slips, treated with normal goat serum (3% in PBS) for 1 h, washed twice, and incubated with anti-GlcCer-3 antiserum (1:50) for 1 h. Subsequently, the sections were washed four times and incubated with a Cy3-conjugated goat anti-rabbit IgG secondary antibody (Dianova, Hamburg, Germany) (1:800) for 1 h. The samples were washed six times, stained for nuclei with 4,6 diamidino-2-phenylindole (5 μg/ml PBS) (Sigma, Deisenhofen, Germany) for 15 min, washed twice with H_2O, fixed on slides with MOWIOL, and analyzed with a Zeiss Axioskop 35 fluorescence microscope.

Acknowledgments

Abbreviations

Anti-GlcCer-2, rabbit anti-glucosylceramide-2 antiserum; anti-GlcCer-3, rabbit anti-glucosylceramide-3 antiserum; Cer, ceramide; EIA, enzyme-immuno assay; GlcCer, glucosylceramide; GlcCer-2, 1-O-(β-D-glucopyranosyl)-N-acyl-sphingosine; GlcCer-3, 1-O-(β-D-glucopyranosyl)-N-acyl-4-hydroxysphinganine; GSL, glycosphingolipids; LB, lamellar bodies.

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