HPTLC immunodetection coupled with nanoESI-QTOF mass spectrometry for full structural characterization of influenza A virus binding isomeric neolacto-series gangliosides from human granulocytes

Jamal Souady[1], Ute Distler[1], Gottfried Pohlentz[1], Iris Meisen[2], Jasna Peter-Katalinić[1], and Johannes Müthing[1]

[1] Institute for Medical Physics and Biophysics, University of Münster, Robert-Koch-Straße 31, D-48149 Münster, Germany
[2] Institute of Occupational Medicine, University of Münster, Robert-Koch-Straße 51, D-48149 Münster, Germany

INTRODUCTION

Gangliosides are a diverse group of glycosphingolipids (GSLs) composed of long-chain aminodiol and fatty acid, which together make up the ceramide portion (*N*-acylsphingosine), and a carbohydrate moiety containing one or more sialic acid residues [1]. Neolacto-series monosialogangliosides with terminal Neu5Acα2-3Galβ1-4GlcNAc- or Neu5Acα2-6Galβ1-4GlcNAc-sequence, known as typical plasma membrane constituents of human granulocytes, are important receptors for influenza A viruses mediating virus attachment [2].

Due to its high resolving power, high-performance thin-layer chromatography (HPTLC) has become the standard tool for separation of ganglioside mixtures [3]. The HPTLC overlay technique (immunodetection) is an easy, rapid, and sensitive method to investigate specific binding of ligands to separated GSLs. Here we report on a combined strategy of preparative HPTLC immunostaining and mass spectrometry for the differentiation of isomeric α2-3- and α2-6-sialylated neolacto-series gangliosides from human granulocytes.

MATERIALS AND METHODS

Gangliosides

The ganglioside mixture was isolated from human granulocytes and purified as described elsewhere [4].

High-performance thin-layer chromatography (HPTLC)

Gangliosides were applied on silica gel precoated HPTLC-plates (10 x 10 cm, thickness 0.2 mm, Merck, Darmstadt, Germany) employing a Linomat IV applicator (CAMAG, Muttenz, Switzerland) and separated for 20 min using a mixture of chloroform/methanol/water (120:85:20) (v/v) containing 0.2 mg/ml $CaCl_2$. GSL detection was performed by orcinol staining and the more specific and non-destructive overlay technique (immunostaining).

Overlay assay

After separation of GSLs by HPTLC the silica gel was fixed with polyisobutylmethacrylate (plexigum). After incubation overnight in phosphate buffered saline (PBS) the plates were blocked with 1% bovine serum albumin (BSA) in PBS. The primary antibody (pAB) was allowed to bind, followed by three washing steps with Tween 21 in PBS and the binding of a secondary antibody (sAB). The sAB is a conjugate of an antibody directed against the pAB´s constant region and the enzyme alkaline phosphatase that catalyzes the dephosphorylation of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to yield a blue precipitate under alkaline conditions (Figure 1).

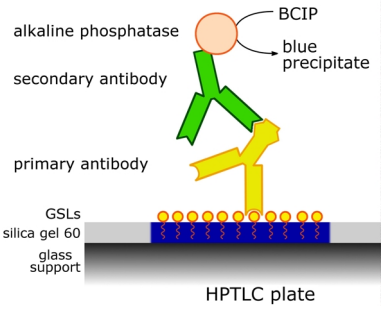

Figure 1: *Scheme of the overlay technique with BCIP staining.*

Extraction of GSLs from silica gel

Preceding the GSL-extraction the plexigum was removed by dipping the plates into chloroform. Then the silica gel of immunostained bands was scraped off and GSLs were extracted by mixing and ultrasonic treatment in extraction solvent chloroform/methanol/water (30:60:8) (v/v) [5]. After centrifugation the supernatant containing the GSLs was transfered to a new vial. The procedure was repeated twice. All supernatants were pooled and dried. After dissolving in methanol the extracts were suitable for MS analysis (Figure 2).

