Preferential binding of the anticancer drug rViscumin (recombinant mistletoe lectin) to terminally α2-6-sialylated neolacto-series gangliosides

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Production of biochemically defined recombinant mistletoe lectin was achieved by cloning and separate expression of the single catalytically active A-chain and the B-chain with carbohydrate binding properties in Escherichia coli, yielding an active heterodimeric protein named rViscumin (Eck et al. [1999] Eur. J. Biochem., 265, 788–797). Employing solid phase binding assays, rViscumin was shown to preferentially bind to terminally α2-6-sialylated neolacto-series gangliosides IV₆Neu5Ac-nLc₄Cer, VI₆Neu5Ac-nLc₄Cer, and VIII₆Neu5Ac-nLc₈Cer isolated from human granulocytes. Only marginal binding of rViscumin to galactose-terminated neutral GSLs was determined, whereas reinvestigation of ricin specificity demonstrated this lectin as a galactose-binding protein. Human promyelocytic HL-60 cells exhibited an IC₅₀ value (half maximum cytotoxicity) of 1.16 pM and human bladder carcinoma 5637 cells of 12.1 pM rViscumin; CHO-K1 cells were resistant to rViscumin treatment up to a concentration of 5.26 nM tested. Quantification of the predominant receptor ganglioside IV₆Neu5Ac-nLc₄Cer by means of a specific anti-Neu5Acα2-6Galβ1-4GlcNAc-R antibody revealed 3.68 × 10⁶ and 1.54 × 10⁶ receptor molecules per HL-60 and 5637 cell, respectively; CHO-K1 cells were negative, lacking α2-6-sialylated gangliosides. The data imply a direct correlation of rViscumin cytotoxicity and the expression of receptor gangliosides. Moreover, CHO-K1 cells were rendered susceptible toward rViscumin cytotoxicity after exogenous application of human granulocyte gangliosides. Thus, (1) rViscumin has to be considered as a sialic acid–specific rather than a galactose-specific type II ribosome-inactivating protein, and (2) neolacto-series gangliosides with Neu5Acα2-6Galβ1-4GlcNAc-terminus are true functional and physiologically relevant rViscumin receptors.

Key words: gangliosides/glycosphingolipids/recombinant viscumin/ricin/TLC immunostaining

Introduction

Proprietary mistletoe extracts are widely used as therapeutic immunomodulatory agents, mainly for oncological application in adjuvant therapy but also for general immunostimulation (Bocci, 1993; Hajto et al., 1990; Beuth, 1997). Mistletoe lectin (ML) is a ribosome-inactivating protein of type II like ricin, abrin, and others (Barbieri et al., 1993). Although these toxic proteins are of different phylogenetic origin, they share similar activities and structural properties (Eschenburg et al., 1998), being potent inhibitors of eukaryotic protein synthesis at the ribosomal level (Glück et al., 1992).

Three MLs with different carbohydrate specificities (ML-I, ML-II, and ML-III) have been isolated from mistletoe leaves. ML-I (also known as viscumin) was identified as the main therapeutic principle (Hajto et al., 1989; Beuth et al., 1993). Unlike ricin, ML-I-containing extracts are medically applied for the treatment of human cancer. Viscumin is categorized as heterodimeric two-chain (type II) ribosome-inactivating protein, composed of a catalytically active A chain with RNA N-glycosidase activity and a B chain with carbohydrate binding specificities (Olsnes et al., 1982; Franz, 1986) to yet undefined receptors on the surface of target cells. Recently, biotechnological production of recombinant ML (rViscumin) was achieved after cloning and separate expression of the single chains in Escherichia coli (Eck et al., 1999a,b), yielding the active rML-heterodimer (Eck et al., 1999b). The enzymatic A-chain activity and the carbohydrate binding activity of the B-chain are both essential for the cytotoxic apoptosis-inducing effect of the holoprotein as an anticancer drug (Langer et al., 1999).

In numerous studies ML has been reported to belong to the group of galactoside-specific/lactose-binding lectins, which bind and cross-link certain multitannenary oligosaccharides, glycopeptides, and glycoproteins (Hajto et al., 1989; Gabius et al., 1992; Barbieri et al., 1993; Gupta et al., 1996). Due to the lack of data concerning the potency of glycosphingolipids (GSLs) as lectin receptors, we investigated the binding specificity of rViscumin in comparison to ricin toward neutral GSLs and gangliosides.

GSLs are amphipathic molecules consisting of a hydrophilic oligosaccharide chain and a hydrophobic component named ceramide (Stults et al., 1989). Gangliosides are characterized by the presence of one or more sialic acids in the oligosaccharide moiety, which are known to play crucial roles in various biological functions (Varki, 1992; Schauer et al., 1995).
Gangliosides are involved in cell–cell recognition (Hakomori et al., 1998; Hakomori, 2002) and adhesion by counterpart lectins (Schnaar, 1991; Feizi, 2001) and are well-characterized cellular attachment sites for viruses, bacteria, and exotoxins (Suzuki, 1994; Karlsson, 1989). Employing naturally occurring GSLs of mammalian origin, we were able to show herein the preferential binding of rViscumin to α2-6-sialylated neolacto-series gangliosides and only marginal binding to galactose-terminated neutral GSLs. Consequently, rViscumin has to be considered as a sialic acid–specific rather than as a galactose-specific type II ribosome-inactivating protein, whereas reinvestigation of ricin specificity confirmed this lectin as galactose-specific.

Results

The first approach to obtain preliminary structural information regarding GSL binding specificities of rViscumin was to perform a screening by use of microwells coated with GSL mixtures of well-defined structures. Human erythrocytes are known to express globo-series neutral GSLs Gb4Cer and Gb3Cer, the former being the dominant one. Neutral GSLs of human granulocytes preferentially contain LacCer and the neolacto-series GSLs nLc4Cer, nLc6Cer, and to a lesser extent fucosylated derivatives of, for example, nLc6Cer, the so-called Lewis^x^-GSLs. By searching for certain ganglio-series neutral GSLs as potential receptors, a GSL mixture of MDAY-D2 cells was chosen due to its high content of Gg3Cer and Gg4Cer. Human brain gangliosides are suitable to investigate binding specificities of ganglio-series gangliosides, and human granulocyte gangliosides (HGGs) are the ideal candidates exploring the binding potency of terminally α2-3- and α2-6-sialylated neolacto-series monosialogangliosides.

Binding of rViscumin toward microwell adsorbed neutral GSLs and gangliosides

The microwell adsorption assays of rViscumin with three reference neutral GSL preparations (A, B, and C) and two ganglio-series mixtures of well-known structures (D and E) are shown in Figure 1. The only positive reaction within the neutral GSL fractions could be detected for neutral GSLs from human granulocytes, consisting of LacCer and nLc4Cer as the main compounds (Figure 1B). Of the two ganglioside fractions, human brain gangliosides were negative, but HGG revealed strong binding interaction with rViscumin (Figure 1E).

