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Review

On the character and functions of sphingolipids*

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Sphingolipids form a large group of membrane lipids showing a diversity of molecular species. Specific functions associated with the saccharide part of glycosphingolipids including co-receptor functions, cell homing phenomena, and attachment by microbes and microbial toxins may not be unique for sphingolipids. However, there are saccharides which appear only in ceramide-bound form and not in other glycoconjugates, and such glycolipids have often been selected as attachment sites by microbes. During the last few years convincing evidence has been presented in favor of ceramide and sphingosine being signaling molecules for various cell functions. The influence of sphingolipids (ceramide) on the properties of the membrane bilayer is still largely unknown. However, based on the structure of ceramide and some experimental evidence one may formulate its role in membrane stability and barrier properties determined by hydrogen bonding in the amide region of ceramide. Furthermore, a natural variation in the number of hydroxyl groups (of fatty acid and long-chain base) may be important for regulation of the potential hydrogen bonds.

The amphipatic sphingolipid is mainly localized to the outer leaflet of the eukaryotic surface membranes, and the large diversity of various components (fatty acids, long-chain bases and polar head groups) may combine into an almost endless number of different sphingolipids. In contrast to the knowledge of structure, the biological meaning of this diversity is largely unknown. It is, however, of interest that the initiator of a more precise sphingolipid science, J. Ludwig W. Thudichum, as early as one hundred years ago re-

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Abbreviations: MALDI-TOF, matrix-assisted laser desorption time-of-flight mass spectrometry; NAP, neutrophil-activating protein.

ported on the medical use of cerebroside and its degradation components, including sphingosine nitrate and psychosine. He reported on a "besonderen Wirkungskraft in Krankheiten des Nervensystems", or "special healing power for diseases of the nervous system" after intradermal injections to his patients [1]. The observed effects may be related to the recent findings that ceramide and sphingosine and derivatives thereof, and also intact sphingolipids, are signaling molecules and messengers with diverse effects on cell function [2].

The function of saccharide part of glycosphingolipids and other glycoconjugates has been the subject of studies for decades [3]. The documented change occurring on transformation and tumor formation is still being studied in a search for tumor-associated antigens of potential use in tumor therapy. Moreover, the importance of host glycoconjugates for microbial attachment and infections has come up as a possible new therapeutic approach. In fact, there are three current clinical trials on humans with receptor saccharide or its analogues. One concerns the treatment of gastritis caused by *Helicobacter pylori* by sialyllactose [4], and two synthetic sialic acid analogues are being tested against influenza [5, 6]. A third area of great importance and potential is the targeting of inflammatory and other cells based on a specific protein recognition of carbohydrate, e.g. E-selectin homing of neutrophils by interaction with sialic acid- and fucose-containing complex glycolipids [7]. A potentially harmful effect of a surface saccharide antigen may be due to the induction of an immune response following transplantation from non-related donors, for example pig-to-human xenotransplantation. To improve transplant survival, current efforts attempt to modify surface antigens, including the use of transgenic animals and knock-out of specific glycosyltransferases [3].

However, the surface saccharide functions may be linked also to glycoconjugates other than glycolipids. To focus on sphingolipids and their saccharide part one may ask what is unique to their presence in biological membranes? It is known that the unique part of a sphingolipid is ceramide, or rather sphingosine, but there are also saccharide epitopes linked to ceramide which are not found in other glycoconjugates.

The review is concentrated on the glycolipid-specific binding epitopes for microbes. In addition, the properties of ceramide in the membrane is discussed, which although an old subject, will be brought up in view of some recent data on sphingolipid microdomains and messenger functions.

The methods used to analyse and characterize microbial recognition of glycolipids have been covered by recent papers, e.g. one on *H. pylori* recognition of sialic acid-containing epitopes [8], and one on the carbohydrate-binding specificity of a neutrophil-activating protein, NAP, of *H. pylori* [9]. In principle, overlay of radiolabeled ligand on thin-layer plates with separated glycolipids, or on membrane blots after electrophoretic separation of glycoproteins, are used to detect a specific protein-carbohydrate binding. A detected receptor-active glycoconjugate is prepared by established techniques followed by structural characterization by mass spectrometry and NMR spectroscopy. A specifically adapted peracetylation procedure was used for the preparation of polyglycosylceramides [10]. Molecular modeling is being used to help in the interpretation of distinct binding epitopes [11].

