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Review

High-resolution thin-layer chromatography of gangliosides

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Abstract

In this review an updated overview of current improvements on thin-layer chromatography (TLC) of gangliosides over the past decade is provided. Basic general techniques and special advice is given for successful separation of glycosphingolipids. New approaches concerning continuous and multiple development, and several preparative TLC methods are also included. Emphasis is placed on TLC immunostaining and related techniques, i.e. practical applications of carbohydrate-specific antibodies, toxins and bacteria, viruses, lectins and eukaryotic cells. Thus, this review on ganglioside TLC summarizes its power as an analytical tool for a wide range of purposes.

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1. Introduction

Gangliosides are a diverse group of glycosphingolipids (GSLs)[1] composed of long-chain base and fatty acid, which together make up the ceramide portion (N-acylsphingosine), and carbohydrate moieties [1]. Structures and functions of GSLs have been widely reviewed [2–15]. Gangliosides are characterized by the presence of one or more sialic acid units in the oligosaccharide chain. The parent compounds are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc), which are known to play crucial roles in various biological functions [16–18]. Gangliosides, as well as related GSLs, are located primarily in the outer leaflet of the plasma membrane, where they represent a small percentage of total lipids, but are also found in association with intracellular organelles [19–21].

Along with the development of sophisticated methods for detection and structural characterization of GSLs, many different molecular species of gangliosides have been described in all vertebrate species, with highest concentrations in neural and in lower amounts in extraneural tissues. Due to the important biological role of gangliosides much effort has been spent on their isolation and structural elucidation. After extraction from biological source by standard procedures [2,22], anion-exchange chromatography is generally the method of choice as first purification step from crude GSL extracts. Since the first report [23], the separation of gangliosides by DEAE-linked matrices has become a well established method [24–27]. Recent improvements in the separation of gangliosides are the application of DEAE-Fractogel [28] as well as the strong anion-exchangers like Q-Sepharose [29] and TMAE-Fractogel [30], which enable the isolation of ganglioside positional isomers on a preparative scale. Thereafter, the acidic lipid containing fraction undergoes a mild alkaline treatment to saponify contaminating phospholipids usually followed by further purification on silica gel, e.g. Iatrobeads [2,31]. It should be mentioned, that alkaline labile O-acetyl- or lactone-derivatives of sialic acids are irreversibly disintegrated by this procedure. Now ganglioside fractions are ready for thin-layer chromatography.

Due to its resolving power, high-performance thin-layer chromatography (HPTLC) has become the standard tool for resolution of ganglioside mixtures for analytical and preparative applications. Separation and identification of GSLs by TLC has been the subject of several reviews [2,32–34]. The goal of this review is an up-dated overview on improvements in TLC over the past decade. The most commonly used general techniques are reviewed and some special advice is given to achieve successful TLC. Advances in ganglioside chromatography based on continuous and multiple development are also included. Furthermore, this review deals with some selected enhancements for preparative TLC and techniques of combined TLC/mass spectrometry. Finally, the TLC-immunostaining procedure and its various modifications are described. The practical application of carbohydrate-specific reagents, such as antibodies, toxins, lectins, will prove the overlay technique as a powerful tool in the specific detection of GSLs in complex mixtures as shown by the pioneering work of Magnani et al. [35].

2. General techniques of TLC

2.1. TLC plates

Commercial aluminium, plastic and glass backed plates precoated with silica gel 60 are the most commonly used for TLC of GSLs because of their convenience and superior resolving capacity compared to home-made plates. High-performance TLC (HPTLC) plates from E. Merck (Darmstadt, Germany) are generally recommended for diverse applications [36]. HPTLC plates offer excellent resolution potential for the separation of gangliosides. The author prefers glass backed HPTLC plates (10 cm × 10 cm, thickness 0.2 mm, Cat. No. 5633; E. Merck). Compared to other suppliers, GSL bands are

[1] For abbreviations see list on p. 20.

distinctly sharper and less material is required for visualization and quantitation (see below) of gangliosides in the picomolar range [37].

Before use, the plates are usually activated for 30–45 min at 110°C, cooled down and then stored over heat activated silica gel and auxiliary P_2O_5 in desiccators.

2.2. Application of samples and development of chromatograms

Samples of 5–20 μg of ganglioside mixtures are applied 10–20 mm from the bottom of the plate by means of a 5 μl or 10 μl precision microsyringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) as series of microdroplets with a special microdispenser (PB600-1, Pat. No. 3161323, Hamilton) to form a 5–10 mm band. Best chromatographic results are generally obtained by delivery of samples to TLC plates with commercially available automatic applicators (TLC applicator AS 30; Desaga, Heidelberg, Germany) [30].

The most frequently used TLC developing solvents for gangliosides are mixtures of chloroform, methanol, and aqueous salts. For nearly all applications, mixtures of chloroform–methanol–20 m*M* aq. $CaCl_2$ (120:85:20, v/v/v) [38] or more polar (100:80:20, v/v/v) lead to reproducible separation results. Ammonium hydroxide (1–5 *M*) in the aqueous phase changes the mobilities of several gangliosides, and this alkalization is particularly useful to distinguish Neu5Ac from Neu5Gc substituted gangliosides [39,40]. Other commonly used solvent systems include e.g. *n*-propanol–0.25% aq. KCl (3:1, v/v); methyl acetate–*n*-propanol–chloroform–methanol–0.25% aq. KCl (25:20:20:20:17, v/v/v/v.) [2]. The solvent system acetonitrile–isopropanol–50 m*M* aq. KCl (10:67:23, v/v/v) gives excellent separation of polysialogangliosides [27,41]. Mobilities and compactness of ganglioside bands are markedly influenced by the presence of salts or ammonia. Halogen salts in the aqueous phase produced excellent separations, while non-halogen salts gave poor resolutions [41]. It is assumed that strongly ionized cations effectively associate with gangliosides and that such GSL–ion complexes are jointly separated on silica gel.

The solvent mixture (ca. 100 ml) is usually added into a standard developing tank (inner dimensions: length, 21 cm; width, 9 cm; height, 21 cm; Desaga) lined with filter paper. The closed chamber should be allowed to equilibrate for at least 2–3 h (preferably overnight) with fresh solvent before inserting the plate. To assure that the chamber is properly closed, it is advisable to seal the tank by means of a weight on the top of the glass cover. Silicone sealants should be used sparsely. Chromatography is finished when the solvent front has reached a line approximately 10 mm below the top of the plate. Then the plate has to be removed and the solvent is allowed to evaporate in a fume hood at ambient temperature. Before initiation of the staining procedure, traces of solvent are removed by incubating the plate under vacuum or by gentle heating with a hair dryer.

A recently introduced development chamber (TLC-MAT, Desaga) allows automatic development of TLC chromatograms without supervision. The operation of this device is controlled by a sensor based on the detection of the solvent front by differences in the light reflecting properties of dry and wet areas of the TLC plate. Chromatography takes place in an environment protected from atmosphere and light. The solvent vapours are removed by an integrated fan when development is completed, and the exact development time is recorded. Simple operation, reduced volume of the mobile phase and a microprocessor controlled system guarantee validated results obtained by a device which is suitable for glass plates and foils.

2.3. Staining and quantification

Specific detection procedures for staining of gangliosides on thin-layer chromatograms have been reviewed in detail by several authors [2,32–34]. Only the most prominent and frequently used destructive reagents for specific analytical purposes are reviewed in this chapter. Some nondestructive dyes and reagents for unspecific detection of lipids on TLC plates can be used for

preparative recovery and will be described along with the procedure of preparative TLC (see below).

The dried, developed plate is placed upright in a protective frame (e.g. spray box, Desaga) for spraying with aggressive reagents. Particularly for quantitative work (see below), even application of the dye solution should be accomplished with a fine mist sprayer (Sprayer SG1, Desaga), initiating the spray stream off the side from the plate surface, moving in a zigzag pattern beyond the chromatogram until the plate is adequately moistened. Some reagents require covering the sprayed plate before heating. Both the chromatogram and a clean plate of equal size are clamped together and placed in an oven or alternatively on a hotplate until maximum color has developed.