TLC overlay assay of rViscumin with granulocyte neutral GSLs and gangliosides

To identify the individual GSLs responsible for rViscumin binding, thin-layer chromatography (TLC) overlay assays with the faint positive neutral GSLs from human granulocytes and the strongly positive HGG were performed. The TLC-binding assay of rViscumin with neutral GSLs of human granulocytes revealed weak reaction with LacCer (Figure 2A, lane b), which represents the dominant GSL in this mixture. While LacCer (Galβ1-4GlcNAc1-1Cer) was found to be at least a low-affinity binding ligand for rViscumin (see Table I), the N-acetyl group of the penultimate GlcNAc completely abolished rViscumin binding toward nLc4Cer and nLc6Cer (both carrying the Galβ1-4GlcNAc-disaccharide at the nonreducing terminus).

Fig. 1. Microwell adsorption assay of rViscumin with neutral GSLs and gangliosides. Amounts applied correspond to bars from left to right for each GSL fraction. (A) Neutral GSLs from human erythrocytes: 0, 1.25, 2.5, 5, and 10 μg; (B) neutral GSLs from human granulocytes: 0, 1.88, 3.75, 7.5, and 15 μg; (C) neutral GSLs from MDAY-D2 cells: 0, 2.5, 5, 10, and 20 μg; (D) human brain gangliosides: 0, 1.25, 2.5, 5, and 10 μg; (E) HGGs: 0, 1.25, 2.5, 5, and 10 μg. The orcinol-stained thin-layer chromatogram of neutral GSLs (A, B, and C) is shown in Figure 8; the resorcinol-stained TLC runs of both human brain gangliosides (D) and HGG (E), are displayed in Figures 3A and 6A. For structures see Tables I and II.

Fig. 2. TLC overlay binding assay of rViscumin with neutral GSLs (A) and gangliosides (B) from human granulocytes. (A) Lane a: orcinol-stained chromatogram of 15 μg neutral GSLs; lane b: corresponding rViscumin overlay assay. (B) Lane a: resorcinol-stained chromatogram of 15 μg HGGs; lane b: corresponding rViscumin overlay assay.
Ganglioside binding specificity of recombinant Viscumin

Fucosylated neolacto-series GSLs (Lewis*-GSLs) were negative, too. It should be mentioned that this faint binding of LacCer could only be obtained by "overstaining" the TLC plate (20 h overnight dye incubation), indicated by the intensive background staining (Figure 2A, lane b).

On the other hand, a fast and clear reaction could be achieved with HGG, uncovering two strongly stained double bands (Figure 2B, lane b). Compared with the resorcinol stain of the respective lipids, the terminally \( \alpha_2-6 \)-sialylated neolacto-series gangliosides \( \text{IV}^{n} \text{Lc4Cer} \) and \( \text{VI}^{n} \text{Lc6Cer} \) were suggested to represent the preferential receptors of rViscumin.

TLC overlay assays of anti-\( \text{IV}^{n} \text{Lc4Cer} \) antibody and rViscumin with HPLC-separated neolacto-series gangliosides

To confirm this hypothesis, rViscumin binding assays were performed with high-performance liquid chromatography (HPLC)-separated HGG-fractions. Ganglioside fractions (Figure 3A) comprising \( \text{IV}^{n} \text{Lc4Cer} \) (HGG1, lane c), \( \text{VI}^{n} \text{Lc6Cer} \) (HGG2, lane d), \( \text{IV}^{n} \text{Lc4Cer} \) (HGG3, lane e), and \( \text{IV}^{n} \text{Lc4Cer} \) plus \( \text{VI}^{n} \text{Lc6Cer} \) and \( \text{VIII}^{n} \text{Lc8Cer} \) (HGG4, lane f) were chromatographed and overlayed with an anti-Neu5Ac\( \alpha_2-6 \)Gal\( \beta_1-4 \)GlcNAc-R antibody, which has been raised by immunizing a chicken with \( \text{IV}^{n} \text{Lc4Cer} \). As shown in Figure 3B, the antibody specifically bound to \( \text{IV}^{n} \text{Lc4Cer} \), \( \text{VI}^{n} \text{Lc4Cer} \), and \( \text{VIII}^{n} \text{Lc8Cer} \) of the HPLC fractions as well as to these gangliosides in the total HGG fraction. The isomeric structures \( \text{IV}^{n} \text{Lc4Cer} \) and \( \text{VI}^{n} \text{Lc6Cer} \) were not detected by this antibody. A further indication for the antibody specificity is shown in lane a of Figure 3B, where no cross-reaction could be observed with any ganglio-series gangliosides. Exactly identical binding patterns compared to the anti-Neu5Ac\( \alpha_2-6 \)Gal\( \beta_1-4 \)GlcNAc-R antibody were obtained in the TLC overlay binding assay with rViscumin (Figure 3C), exhibiting its high specificity toward monosialogangliosides with Neu5Ac\( \alpha_2-6 \)Gal\( \beta_1-4 \)GlcNAc-termini (see Table II). Due to assaying ganglioside fractions isolated by gradient anion-exchange HPLC, any cross-reactivities to, for example, "underlying" disialogangliosides or other lipid contaminants, which are absent in the HPLC eluates but might occur in the total HGG fraction, could be excluded. Thus, the most important and unexpected result of these binding assays is the fact that rViscumin is not a galactose-specific but a sialic acid–specific lectin with strict preference of the Neu5Ac\( \alpha_2-6 \)Gal\( \beta_1-4 \)GlcNAc configuration.

NanoESI-QTOF MS/MS of \( \alpha_2-6 \)-sialylated gangliosides

The molecular ions of terminally \( \alpha_2-6 \)-sialylated neolacto-series gangliosides obtained from the mass spectrometry (MS) spectrum of fraction HGG4 are listed in Table III. The most

### Table I. Binding of rViscumin and ricin toward neutral GSLs

<table>
<thead>
<tr>
<th>Structure</th>
<th>Abbreviation</th>
<th>rViscumin*</th>
<th>Ricin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galβ1-4Glcβ1-1Cer</td>
<td>Lc2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Galα1-4Galβ1-4Glcβ1-1Cer</td>
<td>Gb3</td>
<td>–</td>
<td>(+)</td>
</tr>
<tr>
<td>GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer</td>
<td>Gb4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GalNAcβ1-4Galβ1-4Glcβ1-1Cer</td>
<td>Gg3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Galβ1-4GalNAcβ1-3Galβ1-4Glcβ1-1Cer</td>
<td>Gg4</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer</td>
<td>nLc4</td>
<td>–</td>
<td>++++</td>
</tr>
<tr>
<td>Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer</td>
<td>nLc6</td>
<td>–</td>
<td>++++</td>
</tr>
<tr>
<td>Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer</td>
<td>Lewis*</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

For TLC immunostain of rViscumin see Figure 2A and of ricin see Figure 8. *Appearance on immunostained TLCs graded from – (negative), (+) trace positivity, + weak binding up to +++ highest intensity.

