Automated multiple development (AMD) and analysis of CD15 glycosphingolipids directly from HPTLC plates by infrared matrix-assisted laser desorption/ionization orthogonal time-of-flight mass spectrometry

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INTRODUCTION

Glycosphingolipids (GSLs) are amphipathic molecules comprising of two structural elements: a lipophilic membrane anchor, the ceramide portion, formed by a long-chain amino alcohol and a fatty acid, and a hydrophilic carbohydrate moiety [1]. Neutral GSLs of the neolacto-series with the terminal trisaccharide motif Galβ1-4(Fucα1-3)GlcNAc-R are known as Lewis^X - or CD15-GSLs. Prolongation of the core-structure by Galβ1-4GlcNAc-repeats and additional fucosylation at subterminal and internal GlcNAc-positions result in highly polar CD15-GSLs with nLc6Cer, nLc8Cer, nLc10Cer, and nLc12Cer core structures. These components are supposed to play crucial roles in carbohydrate-protein mediated interactions of cells of the immune system.
Analytical and preparative high-performance thin-layer chromatography (HPTLC) are widely used for separation and identification as well as for isolation and purification of individual GSLs [1]. One-dimensional HPTLC using silica gel-precoated HPTLC plates is the simplest and still commonly applied method for separating GSL mixtures. However, increased separation of complex mixtures of CD15-GSLs by automated multiple developmnet (AMD) is required for their structural characterization. In this study an improved separation of CD15-GSLs from human granulocytes by AMD combined with their structural characterization by mass spectrometry is described.

MATERIALS AND METHODS

High-performance thin-layer chromatography (HPTLC)
A mixture of neutral GSLs from human granulocytes, containing lactosylceramide (Lc2Cer) and neolactotetraosylceramide (nLc4Cer) as major and various CD15-GSLs as minor components, was separated on silica gel precoated HPTLC plates (Merck, Art. 5633) and stained with orcinol.

Conventional HPTLC
Single HPTLC was performed in a conventional glass tank for 20 min in the solvent chloroform/methanol/water (120:70:17, v/v/v).

Automated multiple development (AMD)
Multiple development of GSLs was performed in the same solvent employed for conventional HPTLC using the AMD 2 system from CAMAG (Muttenz, Switzerland). Triplicate runs were performed for about 45 min each run with intermediate drying periods of 10 min (cf. Figure 1).

Overlay assay
CD15 GSL-species were immunodetected with monoclonal mouse IgM anti-CD15 antibodies (Clone 80H5). As secondary antibodies alkaline phosphatase labeled goat anti-mouse antibodies were used. The HPTLC immunostaining procedure (overlay assay) was performed as reported previously [2].

Mass spectrometry
The CD15-positive GSLs were further structurally characterized directly on the HPTLC plate by use of infrared matrix-assisted laser desorption/ionization orthogonal time-of-flight mass spectrometry (IR-MALDI-o-TOF-MS), Sciex (Concord, Canada), in the positive ion mode using glycerol as the matrix [3] .

Figure 1: *The AMD 2 from CAMAG (Muttenz, Switzerland) is equipped with an EMF adjustable gas ballast oil drain (Edwards, Crawley, UK) and controlled with the software winCATS.*

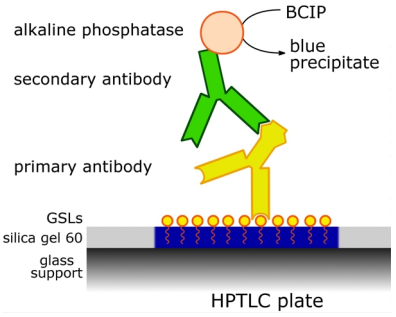

Figure 2: *Scheme of the overlay technique with BCIP staining.*

RESULTS

Identification and structural characterization of CD15-GSLs in a mixture of neutral GSLs from human granulocytes
Figure 3A shows the orcinol stain and Figure 3B the corresponding overlay assay with the monoclonal anti-CD15 antibody of conventional HPTLC- (lanes a) and of AMD-separated (lanes b) neutral GSLs. The immuno-positive CD15 bands were further analyzed by use of IR-MALDI-o-TOF MS (cf. Figure 4).