Resorcinol spray is specific for sialic acid and therefore remains the most selective and sensitive tool for ganglioside detection on TLC plates [42]. Resorcinol–HCl–Cu^{2+} spray reagent is prepared by mixing 10 ml of 2% aqueous resorcinol solution with 80 ml concentrated HCl and 0.25 ml of 0.1 *M* copper sulfate. The total volume is filled up to 100 ml with water. The reagent should be kept in the dark at 4°C and remains stable for at least 2 weeks. The plate is sprayed moderately with a fine mist of resorcinol reagent, covered with a glass plate and heated in an oven at 100°C for ca. 20–30 min. Gangliosides produce a characteristic blue-violet color, whereas neutral and sulfated GSLs give less intense yellow-brown color. Overheating leads to discoloration of the gangliosides.

All GSLs can be visualized with a number of general carbohydrate stains. Orcinol–sulfuric acid spray is the most commonly used for detection of lipid bound carbohydrates [43] and applied as a 0.2% solution of orcinol in H_2SO_4–H_2O (3:1, v/v). The dye solution is stable for at least 2 weeks and should be stored at 4°C in the dark. The plate is thoroughly sprayed until becoming obviously moist followed by heating at 100°C for ca. 10–15 min. Neutral and sulfated GSLs, as well as gangliosides, will give pinkish violet spots on a white background, while the color intensity is proportional to the number of monosaccharides in the carbohydrate moieties.

Direct densitometric scanning of resorcinol positive bands is a simple but rapid method for quantification of relatively small amounts of GSLs [2,36]. Ganglioside patterns on HPTLC plates can be scanned with a densitometer (Densitometer CS-910, Shimadzu, Kyoto, Japan; Densitometer CD 60, Desaga), operating in the transmittance mode with incident light at 580 nm, giving linear response in the range of 0.15–3.0 μg sialic acid per band [36]. This scanning technology has been proved to be convenient for a reproducible quantitation of picomolar amounts of individual ganglioside species [37] and its usefulness for clinical purposes has also been demonstrated [44].

3. Continuous and multiple development

3.1. Short-bed continuous development

One-dimensional TLC is the simplest and still commonly used method for analysis of ganglioside mixtures. This method has been continuously optimized with emphasis on enhanced resolution, because HPTLC plates have the disadvantage that the bands are sometimes very closely spaced, although distinctly resolved. Higher resolution by TLC can be achieved by reduction of the solvent strength. This basic theory provides the approach for short-bed continuous development TLC [45]. Its application is limited by an exponential reduction of GSL migration mobility at decreasing solvent strength. This circumstance can be solved by high solvent velocities achieved by continuous development in a short-bed chamber. In this sort of development, volatile solvent is continuously removed from one end of the adsorbent layer by vaporization. This generates a continuous flow of solvent through the silica gel and results in e.g. improved separation as shown with G_{M1b}-type gangliosides of mouse lymphoma cells [46,47]. However, the special chamber provided by Regis Chemical Co. (Morton Grove, IL, USA) is very

basic design and does not work properly. In principle, chromatography can be performed, but the realization of the excellent idea of short-bed continuous development needs fundamental technical amendments which are highly recommended for successful operation. It is suggested that with such design improvements this approach may be of benefit for thin-layer chromatography.

3.2. Multiple development

Multiple development can be carried out in three ways: in the original direction of development with the same or different solvents, or at right angles to the original direction of the chromatography and with a different solvent (= two-dimensional chromatography). All three alternatives increase the capability of TLC by affording separations, not otherwise either adequate or attainable, and will be evaluated in this review.

Normally, a single run is sufficient for the desired resolution, although drying and redevelopment in one dimension in the same or in a different solvent can improve resolution. Limited availability of material frequently prevents extensive purification. Therefore, sensitive HPTLC microanalyses of complex tissue lipids have been developed, using e.g. three or four consecutive solvent systems [48] and not involving preceding column chromatography. More than 20 different lipid subclasses can be separated with this procedure. Unidirectional ascending chromatography in three different solvent systems has been reported for accelerated separation of gangliosides within total lipid extracts [49]. This TLC technique was applied for the resolution of brain and retinal gangliosides without any need for previous purification of gangliosides.

Excellent separation of multisialogangliosides containing more than three sialic acid residues was obtained with two one-dimensional runs in chloroform–methanol–12 m*M* aq. $MgCl_2$–15 *M* ammonium hydroxide (60:35:7.5:3, v/v/v/v) followed, after drying in warm air, by a second chromatography in chloroform–methanol–12 m*M* aq. $MgCl_2$ (58:40:9, v/v/v). Runs were performed at 38°C [50]. Ammonia was found to be necessary in the first run to give sharp resolution of polysialogangliosides; control runs at room temperature resulted in poorer separation. By application of this system, several gangliosides were detected in embryonic brain moving below G_{Q1b}, suggesting gangliosides with six, seven, or even more sialic acid residues per GSL molecule. The bands were sufficiently concentrated for reproducible densitometric quantification.

3.3. Automated multiple development

In another approach to multiple development, called programmed multiple development, TLC plates are automatically cycled through a preset number of developments [51]. As mentioned above, increased separation by TLC can be achieved by decreasing solvent strength, which is the basic theory for short-bed continuous, as well as multiple development. An improved TLC separation of gangliosides by automated multiple development (AMD) was published recently [52]. The AMD equipment (Camag, Muttenz, Switzerland) consisted of a development unit, a control unit and a vacuum pump. Conventional single-step HPTLC is generally performed in the common three-compound solvent chloroform–methanol–20 m*M* aq. $CaCl_2$ (120:85:20, v/v/v) due to its high resolution power and its wide applicability for standard ganglioside separation. An enhanced resolution of mono- and disialogangliosides was obtained by three consecutive runs with intermediate drying periods in a solvent with reduced polarity compared to the routine separation of the gangliosides in question. Three times repeated chromatography of a complex mixture of neolacto-series monosialogangliosides from human granulocytes (Table 1) in the less polar solvent system chloroform–methanol–20 m*M* aq. $CaCl_2$ (120:85:14, v/v/v) resulted in a ca. three-fold increase in separation distance of e.g. α2–3 and α2–6 sialylated ganglioside isomers compared to conventional single chromatography in the stan-

Table 1
Structures of neolacto-series monosialogangliosides from human granulocytes

No.[a]	Fatty acid	Structure
1	24:1, 22:0	II^3Neu5Ac-LacCer (G_{M3})
2	16:0	II^3Neu5Ac-LacCer (G_{M3})
3	24:1, 22:0	IV^3Neu5Ac-nLcOse$_4$Cer
4	16:0	IV^3Neu5Ac-nLcOse$_4$Cer
5	24:1	IV^6Neu5Ac-nLcOse$_4$Cer
6	16:0	IV^6Neu5Ac-nLcOse$_4$Cer
7	24:1	VI^3Neu5Ac-nLcOse$_6$Cer
8	16:0	VI^3Neu5Ac-nLcOse$_6$Cer

[a] According to Fig. 1.

dard solvent as shown in Fig. 1. Disialogangliosides were developed three times in chloroform–methanol–20 m*M* $CaCl_2$ (120:85:16, v/v/v) leading to more than two-fold increase of resolution, and chloroform–methanol–20 m*M* $CaCl_2$ (120:85:22, v/v/v) was the solvent of choice for AMD of polysialogangliosides [52]. These examples should serve as a starting point for future developments.

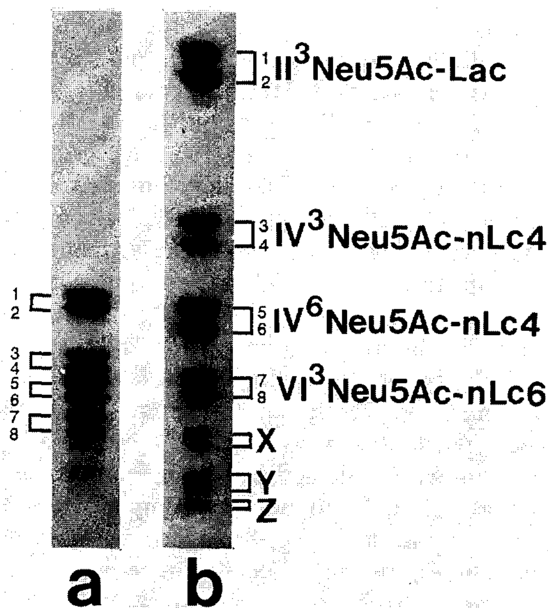

Fig. 1. AMD chromatography of neolacto-series monosialogangliosides from human granulocytes. 15 μg gangliosides were chromatographed in chloroform–methanol–20 m*M* aq. $CaCl_2$ (120:85:20, v/v/v) (lane a) and separated by threefold automated multiple development in chloroform–methanol–20 m*M* aq. $CaCl_2$ (120:85:14, v/v/v) (lane b). Gangliosides were visualized by resorcinol [42]. Structures of gangliosides are listed in Table 1. Lac, lactosylceramide; nLc4, nLcOse$_4$Cer; nLc6, nLcOse$_6$Cer.