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**Fig. 3.** Resorcinol stain (A), anti-\( \text{IV}^{n} \text{Lc4Cer} \) antibody TLC overlay assay (B), and rViscumin TLC overlay assay (C) with HPLC-purified \( \alpha_2-3 \)- and \( \alpha_2-6 \)-sialylated neolacto-series monosialogangliosides. Lanes a: 15 µg human brain gangliosides; lanes b: 15 µg human HGGs; lanes c: 4 µg \( \text{IV}^{n} \text{Lc4Cer} \) (HGG1); lanes d: 4 µg \( \text{VI}^{n} \text{Lc6Cer} \) (HGG2); lanes e: 4 µg \( \text{IV}^{n} \text{Lc4Cer} \) plus \( \text{VI}^{n} \text{Lc6Cer} \) and \( \text{VIII}^{n} \text{Lc8Cer} \) (HGG4).
prominent ions (m/z 1516.83 and m/z 1626.93) belong to the monosialylated neolacto-series gangliosides of fraction HGG4 investigated in negative ion mode.

For TLC immunostain of rViscumin see Figures 2B and 3C and of ricin see Figure 9.

A series of high abundance Y-type ions is present in the tandem mass spectrometry (MS/MS) spectrum of the molecular ion (m/z 1881.95 and m/z 2247.10, see Table III) were selected for low-energy collision-induced dissociation experiments. Their masses, together with the TLC immunostaining data were consistent with their assignment as being the gangliosides VP<sub>n</sub>Neu5Ac-nLc6Cer- and VPII<sub>n</sub>Neu5Ac-nLc8Cer-species, respectively, containing C<sub>16:0</sub> fatty acid in the ceramide portion.

Table II. Binding of rViscumin and ricin toward gangliosides

<table>
<thead>
<tr>
<th>Structure</th>
<th>Abbreviation</th>
<th>rViscumin</th>
<th>Ricin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5Acα2-3Galβ1-4Glcβ1-1Cer</td>
<td>GM3</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer</td>
<td>GM1</td>
<td>–</td>
<td>(+)</td>
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<tr>
<td>Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer</td>
<td>GD1a</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer</td>
<td>GD1b</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glcβ1-1Cer</td>
<td>GT1b</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer</td>
<td>IV&lt;sub&gt;n&lt;/sub&gt;LnC4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer</td>
<td>IV&lt;sub&gt;n&lt;/sub&gt;LnC4</td>
<td>++++</td>
<td>–</td>
</tr>
<tr>
<td>Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer</td>
<td>VI&lt;sub&gt;n&lt;/sub&gt;LnC6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer</td>
<td>VI&lt;sub&gt;n&lt;/sub&gt;LnC6</td>
<td>++++</td>
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Table III. Molecular ions of terminally α2-6-sialylated neolacto-series gangliosides of fraction HGG4 investigated in negative ion mode

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Structure</th>
<th>[M-H]&lt;sup&gt;–&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV&lt;sub&gt;n&lt;/sub&gt;LnC4</td>
<td>IV&lt;sup&gt;n&lt;/sup&gt;Neu5Ac-nLc4Cer (d18:1/16:0)</td>
<td>1516.83</td>
</tr>
<tr>
<td>IV&lt;sub&gt;n&lt;/sub&gt;LnC4</td>
<td>IV&lt;sup&gt;n&lt;/sup&gt;Neu5Ac-nLc4Cer (d18:1/24:1)</td>
<td>1626.93</td>
</tr>
<tr>
<td>VP&lt;sub&gt;n&lt;/sub&gt;LnC6</td>
<td>VP&lt;sup&gt;n&lt;/sup&gt;Neu5Ac-nLc6Cer (d18:1/16:0)</td>
<td>1881.95</td>
</tr>
<tr>
<td>VP&lt;sub&gt;n&lt;/sub&gt;LnC6</td>
<td>VP&lt;sup&gt;n&lt;/sup&gt;Neu5Ac-nLc6Cer (d18:1/24:1)</td>
<td>1992.06</td>
</tr>
<tr>
<td>VII&lt;sub&gt;n&lt;/sub&gt;LnC8</td>
<td>VIII&lt;sup&gt;n&lt;/sup&gt;Neu5Ac-nLc8Cer (d18:1/16:0)</td>
<td>2247.10</td>
</tr>
<tr>
<td>VII&lt;sub&gt;n&lt;/sub&gt;LnC8</td>
<td>VIII&lt;sup&gt;n&lt;/sup&gt;Neu5Ac-nLc8Cer (d18:1/24:1)</td>
<td>2357.10</td>
</tr>
</tbody>
</table>

A series of high abundance Y-type ions is present in the tandem mass spectrometry (MS/MS) spectrum of the molecular ion (m/z 1881.95 and m/z 2247.10) of the heptasaccharide ceramide (Figure 4A), starting with loss of sialic acid (Y<sub>6</sub> at m/z 1590.83) and followed by consecutive glycosidic cleavage of the other monosaccharide residues (Figure 4B). The mass differences of 162 Da between Y<sub>6</sub> (m/z 1590.83) and Y<sub>5</sub> (m/z 1428.80), and 200 Da between Y<sub>5</sub> (m/z 1428.80) and Y<sub>4</sub> (m/z 1225.69), are typical of the loss of either a hexose (e.g., galactose) or N-acetylhexosamine (e.g., N-acetylgalactosamine), respectively. The sequence is completed by the ceramide ion Y<sub>3</sub> at m/z 536.50. Confirmatory evidence for the nLc6 core–containing terminal sialic acid is provided by the full series of B-type ions from the nonreducing end of the sugar chains. The B<sub>2</sub> ion (m/z 452.14) is assigned to the disaccharide sequence Neu5Ac-Hex. In this way the information obtained from Y-type ion series is supported by the B series ions up to the B<sub>2</sub> ion (m/z 1344.45) that represents the complete oligosaccharide core without ceramide. Additionally, a number of internal double cleavages give evidence for the sequence of oligosaccharide units in the chain like the Y<sub>6</sub>/B<sub>3</sub> ion at m/z 364.12 (HexNAc-Hex), the Y<sub>5</sub>/B<sub>2</sub> ion at m/z 729.25 (Hex<sub>3</sub>HexNAc<sub>2</sub>) or the low abundant Y<sub>5</sub>/B<sub>0</sub> ion at m/z 891.32 (Hex<sub>3</sub>HexNAc<sub>2</sub>).

The fragmentation pattern in the spectrum of the molecular ion (m/z 2247.10) of the nonasaccharide ceramide is very similar to that of the heptasaccharide. A prominent Y-type ion series and a supporting but weaker B-type ion series provide corroborative evidence of an nLc8 core structure carrying a sialic acid moiety on its nonreducing terminus.