SPECIFIC GLYCOLIPID EPITOPES

Table 1 summarizes glycolipid-specific epitopes recognized by microbes and microbial proteins, which are not being found in glycoproteins. The high-affinity G_{M1} epitope, defined for cholera toxin more than 20 years ago, has not yet been detected in glycoproteins. Lactose in bound form in animals has only been found as lactosylceramide, which is recognized by a large number of normal and

pathogenic bacteria [12]. Interestingly, mainly the molecular species with hydroxy fatty acid or phytosphingosine are being recognized; this proposed to be due to a preferred conformation/presentation of lactose at the membrane monolayer [13]. In non-transformed human tissue Galα4Gal has been found only in glycolipids [14], and this epitope is recognized by uropathogenic *Escherichia coli* and by Shiga and Vero toxins [13]. The binding of several viruses to simple one-sugar glycolipids such as GalβCer is probably dependent on ceramide characteristics [13,15]. The binding of the neutrophil-activating protein, NAP, of *H. pylori* has for neutrophils so far been found in a particular extended ganglioside (Table 1) and not in glycoproteins [9]. Finally, the recognition by *H. pylori* cells of polyglycosylceramide is apparently glycolipid-specific, since no binding of glycoproteins has so far been detected [8]. The detailed epitope has, however, not yet been identified although it contains sialic acid. This glycoconjugate class is highly heterogeneous, with from 15 up to possibly 50 monosaccharides linked to ceramide. We are at present working on establishing the complete structure of the human erythrocyte and leukocyte polyglycosylceramides. Of great help in characterization of intact molecules are their partial degradation products, and modified molecules testing their structure-activity relationship. For this purpose the matrix-assisted laser desorption time-of-flight mass spectrometry with delayed extraction is valuable, as illustrated in Fig. 1. The accuracy of the technique, close to one mass unit, allows calculation of the composition of individual monosaccharides. The partial spectrum shows a peak at *m/z* 7493 which corresponds to 20 hexoses, 18 *N*-acetylhexosamines, 2 fucoses and 1 sialic acid, all together 41 sugars. We are now focussing on the high-mass interval of the polyglycosylceramide preparations, to specifically test the size limit of polyglycosylceramides.

GLYCOLIPID-SPECIFIC BINDING EPITOPES FOR MICROBES

There may be advantages for a microbe to select a sphingolipid binding site.

The sphingolipid is membrane bound

The sphingolipid provides a strictly membrane-bound epitope (except when shed, e.g. on aged epithelial cells extruded from the intestinal cell layer) in contrast to glycoproteins, which may appear in secretions and compete with microbe-host cell adhesion. Adhesion of microbes allows to avoid elution, but

Table 1. Glycolipid-specific epitopes recognized by microbes and microbial proteins

G_{M1} ganglioside	
Galβ3GalNAcβ4(NeuAcα3)GalβGlcβCer	Cholera toxin
Lactosylceramide	
Galβ4GlcβCer	Many bacteria
Globo glycolipids	
R-Galα4Galβ4GlcβCer	Uropathogenic *E. coli*
	Shiga and Vero toxins
One-sugar glycolipids	Several viruses
NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer	Neutrophils and neutrophil-activating protein, NAP, of *H. pylori*
Polyglycosylceramides	
Epitope still unidentified	*H. pylori*

R: Extended saccharide chain.