3.4. Two-dimensional TLC

Considerable complexity of ganglioside mixtures has prompted the research demand for improved methods with the goal of high resolution chromatography being capable to separate and detect minor gangliosides beside the major species. Two-dimensional TLC procedures are successful in resolving complex mixtures of gangliosides and can be carried out with small sample amounts. Chromatographies are preferably performed on HPTLC glass-backed plates (10 cm × 10 cm; E. Merck, Germany). The sample is spotted as a small droplet in the lower left-hand corner of a heat-activated plate (see above). The first run is performed in the desired solvent. Thereafter, the plate is carefully dried, turned left vertically and placed in the second solvent system with different composition. After solvent evaporation GSLs are visualized by staining as described above.

The microanalysis of a 500 μg brain lipid extract using multiple two-dimensional TLC rendered quantitation of cholesterol, cerebrosides, sulfatides, phospholipids and gangliosides [53]. Approaches utilizing two-dimensional TLC together with conventional spray methodology [54] and combined with autoradiography [55] have been reported. The radiolabelling revealed minor brain ganglioside species that can not be visualized with conventional spraying. Several previously uncharacterized fucose-containing gangliosides were detected in this study after intracranial injection of [^{14}C]N-acetylmannosamine and [^{14}C]fucose.

Accurate densitometric quantification was attained with brain gangliosides developed by two-dimensional TLC [56]. The spots were quantified by sequential scanning densitometry. Linear response has been obtained for amounts ranging from 0.1 to 6 nmol bound sialic acid. This methodology was exploited to qualitative and

quantitative analysis of alkali-labile gangliosides from the brains of different animals [57] and consists of two-dimensional TLC employing the same solvent, chloroform–methanol–aq. 0.2% $CaCl_2$ (50:40:10, v/v/v), for both chromatographies. Prior to the second run, the plate is exposed to ammonia vapour for 5 h at room temperature to split alkali-labile O-acetylated as well as inner esters (lactones) of sialic acid residues in gangliosides. At the end of the chromatography alkali-stable gangliosides appear along a diagonal line starting from the origin; the spots corresponding to alkali-labile gangliosides are located out of the diagonal. By this procedure up to 15 different labile gangliosides can be recognized in brain samples.

Remarkable variations in the pattern of minor gangliosides of muscle taken from ten vertebrate species were detected by two-dimensional TLC [58]. More than 15 different minor components have been found in bovine and hog muscle owing to the presence of Neu5Gc-substituted GSLs, whereas muscle gangliosides of human, dog, cat, rabbit, and chicken origin showed less complex patterns which is interpreted by the absence or even small amounts of Neu5Gc-type gangliosides. Particularly in leukocytes from murine origin, heterogeneity e.g. in the sialic acid portion raises difficulties in resolution by conventional one-dimensional TLC which can be solved by two-dimensional TLC. Total ganglioside mixtures of B lymphocytes and macrophages from endotoxin-responsive and endotoxin-hyporesponsive mouse strains [59,60] as well as from a murine macrophage-like cell line [61] were investigated by two-dimensional TLC, which facilitated discovering G_{M1b}-type gangliosides (G_{M1b}, GalNAc-G_{M1b}) in macrophages and the related cell line. However, G_{M1b} and GalNAc-G_{M1b} have been previously identified in mouse spleen and T lymphocytes by conventional TLC [62,63].

4. Preparative TLC

Separation and recovery of gangliosides by preparative HPTLC is known to be in many cases unsatisfactory. The "old-fashioned" technique of TLC separation and subsequent extraction of GSLs from scraped silica gel is still a frequently used method [2,22,32–34] and can be carried out as follows. A partially purified GSL sample is dissolved in a minimum volume of chloroform–methanol and applied in a band long up to several centimeters, compatible with good resolution by preventing overloading the plate. After chromatography, gangliosides have to be localized by nondestructive reagents [64]. Then the desired areas are scraped off the plate and the GSLs are extracted from the silica gel with chloroform–methanol–water or 2-propanol–hexane–water. Salts and/or other impurities in the ganglioside fractions are commonly removed by reversed-phase, and/or silica gel chromatography. A few procedures for application of preparative TLC are discussed in this chapter.

A simple but less sensitive reversible staining method is to spray the TLC plate intensively with distilled water. The silica gel becomes translucent while lipids remain white. However, successful isolation of gangliosides has even recently been reported with this method [60].

Iodine vapour detects many lipids, also with low sensitivity and GSLs should be present in relatively high quantities to be detected. The chromatogram is placed in a double-trough TLC chamber containing solid iodine crystals along one side. In several minutes most of the lipids will appear as brown spots on a pale yellow background. Visualization of gangliosides with iodine vapour and elimination of silica gel from the primary ganglioside extract by reversed-phase chromatography with Sep-Pak cartridges has been reported as simple and effective preparative TLC method in ganglioside purification [65].

The most sensitive nondestructive dye for lipids is primuline [32]. The developed plate is sprayed lightly with primuline (e.g. from Aldrich-Chemie, Steinheim, Germany) 0.01% in acetone–H_2O (4:1, v/v). The spots are viewed under long-wave ultraviolet light at 366 nm (HP-UVIS cabinet or MinUVIS, Desaga). Lipids are revealed as light blue or yellowish bands on the dark, blue-violet background. Due to the high sensitivity of this test, primuline has become the

most popular fluorescent dye in preparative TLC [66–68]. After recovery of GSLs by extraction with organic solvent, primuline can be removed by continuous or stepwise gradient elution with isopropanol–hexane–water (55:40:5 to 55:25:20, v/v/v) [69,70].

In vitro propagation of e.g. mammalian cells offers the opportunity to feed growing cells with radioactively labeled precursor molecules, such as ^{14}C-galactose, ^{14}C-glucosamine, ^{14}C-mannosamine, which are incorporated into cellular GSLs. Metabolically labeled gangliosides can be detected by exposure of chromatograms to X-ray film, for instance Hyperfilm-^{3}H (Amersham Buchler, Braunschweig, Germany), and isolated from scraped silica gel as described above [63,71]. Furthermore radioactive labeling in the ceramide portion can be achieved by treatment of GSLs with potassium boro[^{3}H]hydride and palladium as catalyst [72]. The specific radioactivity depends on the unsaturation of the ceramide moiety and the specific activity of the boro[^{3}H]hydride employed. Both methods of radiolabelling require expensive radiochemicals, special laboratories and highly sophisticated equipment.

Nondestructive detection of gangliosides with lipophilic fluorochromes and their employment for preparative TLC was recently reported [73,74]. The nondestructive fluorochrome NBD dihexadecylamine was used for preparative TLC of brain gangliosides in microgram and milligram scale [73]. The fluorescent zones were scraped off and the gangliosides were extracted with a mixture of chloroform–methanol–water (30:60:8, v/v/v). The gangliosides were separated from uncharged NBD dihexadecylamine by anion-exchange chromatography and impurities were removed by Iatrobeads chromatography. The flowsheet of ganglioside isolation according to this procedure is shown in Fig. 2 and purified individual gangliosides are demonstrated in Fig. 3 (for structures of gangliosides see Table 2). A wide spectrum of lipophilic fluorochromes which are listed in Table 3 was selected for preparative TLC [74]. Fluorescent ganglioside spots were located on HPTLC plates under short- or long-wave ultraviolet light (λ = 254 or 366 nm; see