Biological activity of rViscumin

To investigate the cytotoxic ability of rViscumin on the cellular level, three cell lines with different biological background were employed to determine their cell-specific rViscumin sensitivity. Cultures of the human promyelocytic HL-60 cell line, consisting mainly of neutrophilic promyelocytes, exhibited the lowest IC<sub>50</sub> value (highest sensitivity) responding to 1.16 pM rViscumin. Confirmed evidence for the nLc6 core–containing terminal sialic acid is provided by the full series of B-type ions from the nonreducing end of the sugar chains. The B<sub>2</sub> ion (m/z 452.14) is assigned to the disaccharide sequence Neu5Ac-Hex. In this way the information obtained from Y-type ion series is supported by the B series ions up to the B<sub>2</sub> ion (m/z 1344.45) that represents the complete oligosaccharide core without ceramide. Additionally, a number of internal double cleavages give evidence for the sequence of oligosaccharide units in the chain like the Y<sub>6</sub>/B<sub>3</sub> ion at m/z 364.12 (HexNAc-Hex), the Y<sub>5</sub>/B<sub>2</sub> ion at m/z 729.25 (Hex<sub>3</sub>HexNAc<sub>2</sub>) or the low abundant Y<sub>5</sub>/B<sub>0</sub> ion at m/z 891.32 (Hex<sub>3</sub>HexNAc<sub>2</sub>).

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Biological activity of rViscumin

To investigate the cytotoxic ability of rViscumin on the cellular level, three cell lines with different biological background were employed to determine their cell-specific rViscumin sensitivity. Cultures of the human promyelocytic HL-60 cell line, consisting mainly of neutrophilic promyelocytes, exhibited the lowest IC<sub>50</sub> value (highest sensitivity) responding to 1.16 pM rViscumin with half maximum cytotoxicity (Figure 5). The human 5637 cell line, representing small malignant cells with epithelial-like morphology and derived from bladder carcinoma, was one order of magnitude less susceptible corresponding to an IC<sub>50</sub> value of 12.1 pM rViscumin. Chinese hamster ovary (CHO-K1) cells, epithelial cells derived from a hamster ovary, were insensitive toward the cytotoxic action of rViscumin up to the highest concentration of 5.26 nM tested in this series of experiments. At concentrations of 0.175 nM rViscumin, both HL-60 and 5637 cells completely lost viability, whereas CHO-K1

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cells did not show any reduction in cell viability even at 30-fold higher concentrations.

Expression of rViscumin receptor ganglioside IV\textsuperscript{n}Lc4Cer by in vitro propagated CHO-K1, 5637, and HL-60 cells

Gangliosides were isolated from the three cell lines with different sensitivity and their orcinol-stained thin-layer chromatograms are shown in Figure 6A. A simple double-banded GM3 pattern is characteristic for CHO-K1 cells (Figure 6A, lane b), lacking any higher gangliosides. In contrast to CHO-K1 cells, 5637 cells exhibited a couple of complex and yet unknown gangliosides (Figure 6A, lane c). HL-60 cells were found to express rather low levels of gangliosides (Figure 6A, lane d) but resembling the ganglioside profile of human granulocytes (Figure 6A, lane a). The presence of IV\textsuperscript{n}Lc4Cer in the ganglioside fractions of the three cell lines was investigated by use of the anti-IV\textsuperscript{n}Lc4Cer antibody. For direct comparison, ganglioside amounts equivalent to 1 x 10\textsuperscript{7} cells of each cell type were used for the TLC immunostain (Figure 6B). The rViscumin-resistant CHO-K1 cells were found to lack the receptor ganglioside, whereas 5637 and HL-60 cells express IV\textsuperscript{n}Lc4Cer structures, which appear as double bands on the immunostained chromatogram. Owing to their identical TLC positions compared to IV\textsuperscript{n}Lc4Cer of human granulocytes (Figure 6B, lane a), upper bands of both cell lines represent the typical granulocyte gangliosides IV\textsuperscript{n}Lc4Cer with C24-fatty acid (compound 1) and lower bands IV\textsuperscript{n}Lc4Cer with C16-fatty acid (compound 2). Applying 134 ng of total HGG, the absolute amounts of compound 1 (26% of total HGG) and compound 2 (13.3% of total HGG) of lane a in Figure 6B correspond to 34.8 ng and 17.8 ng, respectively. Deduced from the TLC overlay assay, CHO-K1 cells were receptor-negative and HL-60 cells exhibited considerably higher quantities of IV\textsuperscript{n}Lc4Cer compared with 5637 cells. These findings are in excellent agreement with those obtained by negative ion mode MS/MS analysis of ganglioside IV\textsuperscript{n}Neu5Ac-nLc6Cer (d18:1/16:0) with the molecular mass m/z 1881.95 from HGG fraction HGG4. The resorcinol-stained TLC run and TLC immunostains of HGG4 are displayed in Figure 3.
agreement with rViscumin resistance of CHO-K1 cells and the significantly higher rViscumin sensitivity of HL-60 cells in comparison to 5637 cells (see Figure 5), pointing to an obvious correlation of cellular sensitivity and expression of receptor gangliosides.

Quantification of receptor ganglioside IV\textsubscript{6}nLc4Cer in 5637 and HL-60 cells

To calculate the absolute amounts of IV\textsubscript{6}nLc4Cer receptor gangliosides per single cells, TLC immunostained bands of defined cell numbers, ranging from 1 × 10\textsuperscript{6} to 2 × 10\textsuperscript{7} cells, were quantified by densitometry with the aid of HGG calibration curves. Based on the molecular weights of IV\textsubscript{6}nLc4Cer with C24-fatty acid (compound 1, MW = 1626) and IV\textsubscript{6}nLc4Cer with C16-fatty acid (compound 2, MW = 1516) and Avogadro’s constant, the absolute molecule numbers per single cell were determined for 5637 and HL-60 cells (see Table IV). For 5637 and HL-60 cells 1.54 × 10\textsuperscript{6} and 3.68 × 10\textsuperscript{6} IV\textsubscript{6}nLc4Cer (compound 1 plus compound 2) receptor molecules per cell were revealed, respectively. As a final result, the absolute amount of IV\textsubscript{6}nLc4Cer was 2.4-fold higher in HL-60 compared to 5637 cells. This data is in excellent agreement with the enhanced cytotoxicity of rViscumin toward HL-60 cells (see Figure 5).