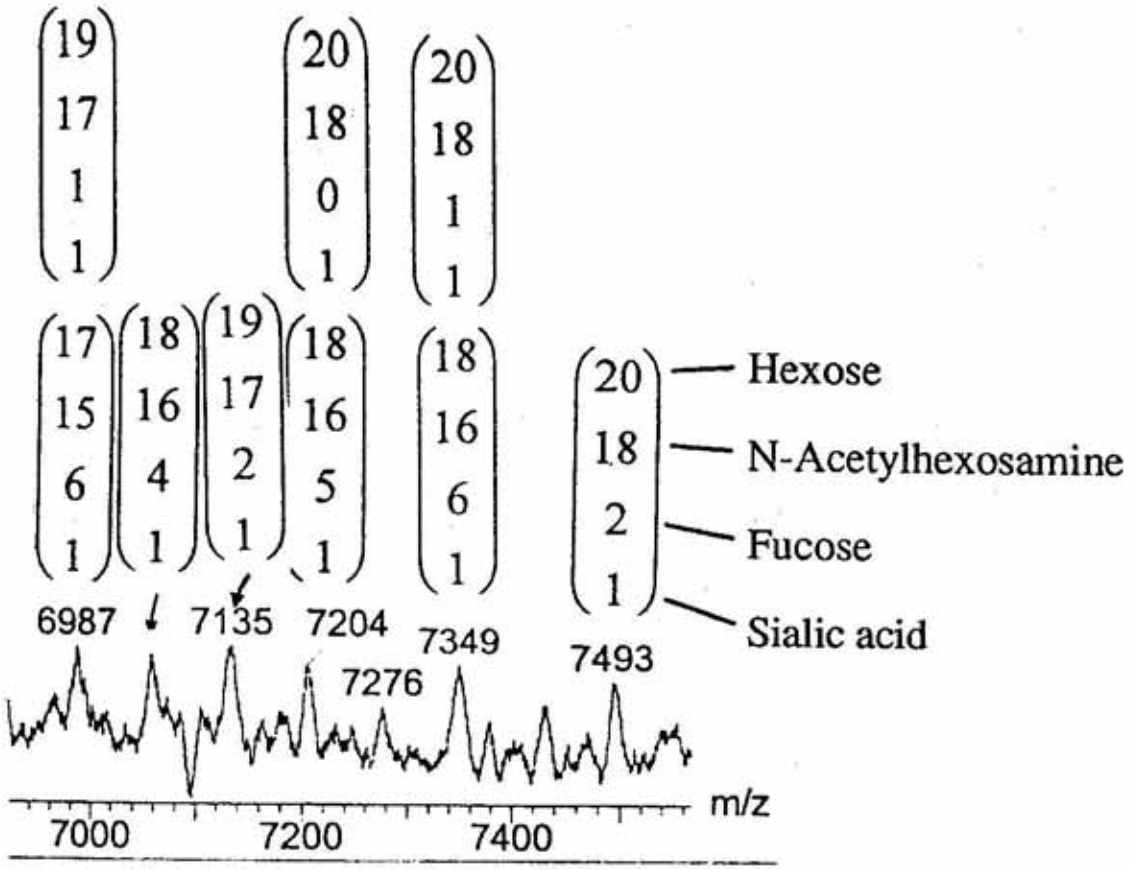

Figure 1. A high-end spectrum of a monosialo subfraction of saccharides released from polyglycosylceramides by endoceramidase and fractionated by Dionex alkaline ion exchange chromatography (H. Miller-Podraza, unpublished).

The figures indicate (from top) number of hexoses, *N*-acetylhexosamines, fucose and sialic acid, respectively. Thus the peak at *m/z* 7493, with an approximate accuracy of 1 mass unit, corresponds to a polyglycosylceramide with 41 monosaccharides. The analysis was performed on a MALDI-TOF instrument (TofSpec-E/SE, Micromass) equipped with delayed extraction device.

is also important for bacterial cell division and nutrient uptake. On the other hand, the bulk of *H. pylori* is in the mucus in human stomach where the cells divide, although they also are found intimately attached to epithelial cells [16].

The lactosylceramide binding (Table 1), can not explain the tissue tropism, since a large number of bacteria with distinct target cells use this specific binding site [12]. Our hypothesis is [12, 13] that this specificity is used for a second-step binding, to assure a membrane-anchoring after first binding to a cell-specific receptor. Also, lactosylceramide may not be directly accessible for binding from the outside, but rather provides a cryptic site, which requires a reorganization (lateral redistribution) at the surface following the first-step interaction. However, the biological relevance of this specificity has not yet been proven.