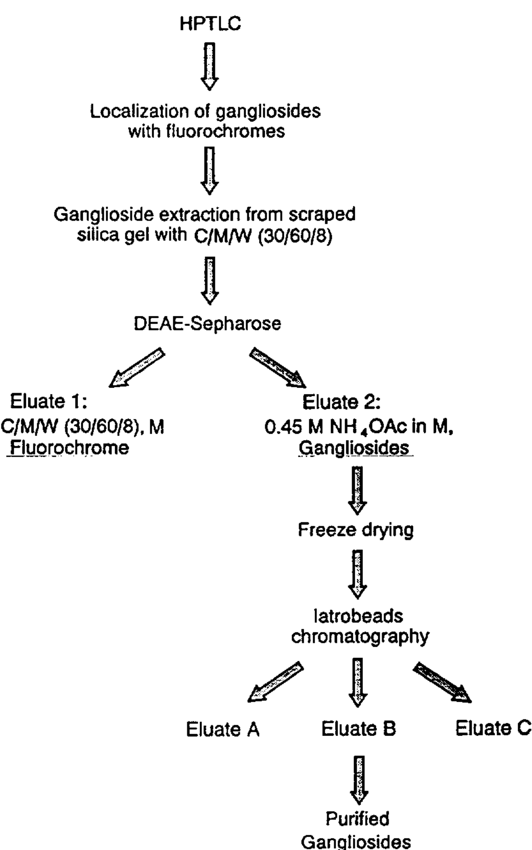

Fig. 2. Flow diagram of the isolation of gangliosides by preparative TLC. Gangliosides were localized on the plates and extracted from scraped silica gel. Fluorochromes were removed by anion-exchange chromatography and impurities were removed by Iatrobeads column chromatography. C, chloroform; M, methanol; W, water; eluate A, C–M (85:15, v/v); eluate B, C–M (1:2, v/v); eluate C, M [73,74].

Table 3) after chromatography in the dark with 0.002% (w/v) fluorochrome in the solvent. Addition of fluorescence dye to the running solvent does not alter ganglioside mobility compared to solvent without fluorochrome [73,74]. Alternatively, the plate can be sprayed after running with 0.002% (w/v) fluorochrome in acetone–methanol (60:40, v/v). The extraction was performed as described above for NBD dihexadecylamine. Both methods [73,74] offer an easy to handle and effective preparative TLC strategy to obtain pure gangliosides in microgram and milligram quantities [75,76]. Several probes can

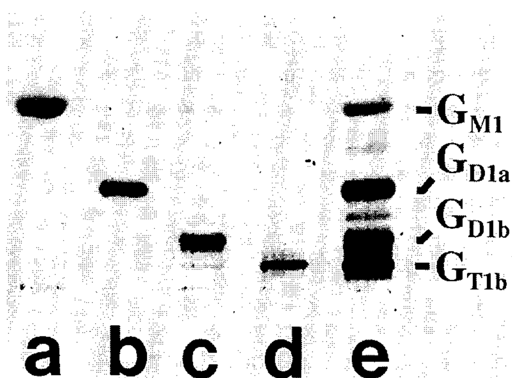

Fig. 3. Thin-layer chromatogram of individual gangliosides isolated from mouse brain by preparative TLC using NBD dihexadecylamine dissolved as reversible stain in the running solvent. Gangliosides were chromatographed in chloroform–methanol–20 m*M* aq. $CaCl_2$ (120:85:20, v/v/v) and visualized by spraying the plate with resorcinol-HCl reagent. Aliquots of TLC purified G_{M1} (a), G_{D1a} (b), G_{D1b} (c), G_{T1b} (d) and 15 μg of whole gangliosides from mouse brain (e) were applied [73].

Table 2
Structures of ganglio-series gangliosides

Symbol[a]	Structure
G_{M1} or G_{M1a}	II^3Neu5Ac-$GgOse_4$Cer
G_{M1b}	IV^3Neu5Ac-$GgOse_4$Cer
GalNAc-G_{M1b}	IV^3Neu5Ac-$GgOse_5$Cer
G_{D1a}	IV^3Neu5Ac,II^3Neu5Ac-$GgOse_4$Cer
G_{D1b}	$II^3(Neu5Ac)_2$-$GgOse_4$Cer
G_{D1c}	$IV^3(Neu5Ac)_2$-$GgOse_4$Cer
$G_{D1\alpha}$	IV^3Neu5Ac,III^6Neu5Ac-$GgOse_4$Cer
G_{T1b}	IV^3Neu5Ac,$II^3(Neu5Ac)_2$-$GgOse_4$Cer
G_{Q1b}	$IV^3(Neu5Ac)_2$,$II^3(Neu5Ac)_2$-$GgOse_4$Cer

[a] According to Svennerholm [234].

be purified simultaneously and the method only requires standard laboratory equipment, therefore being suitable for a wide range of researchers working in this field.

5. Combined TLC and fast atom bombardment-mass spectrometry (FAB-MS)

The analysis of structural parameters of GSLs has been successfully probed by mass spectrometry for two decades, generally using thermal evaporation and electron ionization of appropriate derivatives [77]. The soft ionization methods, especially fast atom bombardment (FAB) and liquid secondary ion mass spectrometry, in combination with the development of high-field instruments have brought substantial progress [78–81].

Following the procedure of preparative TLC (see above), GSLs have to be extracted from the silica gel of TLC plates before analysis by FAB-MS. Along with this procedure GSLs can be characterized from their FAB mass spectra in terms of partial monosaccharide sequence, ceramide composition, and molecular weight [82]. The coupling of soft ionization mass spectrometry and TLC offers new perspectives by direct analysis of GSLs on TLC plates without preceding elution of the sample from the silica gel [83]. This method involves separation on a flexible aluminium- or plastic-backed silica gel TLC plate and nondestructive localization [84]. The area of interest on the plate was cut out and

Table 3
Features of fluorochromes selected for preparative TLC

Fluorochrome	Excitation (nm)	Color	Emission intensity[a]
Acridine orange	254	Yellow	++++
Pyrene-1-aldehyde	254	Light blue	++++
Rhodamine 6G	254	Orange	++++
3,3′-Diethyloxacarbocyanineiodide	254	Yellow	+++
Nile red	254	Red	++
N-Phenyl-2-naphthylamine	366	Light blue	++
N-Phenyl-2-naphthylamine	366	Light blue	+

[a] Appearance on HPTLC plates graded from + to ++++ after chromatography and thorough evaporation of solvents.

the piece was attached to the mass spectrometer probe tip. After addition of a few microliters of methanol and about 2–3 μg matrix liquid, the probe tip was introduced into the mass spectrometer. The probe was handled manually for the scanning of the piece of the TLC plate, and mass spectra were recorded every 0.25 mm along the plate using Xe^+ ions as the primary ion with an energy of 5 kV (for more details, see [83]). The mass spectra obtained were comparable to those obtained by conventional methods [85]. The combination of TLC and mass spectrometry is especially attractive and useful, because it simultaneously provides chromatographic and structural information. The application of this technique for GSL storage disease has been reported [86].

The resolution into molecular species on desorption of GSLs from thin-layer chromatograms, using combined TLC and FAB-MS was demonstrated for sulphatides, G_{M3} and $GgOse_4Cer$ [87]. In this study a mass spectrometer was used with a special FAB probe and an extended solid inlet port. The mounted TLC strip was moved by a motor at 0.25 mm per scanning cycle, which allowed >200 scans per plate (for details, see [88]).

With the development of the overlay-binding assay (to be elucidated in the next chapter), the requirement for improved techniques to identify unknown receptor molecules in a mixture of GSLs became obvious. Although the sensitivity of TLC–MS (μg level) is lower compared to TLC immunostaining (ng level), both methods are complementary for characterizing GSL probes separated on a TLC plate.

6. Overlay technique

The TLC overlay procedure is an easy, rapid and sensitive method to investigate specific binding of ligands to separated GSLs. Overlay techniques are valuable tools for the localization of biologically active compounds on TLC plates. The first proof of selective binding of cholera toxin to gangliosides resolved on a TLC plate [35] led to the development of a huge variety of related TLC overlay methods for direct binding of antibodies, various toxins, lectins and other proteins as well as related compounds. These techniques are also useful in identifying GSL structures as potential receptors for viruses, bacteria and cells.