Generating CHO-K1 cell susceptibility toward rViscumin by exogenous gangliosides

Gangliosides added exogenously to culture medium are taken up by a wide range of cells in vitro (Saqr et al., 1993) and incorporated into the plasma membrane by a time- and concentration-dependent process (Radsak et al., 1982). CHO-K1 cells have been found to express predominantly GM3 (Figure 6A, lane b). Other gangliosides are either absent or present only in trace quantities. Attempts were made to render CHO-K1 cells susceptible toward rViscumin. For that purpose, increasing amounts of HGG harboring the rViscumin receptor IV\textsubscript{6}nLc4Cer as the prevalent ganglioside were applied to CHO-K1 cell cultures and allowed to incorporate. As shown in Figure 7, incubation of CHO-K1 cells with increasing concentrations of HGG prior to rViscumin treatment reduced the cell viability in a concentration-dependent manner. Cells prewashed with serum-free medium before rViscumin application revealed viabilities decreasing from 85.7% down to 31.5% (relative to control cultures without gangliosides and rViscumin) after incubation with 25 µM to 200 µM HGG, respectively. Prewashing with serum-supplemented medium enhanced the cytotoxic effect leading to somewhat diminished viabilities at the respective HGG concentrations. Gangliosides alone had no effect on CHO-K1 cells up to a concentration of 100 µM; after treatment with 200 µM HGG viability was slightly decreased by about 12% compared to control cultures without gangliosides. From this data it was concluded that insertion of the receptor ganglioside IV\textsubscript{6}nLc4Cer from HGG, which represents the major ganglioside with Neu5Ac\textsubscript{α}2-6Gal\textsubscript{β}1-4GlcNAc terminus in...
Ganglioside binding specificity of recombinant Viscumin

the mixture, turned CHO-K1 cells from rViscumin-resistant to -sensitive cells, thereby confirming the biological function of IV6nLc4Cer as a true and physiologically relevant rViscumin receptor.

TLC overlay assay of ricin with neutral GSLs and gangliosides

Ricin (Ricinus communis lectin) is well accepted to represent a type II ribosome-inactivating protein with galactose specificity. This was tested with the same set of GSL references used for exploring rViscumin binding specificity. The orcinol stain (Figure 8A) and the parallel TLC-binding assay of ricin with neutral GSLs disclosed a weak staining of LacCer (Galβ1-4Glcβ1-1Cer) in all three reference mixtures (Figure 8B). The N-acetyl group of the penultimate GlcNAc obviously increased the strength of binding to nLc4Cer and nLc6Cer (both with Galβ1-4GlcNAc terminus) as shown in lane b of Figure 8B (Table I). Fucosylation of the nLc6Cer-core completely abolished ricin binding, indicated by failed recognition of Lewisx-GSL. Significant but less intensive ricin adhesion compared to, for example, nLc4Cer, was observed for Gg4Cer bearing the Galβ1-3GalNAc-disaccharide at the nonreducing end of the oligosaccharide sequence. Gb4Cer (GalNAcβ1-3Gal terminus) was negative and Gb3Cer (Galβ1-4Gal terminus) exhibited weak signals. The order of binding preference of ricin toward neutral GSLs, deduced from TLC immunostainings, were the sequences Galβ1-4GlcNAc > Galβ1-3GalNAc > Galβ1-4Glc > Galα1-4Gal.

Finally, TLC ricin overlay assays performed with gangliosides gave negative results for all terminally sialylated ganglio- and neolacto-series gangliosides (Figure 9B). A faint positive reaction was observed for GM1, a Gg4Cer-core ganglioside with Neu5Ac bound to the internal position II of the tetraosyl-backbone. Sialylation at position IV of Gg4Cer (GD1a) and the disialo group Neu5Acα2-8Neu5Acα2-3R at position II of Table IV.

Table IV. Quantification of rViscumin receptors IV6nLc4Cer (C24-fatty acid) and IV6nLc4Cer (C16-fatty acid) in ganglioside fractions of 5637 and HL-60 cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>IV6nLc4 (C24-fatty acid)</th>
<th>IV6nLc4 (C16-fatty acid)</th>
<th>IV6nLc4 (C24- + C16-fatty acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5637 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ng/10^7 cells</td>
<td>33.1</td>
<td>7.9</td>
<td>41</td>
</tr>
<tr>
<td>mol/10^7 cells</td>
<td>2.036 × 10^-11</td>
<td>5.218 × 10^-12</td>
<td>2.558 × 10^-11</td>
</tr>
<tr>
<td>Molecules/cell</td>
<td>1.23 × 10^6</td>
<td>3.14 × 10^6</td>
<td>1.54 × 10^6</td>
</tr>
<tr>
<td>HL-60 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ng/10^7 cells</td>
<td>84.9</td>
<td>13.5</td>
<td>98.4</td>
</tr>
<tr>
<td>mol/10^7 cells</td>
<td>5.221 × 10^-11</td>
<td>8.885 × 10^-12</td>
<td>6.109 × 10^-11</td>
</tr>
<tr>
<td>Molecules/cell</td>
<td>3.14 × 10^6</td>
<td>5.35 × 10^6</td>
<td>3.68 × 10^6</td>
</tr>
</tbody>
</table>

Performed by TLC immunostaining with anti-IV6nLc4Cer antibody.

*See Figure 6.
GD1b, completely prevented ricin binding. Most important with regard to rViscin specificity, no binding to any neolacto-series gangliosides could be observed as indicated by unstained white bands in the intentionally overstained background of the TLC immunoassay plate (Figure 9B). According to the data of these TLC immunostaining investigations, ricin can be unequivocally considered as a galactose-binding lectin, clearly distinguishable from rViscin, which was shown to represent a sialic acid–specific lectin.

**Discussion**

The first step in the biological action of viscinum (ML-I) is recognition and binding of specific ligands on the surface of target cells. When glycoproteins with a complex pattern of glycosylation serve as source for the presentation of glycoclusters, the individual contribution of each epitope is difficult to discern. Consequently, a huge number of binding and inhibition assays with chemically well-defined model ligands have been performed to better understand the initial process of ML-I adhesion. For that purpose, carbohydrate binding specificity investigations have been performed to discriminate the potential role of each epitope.

Among all the gangliosides tested in this study, terminally sialylated neolacto-series gangliosides with a Neu5Acα2-6Galβ1-4GlcNAc epitope at the nonreducing terminus, which have been isolated from human granulocytes, were the exclusive targets for rViscin-mediated adhesion. The isomeric gangliosides with Neu5Acα2-3Galβ1-4GlcNAc sequences and neutral GSLs with Galβ1-4GlcNAc epitope were devoid of binding activity. LacCer (Galβ1-4Glcβ1-1Cer) was found to be the only, but rather slightly interacting neutral GSL with marginal receptor potency in comparison to α2-6-sialylated gangliosides. However, α2-6-sialylated lacto-series gangliosides with Galβ1-3GlcNAc-core cannot be excluded as possible rViscin ligands at this stage of research. This aspect of further receptor characterization is a part of our future investigations.

Cancer cells are often characterized by the presence of “tumor-associated” GSL-antigens, and major progress has been made over the past two decades on structural identification of these antigens (Hakomori, 1998). None of these structures are truly tumor-specific, and many GSLs to which antitumor antibodies responses are directed are also found in normal tissues. However, a large number of anti-GSL antibodies showed specific or preferential reactivity with tumor cells and no reactivity with normal cells or tissues, based on organizational differences of membrane GSLs in tumor cells versus normal cells. To elucidate the potential role of terminal α2-6-sialylated gangliosides as tumor-associated antigens, careful (re)investigations of rViscin-susceptible tumor cell lines and/or tumor tissues has to be performed with regard to their specific expression.