Mass spectrometry

Nanoelectrospray ionization quadrupole time-of-flight mass spectrometry (nanoESI-QTOF MS) was performed by use of a micromass Q-ToF mass spectrometer (Micromass, Manchester, UK). All MS experiments were carried out in negative ion mode. For structural analysis by low-energy collision induced dissociation (CID) singly charged precursor ions were selected and fragmented using argon as the collision gas.

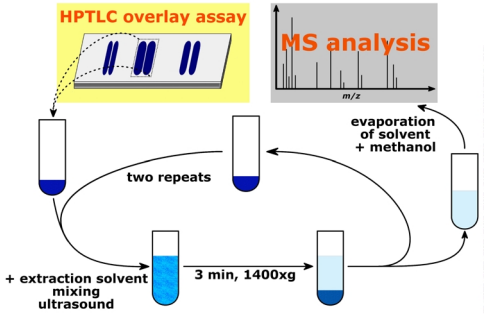

Figure 2: *Workflow of GSL-extraction from silica gel after HPTLC immunostaining and preparation for MS analysis.*

RESULTS

Separation and staining of gangliosides

The ganglioside mixture of human granulocytes contains five major components, GM3 as well as α2-3- and α2-6-sialylated gangliosides with nLc4- and nLc6-core structures, respectively. These are well separated by HPTLC on silica gel plates as shown by orcinol stain (Figure 3, lane a). The separation is primarily based upon the number of sugar units. Due to the substitution of the sphingosine moiety with different fatty acids, mainly C16 and C24, gangliosides containing the same sugar moiety occur as double bands. Furthermore, double bands of two isomeric structures only differing in type of sialylation are completely separated by HPTLC, i.e., IV^3nLc4Cer, Neu5Ac**α2-3**Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer, and IV^6nLc4Cer, Neu5Ac**α2-6**Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer as shown in Figure 3, lane a.

Corresponding overlay assays of the ganglioside mixture were stained separately using anti-Neu5Acα2-3Galβ1-4GlcNAc-R and anti-Neu5Acα2-6Galβ1-4GlcNAc-R antibodies (cf. Figure 3, lane b and c, respectively). Gangliosides with nLc4- and nLc6-core structure exhibiting Neu5Ac in either α2-3- or α2-6-linkage were distinguished unambiguously. For detailed characterization and verification of the structures immunostained bands were scraped off and GSLs extracted from the silica gel prior to nanoESI-QTOF MS analysis.

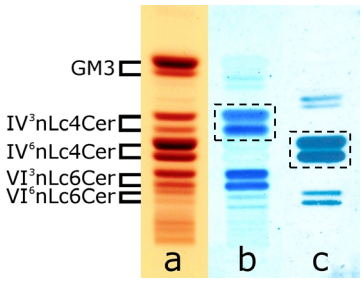

Figure 3: *HPTLC orcinol stain of human granulocyte gangliosides (lane a) and the corresponding overlay assays with primary anti-IV^3nLc4Cer (lane b) and anti-IV^6nLc4Cer-antibody (lane c). Applied gangliosides correspond to 10 µg for orcinol staining (lane a) and 2 µg for immunodetections (lane b and c).*

Mass spectrometric characterization of isomeric gangliosides

In the negative ion mode all molecular species were detected as deprotonated singly charged ions (Figure 4 A and Figure 5 A). In both samples the structures of the major components were assigned to monosialylated nLc4Cer. In MS/MS-experiments of the precursor ions with *m/z* 1626.99 and 1627.04, full sets of Y- and C-ions and some B- and Z-ions were detected confirming the proposed structures (Figure 4 B and Figure 5 B). Moreover, $^{0,2}X_4$-ions of *m/z* 1405.95 and $^{0,4}A_2$- and $^{2,4}A_3$-ions eliminating CO_2 of *m/z* 306.15 and 468.16, respectively (Figure 5 C), were detected. It has been shown recently that these ions are diagnostic for α2-6-linked sialic acid [6].