6.1. General procedure

After chromatography of GSLs some TLC techniques share several common steps. The pretreatment of the chromatograms with plastic, usually polyisobutylmethacrylate (Plexigum P28, Röhm, Darmstadt, Germany), is recommended. Coating of TLC plates can be performed by chromatography in polyisobutylmethacrylate saturated hexane [89] or by dipping the plate, for example, in 0.2% polyisobutylmethacrylate solution [90]. The rationale for plastic coating is to prevent flaking of silica gel from the support during the incubation and washing steps. The hydrophobicity of the plate protection is believed to induce a similar presentation of GSLs as in the plasma membrane. However, plastic- as well as aluminium-backed TLC sheets have been reported to be sufficiently robust alone whereas silica gel of glass-backed HPTLC plates needs plastic fixation. For the next step the plate is overlayed with the primary agent (antibody, toxin, etc.), incubated, washed with buffer and, if necessary, overlayed with secondary detection agents (e.g., secondary labeled antibody). Then the plate is washed again, and dried for autoradiography on X-ray film or treated for secondary color development (e.g., alkaline phosphatase-based detection). A companion plate is chromatographed in the same chamber under identical conditions and subjected to chemical detection (see above). Exact conditions of overlay procedures are explained in detail along with the particular application, examples of which are given in the following sections.

6.2. Antibodies

To study function and expression of glycoconjugates on the cell surface, specific antibodies have been raised against distinct carbohy-

drate determinants [91–95]. TLC overlay methods for defining GSL specificities of mono- and polyclonal antibodies have been developed [96–99] with aid of ^{125}I-labeled secondary antibodies. The procedure is carried out as follows [98]: Gangliosides were chromatographed on aluminium-backed HPTLC plates and the dried chromatogram was soaked in a saturated solution of polyisobutylmethacrylate in hexane. To prevent unspecific binding of antibody, the chromatogram was sprayed after drying with phosphate buffered saline containing 1% bovine serum albumin (buffer A) and immediately soaked in buffer A. The plate was then overlayed with monoclonal antibody solution diluted 1:4 with buffer A and incubated for 3 h at 4°C. After washing in cold phosphate-buffered saline the plate was overlayed with ^{125}I-labeled $F(ab')_2$ rabbit anti-mouse IgG. The plate was washed again and exposed to X-ray film. Following this procedure, e.g. two monoclonal antibodies have been identified reacting with a sialyl derivative of lacto-N-fucopentaose II, a hapten of the human Le^a blood group antigen, which was detected in GSL extracts of several carcinomas [98].

Using specific antibodies [100] against gangliosides G_{M4}, G_{M1}, G_{D3} and $GgOse_4Cer$, the detection of bound primary antibodies was achieved also with ^{125}I-labeled staphylococcal protein A, which has been shown to bind preferentially to the Fc region of human, rabbit and guinea pig IgG. The method allowed positive identification of GSLs on TLC plates and also provides a convenient means of assessing the specificity of anti-GSL antibodies.

To avoid work with radioactive isotopes and to accelerate the immunostaining procedure, assays with peroxidase- and alkaline phosphatase-linked secondary antibodies were established [101–103]. A sensitive enzyme-immunostaining method on TLC plates has proved to be valuable for detection and quantitative determination of Neu5Gc-containing GSLs, Hanganutziu–Deicher antigens [101]. The procedure consists of an immune reaction among gangliosides, affinity-purified chicken anti-G_{M3}(Neu5Gc) and horseradish peroxidase-conjugated rabbit anti-chicken IgG with 4-chloro-1-naphthol as peroxidase substrate. Quantification was achieved by direct densitometric scanning of the enzyme-immunostained spots on the chromatogram. As little as 0.5 pmol of G_{M3}(Neu5Gc), $IV^3Neu5Gc\text{-}nLcOse_4Cer$, and $VI^3Neu5Gc\text{-}nLcOse_6Cer$ (0.64–1.0 ng) have been detected with a good signal-to-noise ratio. Further applications of non-radioactive detection using TLC immunostain have been reported to demonstrate, e.g. the specificities of two sets of monoclonal antibodies against a- and b-pathway ganglio-series gangliosides [104,105].

A complementary method based on peroxidase labeled secondary antibodies, where the GSLs were transferred to a nitrocellulose sheet, has been described [106]: After TLC the separated GSLs are blotted onto nitrocellulose and the replica is incubated with poly- or monoclonal antibodies followed by detection with peroxidase labeled secondary antibodies. Advantages of the procedure are its speed, non-radioactive detection and its suitability for screening applications. This approach has been recently extended to very efficient quantitative blotting of GSLs from HPTLC plates to a polyvinyldine difluoride membrane followed by immunological staining [107]. This technique has been also proved to be a convenient procedure for preparative TLC with GSL yields ranging from 68% to 92%, the mean value being 82.3% [108].

The avidin–biotin enzyme system, which has been used to detect carbohydrate antigens in tissues and isolated cells, was adapted for GSL immunostaining on thin-layer chromatograms and on nitrocellulose blots [109]. This method is based on the extraordinarily high affinity of the egg white protein avidin for biotin. Biotin can be covalently attached to proteins (e.g., antibodies) and these proteins can be detected by the use of a preformed complex of avidin plus biotinylated enzyme. Since avidin has multiple biotin binding sites, several biotinylated enzyme molecules can be bound. The conversion of a soluble substrate into an insoluble product by the biotinylated enzyme provides a highly amplified signal for the location of the antibody binding site. This method is characterized by a high sensitivity, i.e. subnanogram amounts of the GSL can be detected [109].

Another immunochemical detection method originally developed for the detection of glycoproteins on nitrocellulose has been adapted for staining of GSLs on TLC plates. This procedure is based on periodate oxidation of the carbohydrate portion of GSLs [110]. Digoxigenin–X–hydrazide (X = spacer) reacts with the aldehyde groups, and the digoxigenin is recognized by an alkaline phosphatase-labeled polyclonal anti-digoxigenin antibody. This method combines the general applicability of the orcinol and resorcinol stains with the sensitivity of an immunostain. Selective staining of gangliosides could be achieved by the use of low periodate concentrations.

The construction of neoglycolipids as novel approach for determining the antigenicities and receptor functions of minute amounts of oligosaccharides derived from glycoproteins was reported [111]. Unlike hydrophilic oligosaccharides, the neoglycolipids have hydrophobic properties conferred by the lipid moiety, such that ligand-binding assays can be performed by using the oligosaccharides as immobilized probes on solid supports, e.g. TLC plates. Conditions for efficient conjugation of reducing oligosaccharides to dipalmitoyl phosphatidylethanolamine were established, and the potent and specific reactivities of the resulting neoglycolipids with monoclonal antibodies in oligosaccharide-recognition assays on silica gel chromatograms were proved [112]. Development of neoglycolipid technology has been recently reviewed [113].

6.3. Enzymatic modification

The large majority of brain gangliosides belongs to the ganglio-series with $GgOse_3$- and $GgOse_4Cer$ core. Therefore, in some cases it is convenient to analyse gangliosides by combined neuraminidase treatment followed by immunostaining of respective asialo-gangliosides with anti-$GgOse_3Cer$ and/or anti-$GgOse_4Cer$ antibodies [114–117]. After thin-layer chromatography and silica gel fixation, the chromatogram is treated with *Arthrobacter ureafaciens* [114,116,117] or *Vibrio cholerae* neuraminidase [115]. This procedure will remove both external and internal sialic acid residues from the core oligosaccharide backbone. The resulting asialo-gangliosides are then stained with anti-$GgOse_4Cer$ and/or anti-$GgOse_3Cer$ antiserum and secondary detection agents. To remove all sialic acids from ganglio-series gangliosides with *A. ureafaciens* neuraminidase, sodium taurodeoxycholate was found to be required [116,117]. This is incompatible with the first description of this procedure [114]. However, this discrepancy might be due to the probable existence of different isozymes in enzyme preparations from different *A. ureafaciens* strains [118].