Although the conformational parameters of derivatized neutral disaccharide ligands before and after complex formation with the galactose-binding *Viscum album* lectin have been studied in detail (Gilleron et al., 1998), little is known about the interaction of ML-I with naturally occurring sialooligosaccharides and sialoglycoproteins. The precipitability of human glycoprotein and fetuin with ML-I was considerably decreased after desialylation (Wu et al., 1995), but on the contrary the poor reactivity of rat sublingual sialoglycoprotein with ML-I increased substantially after removal of sialic acid. The inhibition of the glycoprotein-lectin interaction by Neu5Acα2-3(α2-6Galβ1-4Glc was taken as evidence that ML-I is specific for sialic acid.

Substitution of the N-acetyllactosamine sequences of oligosaccharides and glycopeptides by sialic acid residues either at O-3 or O-6 of galactose was found to slightly enhance the affinity of ML-I (Debray et al., 1994).

Following the approach with chemically well-defined model ligands (Lee et al., 1992), a panel of strictly defined neoglycoconjugates and synthetic oligosaccharides were systematically tested to determine their individual binding and inhibitory potency (Galanina et al., 1997). Among the two natural isomers of sialyllactose, the α2-6 form displayed a higher level of inhibitory capacity than the α2-3 derivative. However, from all these highly sophisticated binding and inhibition assays performed so far, the natural ligands on immunomodulatory potent and/or tumor target cells being responsible for B-chain-specific binding of the ML remained yet unidentified and rather obscure.

Among all the gangliosides tested in this study, terminally sialylated neolacto-series gangliosides with a Neu5Acα2-6Galβ1-4GlcNAc epitope at the nonreducing terminus, which have been isolated from human granulocytes, were the exclusive targets for rViscin-mediated adhesion. The isomeric gangliosides with Neu5Acα2-3Galβ1-4GlcNAc sequences and neutral GSLs with Galβ1-4GlcNAc epitope were devoid of binding activity. LacCer (Galβ1-4Glcβ1-1Cer) was found to be the only, but rather slightly interacting neutral GSL with marginal receptor potency in comparison to α2-6-sialylated gangliosides. However, α2-6-sialylated lacto-series gangliosides with Galβ1-3GlcNAc-core cannot be excluded as possible rViscin ligands at this stage of research. This aspect of further receptor characterization is a part of our future investigations.

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Following cell surface binding of *V. album* lectin and ensuing signaling, several cellular responses (such as secretion of cytokines, namely, tumor necrosis factor-α, interleukin-1, and interleukin-6 by human peripheral blood mononuclear cells) can be measured in the nontoxic dose range in vitro and in vivo (Hajto et al., 1990; Gabius et al., 1992; Möckel et al., 1997; Stein et al., 1998; Ribéreau-Gayon et al., 1997). Specific immune responses against tumor-associated antigens mediated by T lymphocytes and nonspecific immune responses induced by cells of the mononuclear phagocyte system and natural killer cells appear to participate in naturally acquired resistance against neoplastic diseases. Studies on animals and humans proved that ML-I causes significant increase and activation of natural killer cells and enhances phagocytic activity of granulocytes and monocytes (Hajto et al., 1989, 1990). T cell activation and preferential expansion of CD8+ T cells mediating...
the cytotoxic effect has been reported as well (Baxevanis et al., 1998). The question currently remains unanswered of which type of oligosaccharides, that is, protein- or lipid-bound, is recognized by ML-1 and may be responsible for eliciting the enumerated immunoreponses. Concerning the sialic acid specificity of MLB, amino acid sequencing (Huguet Soler et al., 1998) and molecular cloning of the B-chain in E. coli (Eck et al., 1999b) led to the speculation that rViscumin possesses at least three binding sites, as recently described for ricin (Frankel et al., 1996; Steeves et al., 1999).

Finally, emphasizing the functional importance of receptor clustering described for neoglycoconjugates (Andrè et al., 1997), GSLs and particularly gangliosides are ideal candidates to fulfill the requirements for being effective rViscumin receptors. We presented a body of data that α2-6-sialylated gangliosides might be involved in B-chain-dependent signal triggering, thereby truly resembling cellular targets for rViscumin acting as B-chain-mediated immunomodulator and cytotoxic agent.

**Materials and methods**

**Mass production of cells**

CHO-K1 cells (ATCC CCL-81) and HL-60 cells (promyelocytic human leukemia cell line, ATCC CCL-240) were cultivated with an 1:1 mixture of Dulbecco’s modified Eagle medium (DMEM) and Ham’s F12 medium and the human bladder carcinoma cell line 5637 (ATCC, HTB-9) in RPMI 1640. The cells were routinely passaged in conventional culture flasks (Nunc, Wiesbaden, Germany) at 37°C in a humidified 5% (v/v) CO2 air-atmosphere. The media were buffered with 2.1 g/L NaHCO3 and supplemented with 10% fetal calf serum (v/v). Cells were grown in the presence of 50 mg/L gentamycin and 2.5 mg/L amphotericin B.

Cell production of 5637 cells was performed in 175-cm² culture flasks. HL-60 and CHO-K1 cells were produced in a 1-L SuperSpinner (B. Braun Biotech International, Melsungen, Germany) equipped with a membrane stirrer for optimized oxygen supply of the cells (Heidemann et al., 1996) and Müthing et al. (1996b). The physiological set points for spinner cultivations were: 37°C; pH 7.2; aeration with air; stirrer 40 rpm. After reaching the final cell densities, the cells were harvested and washed twice with aeration with air; stirrer 40 rpm. After reaching the final cell densities, the cells were harvested and washed twice with an 1:1 mixture of Dulbecco’s modified Eagle medium (DMEM) and Ham’s F12 medium and the human bladder carcinoma cell line 5637 (ATCC, HTB-9) in RPMI 1640. The cells were routinely passaged in conventional culture flasks (Nunc, Wiesbaden, Germany) at 37°C in a humidified 5% (v/v) CO2 air-atmosphere. The media were buffered with 2.1 g/L NaHCO3 and supplemented with 10% fetal calf serum (v/v). Cells were grown in the presence of 50 mg/L gentamycin and 2.5 mg/L amphotericin B.

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**Cytotoxicity assay**

Cell viability and in vitro cytotoxicity were assessed using 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1, Roche Diagnostics, Mannheim, Germany). Cytotoxicity of rViscumin was analysed with HL-60, 5637, and CHO-K1 cells. Medium for HL-60 cells was serum-free X-Vivo 15 (Biowhittaker, Walkersville, MO), RPMI 1640 with 2.5% fetal calf serum for 5637, and DMEM/F12 with 1% fetal calf serum for CHO-K1 cells. Indirect measurement of cell viability was achieved by conversion of the soluble sulfonated tetrazolium salt into formazan by cells according to Ishiyama et al. (1993). Samples were tested in six replicates, and the absorbance was determined at 450 nm for each well.