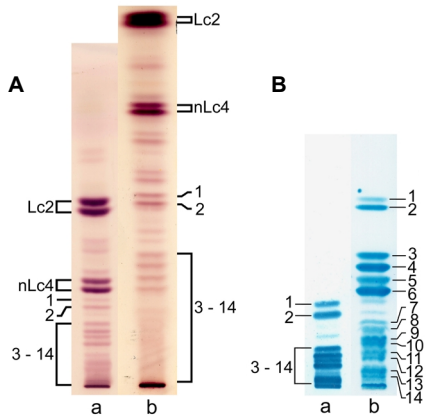

Figure 3: *(A) Orcinol stain of neutral GSLs from human granulocytes separated by conventional HPTLC (lane a) and three consecutive runs performed with the AMD 2 (lane b.) (B) The corresponding overlay assay with anti-CD15 antibody by conventional HPTLC (lane a) and three consecutive runs performed with the AMD 2 system (lane b). 40 µg of the neutral GSL mixture were applied to each lane.*

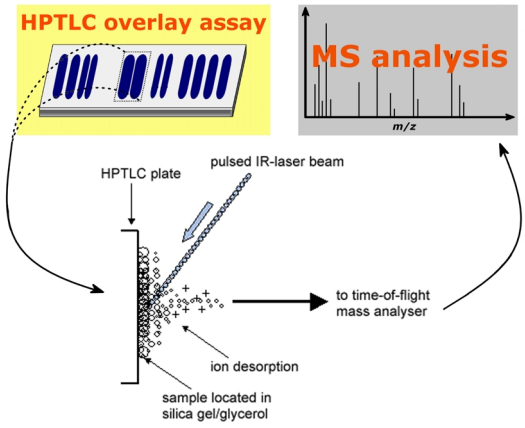

Figure 4: *Scheme of the mass spectrometric analysis directly on the HPTLC plate.*

Mass spectrometric identification of HPTLC-separated neutral GSLs
All molecular species were detected as singly charged monosodiated ions $[M+Na]^+$. Figures 5A and 5B show the mass spectra obtained from band 4 and band 12 as examples. In Table 1 *m/z*-values of the ionic analytes representing CD15 species detected in the corresponding bands are listed. Various CD15-GSLs could be detected and characterized. In lane 1 a CD15 species with nLc6 core structure and C24-fatty acids in the ceramide part could be detected. Band 2 represents the "classical lower band" of band 1 and consists of CD15-nLc6Cer containing a C16-fatty acid. Correspondingly, the major species in band 3 and 4 are CD15 GSLs with nLc6 core and an additional fucose that differ in the fatty acid chain length of their ceramide moiety. The main compounds of bands 5, 6, and 7 have a nLc8 core and varying fatty acids. The corresponding compounds containing an additional fucose residue are found in bands 8 - 10. CD15 species with various fatty acids comprising a nLc10 core ± fucose are detected in bands 11 - 13. The most complex compounds can be found in band 14 as CD15 GSLs containing a nLc12 core and 1 to 3 additional fucose residues. Due to loss of fucoses and/or fragmentation of the molecules during the ionization process additional signals can be observed in a spectrum (cf. Figures 5A and 5B).