For uropathogenic *E. coli* the relevance of the Galα4Gal recognition for pathogenicity has been documented [17]. The PapG adhesin, recognizing this disaccharide, was selectively inactivated (gene knock-out) and bacterial cells with only this change were not able to cause pyelonephritis in monkeys, in contrast to the non-manipulated strain. Thus a binding to the globo series of glycolipids in the urinary epithelium is a specific requirement for the infection. There is also some evidence that the binding (contact) induces expression of virulence genes in the bacterium [18] and a cyto-

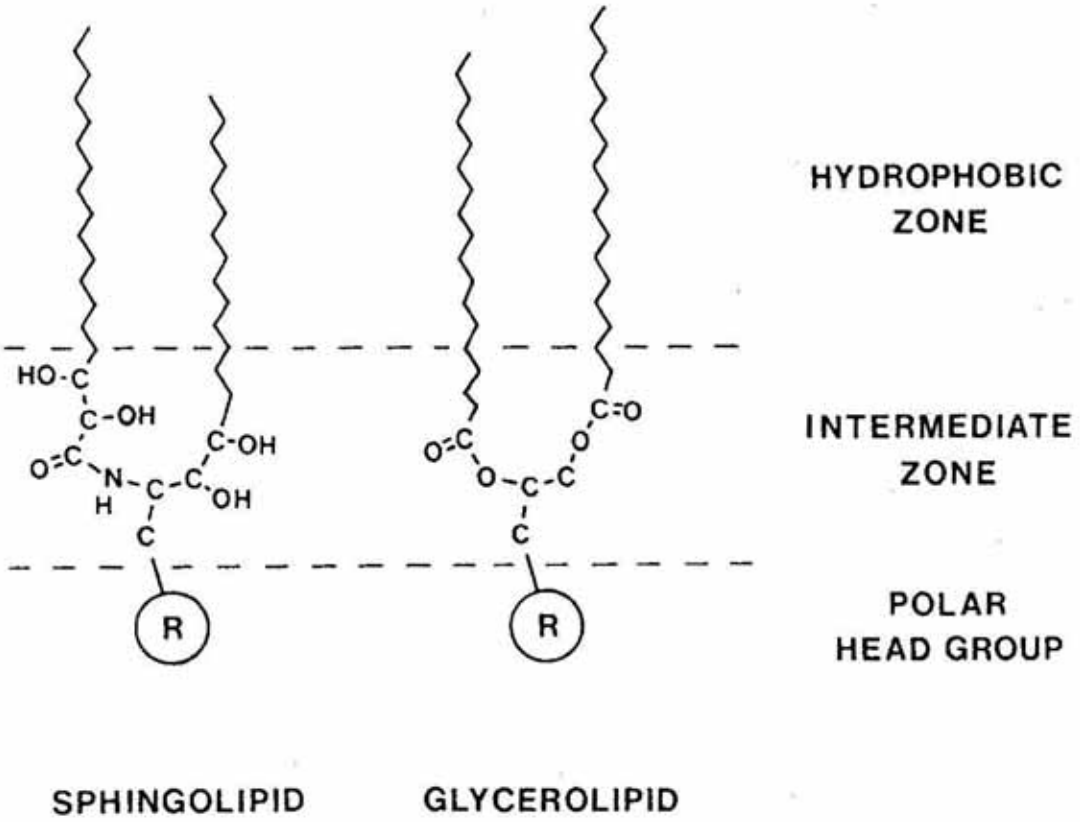

Figure 2. Simplified formulas to indicate the difference between membrane lipids of the intermediate zone.

The sphingolipid has both hydrogen bond donors and acceptors, while glycerolipid has only hydrogen bond acceptors.