To assay cytotoxicity, 8 × 10³ 5637, 1 × 10⁴ CHO-K1, or 1.8 × 10⁴ HL-60 cells, respectively, were seeded per well in 96-well tissue culture plates (Nunc) and incubated with rViscumin in a final volume of 100 µl for 72 h at 37°C. For treatment, an adequate concentration range of rViscumin was chosen according to the sensitivity of the respective cell line (see Figure 5). After 72 h 10 µl WST-1 solution were added to the cell cultures and incubated for 3–4 h at 37°C. The amount of dye, which is proportional to the number of viable cells, was measured in a microwell plate reader (THERMOmax, Molecular Devices, Munich, Germany).

**rViscumin, ricin, and anti-lectin antibodies**

The heterodimeric recombinant ML (rML) rViscumin, composed of rMLA (A-chain) and rMLB (B-chain) (Eck et al., 1999b), was used for analyzing binding specificity toward neutral GSLs and gangliosides in microwell adsorption and TLC overlay binding assay. GSL-bound rViscumin was detected with the mouse IgG monoclonal anti-rMLA antibody TA5 (Tonevitsky et al., 1995).

**Generation of rViscumin-susceptible CHO-K1 cells by exogenous gangliosides**

The rViscumin-resistant CHO-K1 cells were tested for change in sensitivity to rViscumin after exogenous application of gangliosides in cell cultures. Cells were seeded at a density of 5 × 10⁵ cells per well into a 96-well plate in serum-free CHO-1 medium (Biowhittaker, MD) and cultured for 24 h. The medium was then withdrawn and replaced by CHO-K1 medium containing 0 (control), 25, 50, 100, and 200 µM, respectively, of a preparation of total HGG. Gangliosides were allowed to incorporate into cells for 48 h under culture conditions at 37°C. After this incubation the cells were extensively washed either with serum-free or 5% fetal calf serum–containing CHO-K1 medium, followed by a 48-h treatment with 5.26 nM rViscumin. Viability was examined by WST-1 staining as described in comparison to untreated control cultures (without gangliosides and rViscumin). All cell culture assays were performed in triplicate in 100 µl volumes per well.

**Isolation of GSLs from in vitro cultivated cells**

GSLs from in vitro propagated cells were isolated according to standard procedures (Ledeen and Yu, 1982) and as described in detail by Duvar et al. (1997). Gangliosides were isolated by anion exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Freiburg, Germany) as reported by Müthing et al. (1987). The ganglioside fractions were incubated for 1 h at 37°C in aqueous 1 N NaOH to saponify phospholipids followed by neutralization with acetic acid and dialysis. Gangliosides were further purified by adsorption
fractions HGG1 and HGG2 contained IV 6nLc4Cer and neolacto-type monosialogangliosides (HGG3 and HGG4). The cartridge (150 mm detail (Müthing and Unland, 1994; Müthing, 2000). A glass gangliosides HPLC was carried out with the Superformance Four fractions were obtained, comprising terminally with a linear ammonium acetate gradient, pooled, and desalted. TMAE-Fractogel-bound gangliosides were eluted gangliosides from human granulocytes (= HGG, see previous TMAE-650(S) (Merck, No. 20286) was loaded with total gangliosides VI 6nLc6Cer and VIII 6nLc8Cer were structurally and VI 6nLc6Cer plus minor quantities of VIII 6nLc8Cer. All compound in fraction HGG3. HGG4 contained IV 6nLc4Cer and VI 6nLc6Cer as the major constituents was characterized in this study by ESI-QTOF-MS (see the following Anion-exchange HPLC separation of terminally α2-3- and α2-6-sialylated neolacto-series gangliosides For the separation of α2-3- and α2-6-sialylated monosialo-gangliosides HPLC was carried out with the Superformance universal glass-cartridge device of Merck, earlier published in detail (Müthing and Unland, 1994; Müthing, 2000). A glass cartridge (150 mm × 10 mm) filled with Fractogel EMD TMAE-650(S) (Merck, No. 20286) was loaded with total gangliosides from human granulocytes (= HGG, see previous methods). TMAE-Fractogel-bound gangliosides were eluted with a linear ammonium acetate gradient, pooled, and desalted. Four fractions were obtained, comprising terminally α2-3 sialylated (HGG1 and HGG2) and terminally α2-6-sialylated neolacto-type monosialogangliosides (HGG3 and HGG4). The fractions HGG1 and HGG2 contained IV 6nLc4Cer and VI 6nLc6Cer, respectively. IV 6nLc4Cer represents the only compound in fraction HGG3. HGG4 contained IV 6nLc4Cer and VI 6nLc6Cer plus minor quantities of VIII 6nLc8Cer. All gangliosides separate as double bands on thin-layer chromatograms due to substitution of the sphingosine moiety with C24-(upper band) and C16-fatty acid (lower band). The structures of IV 6nLc4Cer, IV 6nLc6Cer, and VI 6nLc6Cer have been determined by fast atom bombardment−MS and methylation analysis, as described elsewhere (Müthing et al., 1993). The terminally α2-3-sialylated gangliosides IV 6nLc4Cer and VI 6nLc6Cer have been reinvestigated by electrospray ionization−quadrupole time-of-flight MS (ESI-QTOF-MS) as recently reported (Metelmann et al., 2000). Terminally α2-6-sialylated gangliosides VI 6nLc6Cer and VIII 6nLc8Cer were structurally characterized in this study by ESI-QTOF-MS (see the following section).

ESI-QTOF-MS Gangliosides of HPLC-purified fractions HGG3 and HGG4 were analyzed by nanoESI-MS and MS/MS using a QTOF mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray manipulator. Negative ion mode was used exclusively for the ganglioside analysis. After selecting the precursor ion of interest with the first quadrupole, collision-induced dissociation was applied to obtain fragment ions for MS/MS sequencing (for details see Metelmann et al., 2001). The nomenclature introduced by Domon and Costello (1988) was used for assignment of fragment ions.

Polyclonal anti-Neu5Acα2-6Galβ1-4GlcNAc-R antibody At the age of 12 weeks a chicken was immunized according to the method of Kasai et al. (1980). HPLC-purified IV 6nLc4Cer was prepared from human granulocytes as described. One milligram of the ganglioside was adsorbed to 1 mg permethylated bovine serum albumin (Serva, Heidelberg, Germany) in PBS. The solution was emulsified with an equal part of Freund’s adjuvant (Difco, Detroit, MI) in a final volume of 1 ml and administered at multiple intramuscular sites. Preimmune serum was taken just before immunization. After 4 weeks, the chicken was boosted and exsanguinated 14 days later.