Table 1: *CD15-GSL species detected from immuno-positive bands directly on the HPTLC plate by use of IR-MALDI-o-TOF MS.*

Lane	m/z	Proposed structure
1	1871,01	CD15 (nLc6; C24:1)
	1873,02	CD15 (nLc6; C24:0)
2	1760,89	CD15 (nLc6; C16:0)
	1760,90	CD15 (nLc6; C16:0)
3	1991,04	CD15 + Fuc (nLc6; C22:0)
	2017,06	CD15 + Fuc (nLc6; C24:1)
	2019,06	CD15 + Fuc (nLc6; C24:0)
4	1760,90	CD15 (nLc6; C16:0)
	1906,96	CD15 + Fuc (nLc6; C16:0)
	1963,98	CD15 + Fuc (nLc6; C20:0)
5	2125,98	CD15 (nLc8; C16:0)
	2236,14	CD15 (nLc8; C24:1)
	2238,13	CD15 (nLc8; C24:0)
6	2126,02	CD15 (nLc8; C16:0)
	2154,07	CD15 (nLc8; C18:0)
	2182,10	CD15 (nLc8; C20:0)
	2210,13	CD15 (nLc8; C22:0)
7	2126,04	CD15 (nLc8; C16:0)
8	2126,04	CD15 (nLc8; C16:0)
	2272,04	CD15 + Fuc (nLc8; C16:0)
	2382,20	CD15 + Fuc (nLc8; C24:1)
	2384,20	CD15 + Fuc (nLc8; C24:0)
9	2126,01	CD15 (nLc8; C16:0)
	2272,07	CD15 + Fuc (nLc8; C16:0)
	2300,09	CD15 + Fuc (nLc8; C18:0)
	2328,14	CD15 + Fuc (nLc8; C20:0)
	2356,15	CD15 + Fuc (nLc8; C22:0)
	2384,19	CD15 + Fuc (nLc8; C24:0)
10	2126,02	CD15 (nLc8, C16:0)
	2272,07	CD15 + Fuc (nLc8; C16:0)
11	2418,16	CD15 + 2 Fuc (nLc8; C16:0)
	2491,16	CD15 (nLc10; C16:0)
	2575,26	CD15 (nLc10; C22:0)
12	2019,65	CD15 + Fuc (nLc6; C24:0)
	2491,15	CD15 (nLc10; C16:0)
	2637,20	CD15 + Fuc (nLc10; C16:0)
	2721,31	CD15 + Fuc (nLc10; C22:0)
	2747,32	CD15 + Fuc (nLc10; C24:1)
	2749,32	CD15 + Fuc (nLc10; C24:0)
13	2637,21	CD15 + Fuc (nLc10; C16:0)
14	3002,35	CD15 + Fuc (nLc12; C16:0)
	3148,40	CD15 + 2 Fuc (nLc12; C16:0)
	3294,47	CD15 + 3 Fuc (nLc12; C16:0)

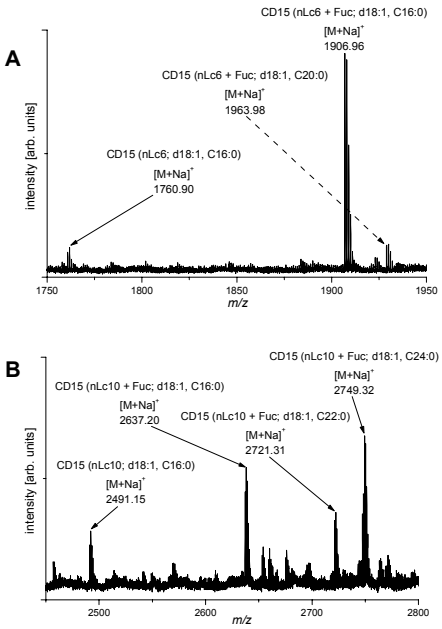

Figure 5: *Examples for the mass spectra of CD15 positive bands measured directly on the HPTLC plate (cf. Figure 3B, lane b) by use of IR-MALDI-o-TOF MS. Mass spectra of band 4 (A) and band 12 (B) are shown.*

CONCLUSIONS

The combined AMD-HPTLC-MALDI-MS method offers a wide range of applications such as the analysis of long chain GSL and tumor-associated GSLs which are currently discussed as targets for clinical applications.

ACKNOWLEDGEMENTS

Supported in parts by the Deutsche Krebshilfe (DKH 106742) and the Deutsche Forschungsgemeinschaft (DR416/5-1).

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