Since terminally sialylated gangliosides such as G_{M1b} and $IV^3Neu5Ac$-$nLcOse_4Cer$ were identified as virus receptors, and certain other terminally sialylated gangliosides were discussed as tumor markers, several overlay procedures have been developed to screen gangliosides from different tissues or cell lines for the presence of such components, especially if only small amount of material is available [89,119,120]. G_{M1b} and $G_{D1\alpha}$ are rendered accessible to anti-$GgOse_4Cer$ stain after *V. cholerae* neuraminidase treatment [89], GalNAc-G_{M1b} is susceptible to anti-$GgOse_3Cer$ binding after *A. ureafaciens* neuraminidase incubation [119,121] and selective detection of terminally α2–3 and α2–6 sialylated neolacto-series gangliosides is possible by combined *V. cholerae* neuraminidase treatment and anti-$nLcOse_4Cer$ immunostaining [120]. The procedure is carried out as follows [120]. After chromatography of the gangliosides the plates were dried for 0.5 h over P_2O_5 in a desiccator equipped with a vacuum pump and then chromatographed twice in hexane saturated with polyisobutylmethacrylate. The plastic solution was prepared by adding excess of polyisobutylmethaccrylate to hexane. After 30 min of stirring at ambient temperature the decanted supernatant was used for silica gel fixation. *V. cholerae* neuraminidase (Behring, Marburg, Germany) treatment was carried out by incubation with 2.5 mU/ml in 0.05 *M* sodium acetate, 9 m*M* $CaCl_2$ (pH 5.5) for 2 h at room temperature. For blocking of unspecific binding the plates were soaked for 15 min in solution A

(phosphate-buffered saline supplemented with 1% bovine serum albumin). Then the plate was overlayed for 1 h with chicken anti-nLcOse$_4$Cer antibody diluted 1:2000 in solution A. Unbound antibodies were removed by washing the plate five times with solution B (phosphate buffered saline, 0.05% Tween 21). Rabbit anti-chicken IgG antiserum (Dianova, Hamburg, Germany) labeled with alkaline phosphatase, was used as the second antibody (dilution of 1:1000 in solution A). After 1 h incubation the plate was washed as described above followed by twofold washing with 0.1 *M* glycine buffer (pH 10.4), supplemented with 1 m*M* $ZnCl_2$ and 1 m*M* $MgCl_2$, to remove phosphate. Bound first antibodies were visualized with 0.05% (w/v) 5-bromo-4-chloro-3-indolyl phosphate (Biomol, Hamburg, Germany) dissolved in the same alkaline buffer [103]. The arising blue color indicated specifically bound antibodies. The specific detection of terminally α2–3 and α2–6 sialylated neolacto-series gangliosides of human granulocytes (for structures, see Table 1) is demonstrated in Fig. 4. Neuraminidase treatment of gangliosides with α2–3 substituted sialic acid is necessary prior to immunostaining (Fig. 4, lane c) whereas α2–6 sialylated gangliosides can be detected without enzyme treatment (Fig. 4, lane b). Steric hindrance of sialic acid bound in position 3 to terminal galactose prevented binding of the antibody to the Galβ1-4GlcNAc sequence whereas sialylation in position 6 does not hinder recognition. The combined method of neuraminidase treatment and immunostaining of neolacto-series gangliosides is a suitable tool for highly specific and sensitive detection down to 10 ng of such gangliosides in complex GSL mixtures.

Not only enzymatic degradation but also solid-phase biosynthesis of GSLs on HPTLC plates has been accomplished [122]. The method takes advantage of the solid matrix for precursor GSLs in biosynthetic experiments after chromatographic development of the precursor sample. The method is simple, fast and sensitive. It minimizes the risk of adding unwanted exogenous precursors and abolishes the need for tedious purification of products after incubation.

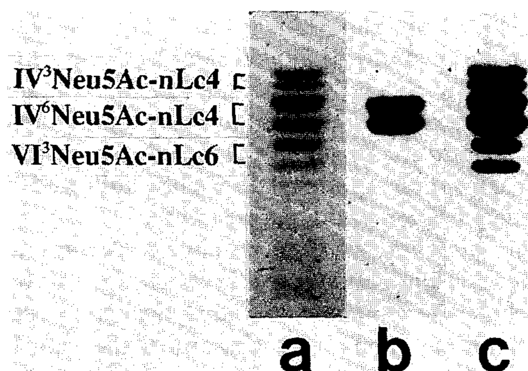

Fig. 4. Selective detection of terminally α2–3 and α2–6 sialylated neolacto-series gangliosides. 3 μg of a G_{M3} depleted ganglioside fraction from human granulocytes per lane were chromatographed as described in Fig. 3. Lane a: resorcinol stain; lane b: immunostain with anti-nLcOse$_4$Cer antibody; lane c: immunostain with anti-nLcOse$_4$Cer antibody with preceding neuraminidase treatment [120]. nLc4 = nLcOse$_4$Cer; nLc6 = nLcOse$_6$Cer. IV^{3}Neu5Ac-nLc4 corresponds to bands 3 and 4, IV^{6}Neu5Ac-nLc4 to bands 5 and 6, and VI^{3}Neu5Ac-nLc6 to bands 7 and 8 of Fig. 1. Structures of neolacto-series gangliosides from human granulocytes are listed in Table 1.

The method opens up new areas for the biosynthetic study of gangliosides by assaying directly and simultaneously the activities of sialyltransferases with multiple acceptor specificity [123]. An acceptor GSL was chromatographed and incubated with an enzyme mixture containing an appropriate radioactive sugar nucleotide. The radiolabeled reaction product was isolated by preparative TLC and the radioactivity determined to measure the activity of rat brain cytidine 5′-monophosphate-*N*-acetylneuraminic acid: LacCer-, G_{M3}-, G_{M1}- or G_{D3}-sialyltransferase.

6.4. Toxins

Ganglioside G_{M1} is the major receptor for cholera toxin on the surface of animal cells [124,125]. G_{M1} substituted with N-acetyl- or N-glycolylneuraminic acid has been reported to exhibit the same toxin receptor capacity [126]. The toxin is a globular 84 kDa protein and consists of two different types of subunits, A and

pentameric B_5 [127]. Its mechanism of action involves binding of the B_5 subunit (= choleragenoid) to ganglioside G_{M1} and other structurally related compounds on the cell surface, followed by a dissociation between subunits and penetration of subunit A into the membrane with activation of adenylate cyclase followed by changes in membrane permeability.

The discovery of G_{M1} as natural receptor for cholera toxin opened the investigation of this binding phenomenon for analytical purposes. Gangliosides that bind cholera toxin can be detected by the direct binding of ^{125}I-labeled toxin to thin-layer chromatograms followed by autoradiography [35,128]. The method is very sensitive detecting 0.1 ng of G_{M1} (70 fmol) on chromatograms of total cellular lipid extracts [35]. A modification of this method has been reported, in which native cholera toxin was used instead of ^{125}I-labeled toxin, followed by incubation with specific antiserum to cholera toxin and species-specific antiserum coupled to horse radish peroxidase [129]. The use of enzyme-labeled secondary antibody was a reliable, reproducible, and sensitive method for G_{M1} detection with limits down to 0.01 ng G_{M1}, corresponding to 6.5 femtomol. To reveal the presence of other members of the gangliotetraose family, i.e., G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b} (for structures see Table 2), *V. cholerae* neuraminidase is employed to convert these gangliosides to G_{M1} prior to treatment with choleragenoid [130], since this enzyme does not cleave the internal sialic acid of G_{M1} linked to galactose at position II (counted from the ceramide portion) of the $GgOse_4Cer$ core. By use of these methods described above, the disialoganglioside G_{D1b} showed considerable binding capacity to cholera toxin (approximately 10:1 ratio of G_{M1}:G_{D1b}) [128–130]. However, TLC combined with sialidase treatment and choleragenoid staining (as well as immunological staining as described above) has found wide application, e.g. determination of gangliotetraose-type gangliosides in human primary medulloblastomas [131], human cerebrospinal fluid [132,133] or in mouse lymphoma [134]. A new ganglioside showing choleragenoid-binding activity in mouse spleen was identified by this technique. Its structure was determined to be Gal-GalNAc-G_{M1b} (IV^3Neu5Gc-$GgOse_6Cer$), which contains a terminal tetrasaccharide structure identical with that of II^3Neu5Gc-$GgOse_4Cer$ (G_{M1}(Neu5Gc)) [135]. The procedure (taken from [134]) can be carried out as follows: A silica gel HPTLC plate coated with polyisobutylmethacrylate was incubated with cholera toxin B_5 subunit (Sigma, No. C-7771, Munich, Germany) at a final concentration of 250 ng ml^{-1} in phosphate buffered saline containing 1% bovine serum albumin (solution A) for 2 h at room temperature. Goat anti-cholera toxin B subunit (choleragenoid) antiserum (Calbiochem, No. 227040, Frankfurt a. M., Germany) and alkaline phosphatase conjugated rabbit anti-goat IgG (Dianova, Hamburg, Germany) antibody (1:1000 dilution in solution A) were used for the immunostaining procedure (1 h at room temperature). The washing steps and the detection of alkaline phosphatase activity on the plate were performed as described above [120]. For detection of G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b}, neuraminidase is employed to convert these gangliosides to G_{M1} prior to treatment with choleragenoid. Plastic coated silica gel plates were incubated with 50 mU ml^{-1} *V. cholerae* neuraminidase for 18 h at 37°C in 0.05 *M* sodium acetate, 9 m*M* $CaCl_2$, pH 5.5. The subsequent immunostaining assay with choleragenoid was performed as described above. The reliability of this technique is shown in Fig. 5. G_{M1} from a human brain ganglioside mixture (and to much lesser extent also G_{D1b}) is immunostained by choleragenoid (Fig. 5, lane a), whereas G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b} are stained after neuraminidase treatment in addition to G_{M1} (Fig. 5, lane b). Conventional resorcinol visualization of human brain gangliosides is shown in Fig. 5 (lane c) in parallel to the combined choleragenoid-immunostains.