kine response in the host cell, presumably mediated by the ceramide signaling pathway [19]. Therefore, it is possible that bacterial adhesion results in glycolipid hydrolysis at the membrane to produce a signaling ceramide. The binding by *H. pylori* to polyglycosylceramides is apparently restricted to neutrophils since these glycolipids were not detected in gastric epithelium (unpublished). Although the details of the binding epitope are not known, the binding is dependent on sialic acid. It may therefore be important for the bacterium to interact selectively with the neutrophil and not with a large number of other cell-bound or soluble sialic acid-containing glycoconjugates that exist in the neighborhood. It has been demonstrated that bacterial cells upon contact with the neutrophils induce a rapid (within seconds) inflammatory burst followed by a slower (within minutes) phagocytosis [20]. The bacteria are not damaged by this process, rather they take advantage of the products of the inflammatory reactions for nutritional purposes [16]. In line with this, *H. pylori* is producing a soluble neutrophil-activating protein, NAP, which, after binding to neutrophils (Table 1), stimulates production of cell adhesion molecules, CD11b/CD18, on the target cells to improve the adhesion to inflamed endothelium. Thus the bacteria actively recruit inflammatory cells for their own use.

The sphingolipid may provide a bilayer-close binding site for membrane penetration

The classical case of G_{M1} and cholera toxin (Table 1) has been studied in some detail for the mechanism of action. Release of the G_{M1} saccharide and coupling it up to various aglycones was used to test the necessity of mem-

Table 2. Apparent relation of ceramide hydroxylation to membrane stability against environment [22]

Sphingolipids with one free hydroxyl group	Erythrocytes (stable environment) of ceramide
Sphingolipids with two free hydroxyl groups	Nerve cells (ionic changes) of ceramide
Sphingolipids with three free hydroxyl groups	Kidney epithelium (varying content and character of urine of kidney tubules)
	Intestinal epithelium (varying lumen content of hydrophilic and hydrophobic substances)
Sphingolipids with four free hydroxyl groups	Yeast cells (nonregulated environment for cell growth)
	Amoeba (nonregulated environment)

brane proximity to mediate the toxin effect [21]. Toxin-resistant target cells were rendered sensitive after coating with G_{M1} or cholesterol-linked G_{M1} saccharide. However, coupling the saccharide to surface protein, or coating saccharide with longer spacers, gave no effect. Therefore, a close location of the saccharide epitope at the bilayer is a prerequisite for the A subunit of the toxin to penetrate the membrane and exert its effect. Lactosylceramide also provides a bilayer-close epitope, and several of the bacteria that carry this binding are invasive [12].

A property in common for several viruses of binding to one-sugar glycolipids [13, 15], may be necessary for virus entry to the host cell. The viruses shown to carry this specificity have also a second virus-characteristic binding to peptide or sugar. Thus the selection of host cell may be through a cell-specific binding, followed by a docking to one-sugar glycolipids to gain proximity for spontaneous membrane-membrane fusion to deposit the genome into the cell for replication [13].

POTENTIAL IMPORTANCE OF CERAMIDE STRUCTURE FOR THE PHYSICO-CHEMICAL PROPERTIES OF MEMBRANES

Although sphingolipids are also components of intracellular membranes, early results from studies on erythrocytes indicated that the sphingolipid occupies only the outer monolayer of the surface membrane (see [22]). This is in accordance with the finding that membrane-bound saccharides, including glycolipids, are exposed on the outside of the cell. However, the diversity of fatty acids and long-chain bases that was revealed when different tissues were analyzed with new techniques, and the distinct appearance of particular molecular species of ceramide, opened up questions about the potential role of the ceramide part of sphingolipids in the surface monolayer.

Sphingolipids differ from glycerolipids in the intermediate zone

Figure 2 shows a simplified representation of a sphingolipid and a glycerolipid. Considering the linkage region in between the polar part and the hydrophobic part, here named the intermediate zone, it is of interest that the glycerolipid carries hydrogen bond acceptors only, while the sphingolipid has both hydrogen bond acceptors and donors.

There is an experimental evidence for hydrogen bonds in the intermediate zone

As summarized already many years ago [22] there is evidence from crystallography and monolayer studies of monoglycosylceramides and ceramides for the existence of laterally oriented hydrogen bonds of potential func-

tional importance. Noteworthy is an early paper by Pascher [23] who summarized the results and provided a testable hypothesis. Unfortunately, very little has been added through the years on this aspect.