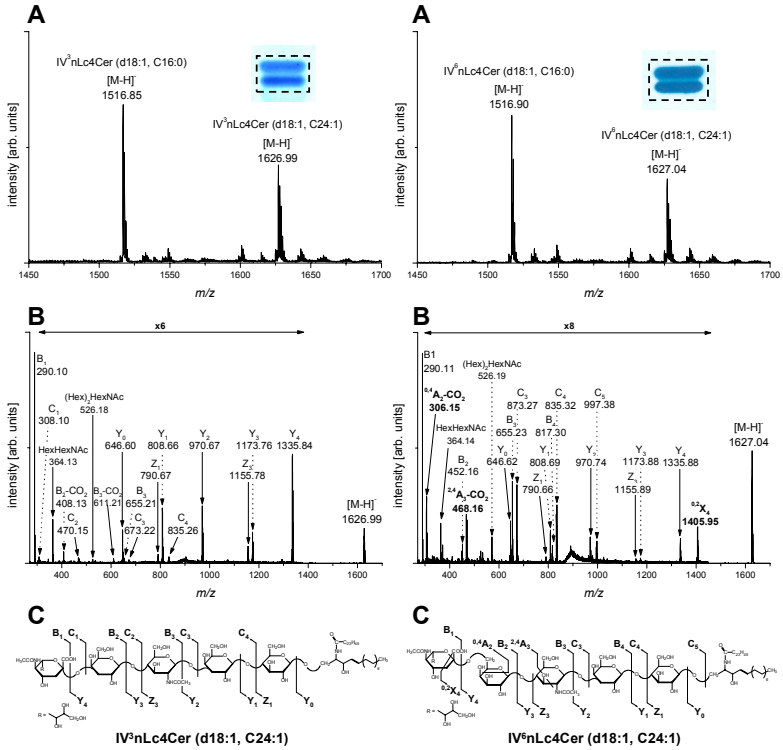

Figure 4: *NanoESI-QTOF mass spectra of a GSL-extract obtained from anti-IV^3nLc4Cer overlay assays (cf. Figure 3, lane b). MS^1 spectrum of GSL extract (A). MS/MS spectrum of IV^3nLc4Cer (d18:1, C24:1) with m/z 1626.99 (B). The analyzed structure and detected fragment ions are depicted in a fragmentation scheme (C).*

Figure 5: *NanoESI-QTOF mass spectra of a GSL-extract obtained from anti-IV^6nLc4Cer overlay assays (cf. Figure 3, lane c). MS^1 spectrum of GSL extract (A). MS/MS spectrum of IV^6nLc4Cer (d18:1, C24:1) with m/z 1627.04 (B). The analyzed structure and detected fragment ions are depicted in a fragmentation scheme (C).*

CONCLUSION

This HPTLC-nanoESI-QTOF-MS joint technology offers a wide range of clinical applications, i.e., in determing the role of influenza viruses in immunologically mediated reactions during infection and recovery.

REFERENCES

[1] Müthing,J. in Glycoscience: Chemistry and Chemical Biology, Vol. 3: Glycolipids, Fraser-Reid,B., Tatsuta,K., Thiem,J., Eds.; Springer, Heidelberg, Germany, 2001; pp. 2220-2249.
[2] Müthing,J., Unland,F., Heitmann,D., Orlich,M., Hanisch,F.-G., Peter-Katalinić,J., Knäuper,V., Tschesche,H., Kelm,S., Schauer,R., and Lehmann,J. (1993) Glycoconjugate J. 10, 120-126.
[3] Müthing,J. in Methods in Molecular Biology, Vol. 76: Glycoanalysis Protocols, Hounsell,E.F., Ed.; Humana Press Inc., Totowa, NJ, 1998; pp. 183-195.
[4] Müthing, J. (2000) Methods Enzymol. 312, 45-64.
[5] Meisen,I., Peter-Katalinić,J., and Müthing,J. (2004) Anal. Chem. 76, 2248-2255.
[6] Meisen,I., Peter-Katalinić,J., and Müthing,J. (2003) Anal. Chem. 75, 5719-5725.