Direct binding analysis of toxin molecules to gangliosides on TLC plates permits clearer identification and sensitive quantification of the receptor structure. For example, TLC overlay technique was employed for screening the binding activity of solubilized protoxin and activated toxin from *Bacillus thuringiensis* [136], type A

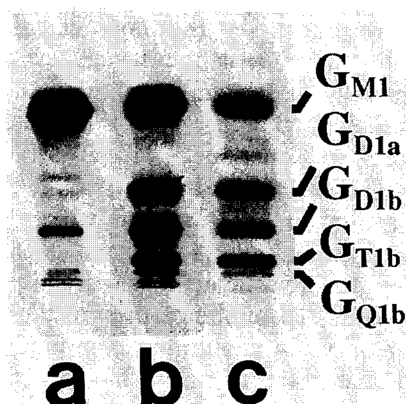

Fig. 5. Detection of gangliotetraose-type gangliosides by combined neuraminidase and choleragenoid-immunostaining assay. 25 ng (lanes a and b, each) and 10 μg of human brain gangliosides (lane c) were chromatographed as described in Fig. 3. Lane a: combined immunostain with choleragenoid and anti-choleragenoid antibody; lane b: *V. cholerae* neuraminidase treatment prior to choleragenoid detection; lane c: resorcinol stain [134]. Structures of gangliotetraose-type gangliosides are listed in Table 2.

neurotoxin from *Clostridium botulinum* [137], toxin A from *Clostridium difficile* [138], Shiga toxin from *Shigella dysenteriae* [139], etc. In the procedures described so far, polyisobutylmethacrylate is generally used to prevent loss of silica gel and to orientate carbohydrate moieties for the binding of various ligands to GSLs. This pretreatment of chromatograms was found to alter the binding behaviour of Verotoxin 1, and therefore caution is advised in analysis of toxin binding to GSLs after treatment with this compound [140].

6.5. Lectins

Lectins are carbohydrate-binding proteins of non-immunoglobulin nature [141], capable of binding to glycolipids and glycoproteins. The use of lectins from plant and animal origin for studying animal cell glycoconjugates has a long and productive history [142]. By definition, most lectins are multivalent proteins having multiple subunits, and the interaction of a glycoconjugate with a lectin is governed by the binding specificity and affinity of each subunit for a glycoconjugate [143]. Today, many different lectins are commercially available and used for purification of glycolipids and glycopeptides and glycolipid- as well as glycoprotein-derived oligosaccharides [144,145] by lectin affinity chromatography. Immunochemical methods and enzyme-linked lectin assays have been developed to detect electrophoretically separated glycoproteins after blotting onto nitrocellulose sheets [146] and to ascertain glycoproteins adsorbed to plastic supports [147]. Binding specificities of lectins against neutral GSLs and gangliosides immobilized on polyvinylchloride microtiter plates have been investigated using an enzyme-linked immunosorbent assay with the biotin–avidin system for detection of bound lectin [148].

At first, ^{125}I-labeled lectins have been described as lectin tools for GSL detection after chromatography on thin-layer chromatograms [149,150]. GSLs that bind radiolabeled lectins can be detected by autoradiography after TLC of GSL standards or crude lipid extracts [151,152]. This technique extends lectin specificity studies from inhibition analyses in aqueous systems to the identification of specific, lectin-binding GSLs in crude lipid mixtures of cell membranes.

Many lectins with well characterized sugar binding activities could be employed for preliminary structural characterization of chromatographically separated GSLs. Alternatively to ^{125}I-labeled lectins, lectin–horse radish peroxidase conjugates or biotinated lectins, followed by incubation with a complex of avidin and biotinated horseradish peroxidase, were used to detect GSLs immobilized on TLC plates by nonradioactive visualization [153,154]. Most of all these overlay procedures are cost effective and less hazardous for laboratory personnel compared to radiolabeled lectins and the results are not dependent upon film exposure time and development.

6.6. Other proteins and related compounds

A number of extracellular matrix proteins, including fibronectin [155,156], laminin [157,158], thrombospondin [159,160] and also von Willebrand factor (coagulation factor VIII related protein) [158,160], have been shown in a

variety of assays to bind either to gangliosides, to sulfatides, or to both (reviewed in [161]). Some recent publications which describe binding studies of GSLs, proteins and/or lipoproteins on thin-layer chromatograms are reported in this chapter.

Mannose-binding protein binds with high affinity to glycoconjugates with terminal mannose or N-acetylglucosamine residues. However, glycoproteins with these terminal residues are relatively rare in normal mammalian tissues and in search for other possible natural receptors, purified mannose-binding protein was found by chromatogram overlay assay to bind with high affinity to $LcOse_3Cer$ and $nLcOse_5Cer$ [162]. Oligosaccharide recognition by three mammalian mannose-binding proteins was investigated by employing a series of structurally characterized neoglycolipids as probes in TLC binding assays [163]. The results illustrated the potential of neoglycolipids in studies of oligosaccharide recognition.

Cytotactin is an extracellular matrix protein that is involved in cell adhesion, migration, and proliferation during embryogenesis. The binding of this glial glycoprotein to a variety of purified GSLs has been investigated and using radiolabeled cytotactin clear evidence was found for binding to sulfatides separated by TLC [164].

Exoenzyme S is an ADP-ribosyl transferase produced by *Pseudomonas aeruginosa* which is in discussion to be, at least in part, responsible for the pathogenesis of this organism in respiratory infection. By employment of the TLC overlay technique it has been shown that exoenzyme S specifically binds to ganglio-series GSLs [165].

Glycolipids have been supposed to act as regulatory molecules in the cellular uptake of low-density lipoproteins [166–168]. For analysis of the ligand-receptor reaction, the TLC immunostaining method greatly facilitated the direct examination of apolipoprotein B binding to various types of acidic and neutral GSLs [169].

6.7. Viruses

During the initial phase of infection, myxoviruses such as influenza and Sendai viruses interact with receptors on the host cell surface, followed by fusion with the surface membrane [4]. Terminally sialylated gangliosides are important receptor binding sites for viruses and are able to mediate virus attachment [75,170,171]. Several assay systems, based on the adsorption to [172], fusion with [173] or infection of target cells [174], have been found convenient to investigate the accessibility of viruses to cells. To minimize the interference of other cell or virus membrane components, direct solid-phase binding assays have been established. These tests offer the opportunity for direct measurement of the interaction between the virus and its ganglioside receptor. Plastic microtiter wells coated with receptor molecules have been used to estimate the avidity of virus binding to a purified ganglioside [175]. This solid-phase approach was extended by employment of virus binding to thin-layer chromatograms on which the GSLs were separated [176]. As an example, the overlay assays of ^{35}S-labeled influenza A/PR/8/34 (H1N1) and Sendai virus (HNF1, Z-strain) on thin-layer chromatograms are shown in Fig. 6. Both viruses were found to bind gangliosides from human granulocytes (for structures see Table 1) to various extent [75]. The following procedure is derived from [75]: Ganglioside chromatography and plastic coating of the TLC plates were performed as described above (Section 6.3). To reduce the amounts of labeled viruses, the plates were cut with a diamond glass cutter into strips of 1.5 cm $\times$ 10 cm per lane. The strips were soaked for 15 min in solution A (phosphate buffered saline, supplemented with 1% bovine serum albumin) to block unspecific binding sites. The solution was thoroughly withdrawn by suction and 80 μl labeled virus preparation were added per lane (about 2×10^5 counts min^{-1}). Then the strips were covered with small pieces of parafilm and kept in a humidified atmosphere for 2 h at 4°C. After incubation the virus suspension was tipped off and the plate was washed six times with phosphate buffered saline. The dried plate was exposed to X-ray film for 20 days at 4°C. Stained parallel ganglioside chromatogram (Fig. 6, lane