Functional consequences of a variation of the intermediate zone

It has been shown for red cells of various animal species that there is a relation between the sphingomyelin:phosphatidylcholine ratio and resistance to osmotic stress and detergents (see [22]). Thus a higher relative level of sphingolipid in the membrane provides stability, which may be due to an improved network of hydrogen bonds. Also, Pascher and coworkers (personal communication during 1997) have studied the uptake of chlorpromazine and deoxycholate into monolayers composed of different membrane lipids. They found that cerebroside, in contrast to phosphatidylcholine, did not expand its monolayer when exposed to these substances. Furthermore, it was shown that the *trans* double bond of sphingosine, as well as extra hydroxyl groups of the fatty acid or long-chain base, promoted a condensation of ceramide monolayers [24].

Moreover, there is a distinct difference in hydroxylation of the intermediate zone between different cells and tissues (Table 2), and this shows an apparent relation to stress affecting the actual cell membrane. Thus, the red cell occupying a stable environment has only one free hydroxyl group, while nerve cells, subjected to fluctuations of ionic levels, usually carry two hydroxyls. Epithelial cells of the small intestine, which has a variable lumen content of hydrophilic and hydrophobic substances, have three hydroxyl groups, and yeast cells may have four. This is a variation which has no counterpart in other membrane lipids.

Of considerable interest are the microdomains of pure sphingolipid that may exist in surface membranes (see [2] and [25]). The evidence for the existence of such domains was first based on freeze fracture electron microscopy. However, it is now possible to prepare these domains by ultracentrifugation after detergent disruption of the membrane. They were shown to contain practically only sphingolipid, but in addition specific proteins which are mediators of the ceramide signaling pathway [2]. Two properties of such microdomains may depend on hydrogen bonds of the intermediate zone. First, the formation of microdomains, this self-assembly, may be due to favored sphingolipid-sphingolipid interactions thus excluding other membrane lipids. Second, this interaction may explain the detergent resistance which allows subfractionation. The selective uptake of proteins may be due to specific laterally oriented peptide-saccharide interactions.

If ceramide (and the first part of polar head group of glycolipids) provides a hydrogen bond-based stability of the surface membrane monolayer, breaking of such bonds may be a necessary prerequisite for membrane penetration. Several viruses bind to one-sugar glycolipids including ceramide dependence (see above). In this case invasion may take place through new bond formations between virus and ceramide, thus destabilizing the monolayer.

As an extension of this explanation, a hypothesis may be set that glycosphingolipids provide receptors for intracellular vesicle homing and fusion. A bilayer-close epitope mediates binding by a specific vesicle protein in a close proximity of the two membranes allowing a spontaneous membrane-membrane fusion.

PROSPECTS FOR STUDIES ON SPHINGOLIPID FUNCTIONS

If we consider functions, in physiological or non-physiological situations, unique for sphingolipids, and which are not cross-carried by other molecules (e.g. many carbohydrate-

mediated processes), the following are the current topics.

Sphingolipid-, ceramide- and sphingosine-mediated signaling pathways

This rapidly growing field [2] is still in its beginning and it will be of great interest to find out if the various cellular effects may be selectively mediated by sphingomyelinases and ceramide glycanases that specifically cleave certain ceramide species with distinct effects.

Glycolipid-specific binding epitopes for microbes, and potentially for physiological intracellular homing events

There are several carbohydrate epitopes which are not expressed in other glycoconjugates and may provide strictly membrane-bound binding sites. If these are ceramide-close they may mediate membrane penetration (proven in the case of G_{M1} and cholera toxin). Two membranes may fuse spontaneously if they approach each other to a few ångström distance. Ceramide-close binding epitopes could thus provide potential vesicle homing and fusion based on specific protein-glycolipid interactions. There are numerous intracellular transport processes based on participation of membrane vesicles and in most cases the homing and sorting processes are considered specific. However, the knowledge of these processes is still fragmentary.

Role of ceramide in surface monolayers

Unfortunately, this aspect is at present a neglected experimental field. It can be expected that the excitement about sphingolipids and their components as signal molecules may result in efforts to define in detail various molecular species of ceramide not only as concerns their biological activity but the physical membrane properties as well. Probes for cell injections or transfections may be designed based on a selective interaction with and breaking of the membrane-stabilizing hydrogen bonds of the intermediate zone.

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