High-performance thin-layer chromatography GSLs were separated on high-performance thin-layer chromatography plates (HPTLC plates, size 10 cm × 10 cm, thickness 0.2 mm, Merck; Art. No. 5633). Neutral GSLs were chromatographed in solvent I (chloroform/methanol/water, 120/70/17, each by volume) and gangliosides in solvent II (chloroform/methanol/water, 120/85/20, each by volume), the latter supplemented with 2 mM CaCl₂. Neutral GSLs were visualized with orcinol (Svennerholm, 1956) and gangliosides with orcinol or resorcinol (Svennerholm, 1957). IV 6nLc4Cer-gangliosides of the HGG fraction were quantified by densitometry with a CD60 scanner (Desaga, Heidelberg, Germany) equipped with an IBM-compatible personal computer and densitometric software. Chromatographed bands were measured in reflectance mode at 580 nm (resorcinol) with a light beam slit of 0.1 × 2 mm.

Neutral GSLs from human granulocytes and MDAY-D2 cells as well as gangliosides from human granulocytes appear as double bands on TLCs as already described.

TLC overlay assay Secondary rabbit anti-chicken IgY, goat anti-mouse IgG and IgM, and goat anti-rabbit IgG antisera, all affinity chromatography-purified and labeled with alkaline phosphatase, were purchased from Dianova (Hamburg, Germany) and used in a 1:2000 dilution (Duvar et al., 1997). The TLC immunostaining procedure was carried out according to Magnani et al. (1982) with some modifications. After TLC of GSLs the silica gel was fixed with polyisobutylmethacrylate (Plexigum P28, Röhm, Darmstadt, Germany) as described by Müthing and Mühradt (1988). Two reviews concerning the details of the TLC immunostaining procedure have been published (Müthing, 1996, 1998).

Terminally α2-6-sialylated neolacto-series gangliosides were detected by the chicken polyclonal anti-Neu5Acα2-6Galβ1-4GlcNAc-R antibody. The HPTLC plate was soaked for 15 min with 1% (w/v) bovine serum albumin in PBS (solution A) and then overlayed for 1 h with the primary antibody diluted 1:1000 in solution A. The plate was then washed three times with 0.05% (v/v) Tween 21 in PBS (solution B) and incubated for 1 h with alkaline phosphate-labeled rabbit anti-chicken IgY antibody diluted in solution A. The plate was then washed three times with solvent B and once with glycine buffer (0.1 M glycine, 1 mM ZnCl₂, 1 mM MgCl₂, pH 10.4) to remove phosphate. Bound antibodies were visualized by color development of blue indigo–like stable stain after incubation
of the plate with 0.05% (w/v) 5-bromo-4-chloro-3-indolyl-phosphate (Biomol, Hamburg, Germany) in glycine buffer.

The rViscumin binding activity toward GSLs was detected after prewashing the plate with 0.1 g/L Tween 80 in PBS (PBS-T80), followed by overlaying the chromatogram for 1 h with 1 μg/ml rViscumin diluted in PBS-T80. The plate was washed with solution B, incubated for 15 min with solution A, and then overlayed with the murine anti-rMLA monoclonal antibody TA5 (1 μg/ml in solution A). The next steps were performed as already described. Secondary alkaline phosphatase labeled anti-mouse IgG and IgM was used for the detection of bound mouse TA5 antibody.

The ricin overlay binding assay was performed according to the protocol described for rViscumin. Ricin was used in a working concentration of 1 μg/ml in PBS-T80; the polyclonal rabbit anti-ricin antibody was employed in 1:200 dilution and detected with alkaline phosphatase labeled secondary anti-rabbit antibody.

**Determination of cellular quantities of IV<sub>n</sub>LnLc4Cer**

The amounts of IV<sub>n</sub>LnLc4Cer expressed by HL-60 and 5637 cells were quantified by scanning of TLC bands stained with the anti-IV<sub>n</sub>LnLc4Cer antibody. Defined quantities of HGG reference IV<sub>n</sub>LnLc4Cer ganglioside (calibration curve) were scanned in comparison to ganglioside fractions of defined cell numbers ranging from 1 × 10<sup>6</sup> to 2 × 10<sup>7</sup> cells. The blue-colored immunostained double bands were scanned in reflectance mode at 630 nm. The numbers of IV<sub>n</sub>LnLc4Cer molecules per cell were calculated by use of Avogadro’s constant (6.02252 × 10<sup>23</sup> molecules per mol) and the known molecular weight of 1626 for IV<sub>n</sub>LnLc4Cer substituted with C24-fatty acid (upper band, compound 1) and by use of Avogadro’s constant (6.02252 × 10<sup>23</sup> molecules per mol) and the known molecular weight of 1516 for IV<sub>n</sub>LnLc4Cer substituted with C16-fatty acid (lower band, compound 2).

**Microwell adsorption assay**

The microwell adsorption assays were carried out with neutral GSLs and gangliosides on polystyrene microtiter plates (MaxiSorp P96 immuno plates; Nunc) at room temperature. Microwells were loaded with GSLs in 100 μl methanol, starting with a defined GSL amount followed by three serial 1:2 dilutions. Neutral GSLs from human erythrocytes: 1.25, 2.5, 5, and 10 μg; neutral GSLs from human granulocytes: 1.88, 3.75, 7.5, and 15 μg; neutral GSLs from MDAY-D2: 2.5, 5, 10, and 20 μg; human granulocyte gangliosides and human brain gangliosides: 1.25, 2.5, 5, and 10 μg. Methanol was evaporated by exposure of the plate in a dry atmosphere for 45 min at 37°C. The solutions used were the same as described for the TLC overlay binding assay.

All incubation steps were performed in volumes of 100 μl per well. The GSL-coated microwells were incubated for 15 min with PBS-T80 and then provided with 1 μg of rViscumin dissolved in PBS-T80 (0.01 μg/μl). After 1 h incubation the wells were washed three times with solvent B, soaked for 15 min in solution A, and then loaded for 1 h with the murine anti-rMLA monoclonal antibody TA5 (1 μg/ml in solution A). The wells were washed three times with solvent B and incubated with alkaline phosphatase labeled anti-mouse IgG and IgM secondary antibody diluted 1:2000 in solution A. After 1-h incubation and threefold washing with solution B, bound antibodies were visualized with di-sodium-4-nitrophenolphosphatehexahydrate (16 mM in 0.1 M glycine buffer pH 10.4). Enzyme activity was recorded after 20 min at 405 nm with an ELISA microplate autoreader (EL311, Bio-Tec Instruments, Winooski, VT).

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**Abbreviations**

CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle medium; ESI-QTOF, electrospray ionization-quadrupole time-of-flight; GSL, glycosphingolipid; HGG, human granulocyte gangliosides; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; ML, mistletoe lectin; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PBS, phosphate buffered saline; TLC, thin-layer chromatography; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate.

The designation of the gangliosides and their core structures follow the IUPAC-IUB recommendations (1998) and the nomenclature of Svennerholm (1963). Structures of neutral GSLs and gangliosides are listed in Table I and II, respectively.

**References**


Ganglioside binding specificity of recombinant Viscumin