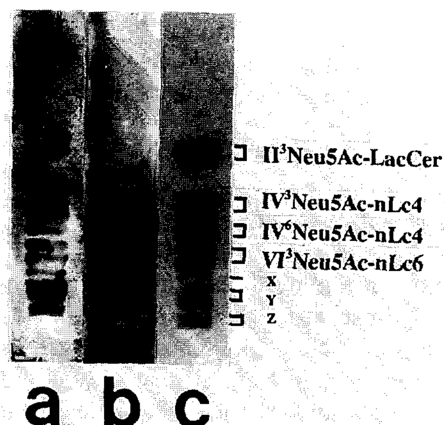

Fig. 6. Autoradiographies of TLC overlay assays of gangliosides from human granulocytes with ^{35}S labeled influenza A/PR/8/34 (lane a) and Sendai virus (lane b), exposure time 20 d. The parallel resorcinol stain is shown in lane c. In each lane 20 μg of gangliosides were applied and chromatographed as described in Fig. 3. Structures of neolacto-series gangliosides from human granulocytes are listed in Table 1 [75]. nLc4 = $nLcOse_4Cer$; nLc6 = $nLcOse_6Cer$; X, Y and Z: unknown structures.

c) and virus autoradiographies (Fig. 6, lanes a and b) can be quantified by densitometry (CD60 scanner, Desaga), and ratios of bound ^{35}S-labeled virus to gangliosides can be calculated [75]. Both, the solid-phase binding assay in microtiter wells and the overlay technique are now well established tools in receptor binding assays [177], and high degree of correlation of values obtained by the TLC overlay technique and the microwell adsorption assay has been recently reported [178].

Influenza C virus uses 9-O-acetyl-N-acetylneuraminic acid as a high-affinity receptor determinant for attachment to cells [179,180]. This unique specificity was used to reveal O-acetylated sialic acids residues on glycoproteins after polyacrylamide gel electrophoresis and transfer onto nitrocellulose sheets and as constituents of gangliosides after thin-layer chromatography [181,182], thus providing a new analytical tool. The TLC overlay and the microwell adsorption assay allowed specific and sensitive detection with a limit of 65 fmol 9-O-acetylated sialic acid in 9-O-acetylated ganglioside G_{D1a}.

6.8. Bacteria

There is a rapidly growing interest in the molecular aspects of microbe association to animal surface cells. Attachment to the host is the initial event of the infection process where membrane-close lipid-linked oligosaccharides are of particular interest. The recently published solid-phase methods (see above) have opened the new possibilities for the detection and characterization of carbohydrate receptors for microbes [9,177,183].

Conditions have been adapted for the binding of intact bacteria to GSLs on thin-layer chromatograms [184]. Externally with ^{125}I or with other isotopes metabolically labeled bacteria are layered on the plate and after repeated washing, the bound bacteria are detected by autoradiography. Using this technique, several kinds of bacteria have been shown to adhere to the plate in a carbohydrate-specific way [185–195]. One of the advantages of this solid-phase overlay approach is the possibility to detect a minor receptor component within a complex GSL mixture. In addition, the multivalent solid-phase presentation of the carbohydrate receptor also reveals low-affinity binding sites, which probably might remain undetectable in the classical inhibition experiments with soluble oligosaccharides [174].

6.9. Cells

Cell–cell recognition and adhesion is mediated by cell surface carbohydrates and complementary carbohydrate receptors [13,196–198]. A method has been reported which detects carbohydrate-specific adhesion of intact eukaryotic cells directly to GSLs separated on TLC plates [199]: After chromatography and plastic coating, plates were mounted in a specially designed plexiglass chamber. To achieve contact between cells and TLC separated GSLs, radiolabeled cells were added into the chamber, which was then

sealed and gently centrifuged. Adherent cells were detected by autoradiography. This method can be used to test GSLs as cell surface recognition markers for a variety of cell types (for more details, see [200]).

7. Conclusion and perspectives

The combination of TLC separation, conventional chemical staining of GSL bands and direct overlay binding can rapidly generate detailed information on ganglioside structure and function without large investments in instrumentation. On the other hand, binding studies of antibodies, toxins or lectins to GSLs provide information of unknown carbohydrate specificities when highly purified GSL standards are used as references. Purification of a GSL to a single band, even in several different solvent systems, does not always ensure homogeneity and purity. Certain complex ganglioside mixtures are difficult to resolve by TLC, while some GSLs, although having different monosaccharide moieties and/or variations in their ceramide parts, chromatograph with identical TLC R_F values. Structural assignments gained by immunochemical methods are also limited and should be carefully interpreted, because underlying minor gangliosides can simulate adsorption to dominant gangliosides in crude fractions as well as in insufficiently purified probes. However, premature conclusions might lead to misinterpretation of antibody binding specificity.

Therefore, even though TLC-based techniques are powerful tools for GSL analysis, they should be combined with other analytical and preparative methods for unambiguous structural determination. For this, a multidisciplinary approach is needed, including preparative purification as well as analytical determination of GSLs by high-performance liquid chromatography [201,202], oligosaccharide sequencing with specific exoglycosidases and endoglycoceramidases [203,204], determination of sialic acids [205–211], FAB-MS [79–82,212–217], GC–MS [218–221] and nuclear magnetic resonance spectroscopy [222–227], or combinations of technologies [83–88,228–231]. Merging of TLC with these techniques enhances its power as an analytical tool for wide range of application.

8. List of abbreviations

AMD, automated multiple development; FAB-MS, fast atom bombardment-mass spectrometry; GC–MS, gas chromatography–mass spectrometry; GSL(s), glycosphingolipid(s); HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid [232]. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations [233] and the nomenclature of Svennerholm [234]. Lactosylceramide or LacCer, Galβ1-4Glcβ1-1Cer; gangliotriaosylceramide or GgOse$_3$Cer, GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliotetraosylceramide or GgOse$_4$Cer, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliopentaosylceramide or GgOse$_5$Cer, GalNAcβ1-4Gal-β1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliohexaosylceramide or GgOse$_6$Cer, Galβ1-3GalNAcβ1-4Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; lactotriaosylceramide or LcOse$_3$Cer, GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; lacto-N-*neo*tetraosylceramide or nLcOse$_4$Cer, Galβ1-4GlcNAc-β1-3Galβ1-4Glcβ1-1Cer; lacto-N-pentaosylceramide or nLcOse$_5$Cer, GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; lacto-*N-nor*hexaosylceramide or nLcOse$_6$Cer, Galβ1-4GlcNAc-β1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; G_{M4}, I^3Neu5Ac-Galβ1-1Cer; G_{M3}, II^3Neu5Ac-LacCer; G_{M2}, II^3Neu5Ac-GgOse$_3$Cer; G_{M1} or G_{M1a}, II^3Neu5Ac-GgOse$_4$Cer; G_{M1b}, IV^3Neu-5Ac-GgOse$_4$Cer; GalNAc-G_{M1b}, IV^3Neu5Ac-GgOse$_5$Cer; G_{D3}, II^3(Neu5Ac)$_2$-LacCer; G_{D1a}, IV^3Neu5Ac, II^3Neu5Ac-GgOse$_4$Cer; G_{D1b}, II^3(Neu5Ac)$_2$-GgOse$_4$Cer; G_{D1c}, IV^3(Neu-5Ac)$_2$-GgOse$_4$Cer; $G_{D1\alpha}$, IV^3Neu5Ac, III^6Neu-5Ac-GgOse$_4$Cer; G_{T1b}, IV^3Neu5Ac, II^3(Neu-5Ac)$_2$-GgOse$_4$Cer; G_{Q1b}, IV^3(Neu5Ac)$_2$, II^3-(Neu5Ac)$_2$-GgOse$_4$Cer.

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