Glycoconjugate Journal (1997) **14:** 19–28

Glycosphingolipid expression in human skeletal and heart muscle assessed by immunostaining thin-layer chromatography

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In this study the comparative TLC immunostaining investigation of neutral GSLs and gangliosides from human skeletal and heart muscle is described. A panel of specific polyclonal and monoclonal antibodies as well as the G_{M1}-specific choleragenoid were used for the overlay assays, combined with preceding neuraminidase treatment of gangliosides on TLC plates. This approach proved homologies but also quantitative and qualitative differences in the expression of ganglio-, globo- and neolacto-series neutral GSLs and gangliosides in these two types of striated muscle tissue within the same species. The main neutral GSL in skeletal muscle was LacCer, followed by $GbOse_3Cer$, $GbOse_4Cer$, $nLcOse_4Cer$ and monohexosylceramide, whereas in heart muscle $GbOse_3Cer$ and $GbOse_4Cer$ were the predominant neutral GSLs beside small quantities of LacCer, $nLcOse_4Cer$ and monohexosylceramide. No ganglio-series neutral GSLs and no Forssman GSL were found in either muscle tissue. G_{M3}(Neu5Ac) was the major ganglioside, comprising almost 70% in skeletal and about 50% in cardiac muscle total gangliosides. G_{M2} was found in skeletal muscle only, while G_{D3} and G_{M1b}-type gangliosides (G_{M1b} and $G_{D1\alpha}$) were undetectable in both tissues. G_{M1a}-core gangliosides (G_{M1},G_{D1a},G_{D1b} and G_{T1b}) showed somewhat quantitative differences in each muscle; lactosamine-containing IV^{3}Neu5Ac-$nLcOse_4Cer$ was detected in both specimens. Neutral GSLs were identified in TLC runs corresponding to e.g. 0.1 g muscle wet weight ($GbOse_3Cer$, $GbOse_4Cer$), and gangliosides G_{M3} and G_{M2} were elucidated in runs which corresponded to 0.2 g muscle tissue. Only 0.02 g and 0.004 g wet weight aliquots were necessary for unequivocal identification of neolacto-type and G_{M1}-core gangliosides, respectively. Muscle is known for the lowest GSL concentration from all vertebrate tissues studied so far. Using the overlay technique, reliable GSL composition could be revealed, even from small muscle probes on a sub-orcinol and sub-resorcinol detection level.

Keywords: **Antibodies, choleragenoid, gangliosides, neuraminidase, neutral glycosphingolipids, TLC immunostaining**

Abbreviations: **ATCC, American Type Culture Collection; GSL(s), glycosphingolipid(s); HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid [78]; PBS, phosphate buffered saline. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations [79] and the ganglioside nomenclature system of Svennerholm [80]. Lactosylceramide or LacCer, Galβ1-4Glcβ1-1Cer; gangliotriaosylceramide or $GgOse_3Cer$, GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliotetraosylceramide or $GgOse_4Cer$, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; globotriaosylceramide or $GbOse_3Cer$, Galα1-4Galβ1-4Glcβ1-1Cer; globoside or globotetraosylceramide or $GbOse_4Cer$, GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer; Fo or Forssman GSL, GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer; paragloboside or lacto-*N*-*neo*tetraosylceramide or $nLcOse_4Cer$, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; lacto-*N*-*nor*hexaosylceramide or $nLcOse_6Cer$, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; G_{M3},II^{3}Neu5Ac-LacCer; G_{M2},II^{3}Neu5Ac-$GgOse_3Cer$; G_{M1} or G_{M1a},II^{3}Neu5Ac-$GgOse_4Cer$; G_{M1b},IV^{3}Neu5Ac-$GgOse_4Cer$; G_{D3},II^3(Neu5Ac)$_2$-LacCer; G_{D1a},IV^{3}Neu5Ac,II^{3}Neu5Ac-$GgOse_4Cer$; G_{D1b},(II^{3}Neu5Ac)$_2$-$GgOse_4Cer$; $G_{D1\alpha}$,V^{3}Neu5Ac,II^{3}Neu5Ac-$GgOse_4Cer$; G_{T1b},V^{3}Neu5Ac,II^3(Neu5Ac)$_2$-$GgOse_4Cer$; G_{Q1b},V^3(Neu5Ac)$_2$,II^3(Neu5Ac)$_2$-$GgOse_4Cer$.**

Introduction

Glycosphingolipids (GSLs) generally consist of an oligosaccharide chain linked to a long chain base (sphingosine or related compound) which is N-acylated by a long chain fatty acid. Gangliosides are glycosphingolipids containing one or more sialic acid residues [1, 2]. Structures and functions of GSLs have been widely reviewed [3–10]. GSLs are now known to be ubiquitous compounds of all mammalian tissues studied so far and have been implicated in a number of biological processes. They are involved in e.g. cell-cell recognition phenomena [11], the regulation of cell growth [12], differentiation [13] and signal transduction [14]. GSLs

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are primarily located in the outer leaflet of the plasma membrane, but are also found in association with intracellular organelles [15].

Although muscle tissue makes up almost half of total body weight, there have been relatively few studies of the GSL expression in mammalian skeletal muscle, probably because of their low concentrations compared with that in neural tissue. Some variations in the ganglioside patterns of muscle were observed in an interspecies comparison performed by Nakamura *et al.* [16] and in a recent study of mouse and rabbit skeletal muscle GSLs published by Müthing *et al.* [17], giving evidence for the predominance of G_{M3} ganglioside and some minor neolacto- and ganglio-series gangliosides. There is even less data on GSLs of human skeletal muscle [18, 19] and relatively little is known about the GSL composition especially in cardiac muscle [20]. In a very recent paper, we have investigated the cellular distribution of GSLs in human skeletal and heart muscle by immunostaining of cryosections with anti-GSL antibodies [21].

The combination of TLC separation, conventional chemical staining of GSL bands and direct overlay binding with antibodies, toxins and/or lectins, can rapidly generate detailed information on GSL structure and function without large investments in instrumentation [22, 23]. Improvements in purification technologies for preparation of homogeneous GSLs, which is the prerequisite for generating specific polyclonal antibodies, the facility of producing monoclonal antibodies by the hybridoma technology [24–26], and furthermore the knowledge of bacterial toxin and lectin carbohydrate specificities led to powerful TLC-based staining methods as recently reviewed [23].

In this study, we performed a comparative TLC immunostaining analysis of neutral GSLs and gangliosides of human skeletal and heart muscle, using a series of monoclonal and polyclonal antibodies as well as G_{M1}-specific choleragenoid. This approach, combined with preceding neuraminidase treatment of ganglioside fractions on TLC plates, proved homologies but also differences in the expression of ganglio-, globo- and neolacto-series neutral GSLs as well as gangliosides in these two types of striated muscle tissues within the same species. Two newly prepared polyclonal antibodies against $GbOse_3Cer$ and G_{M2} are described in this paper.

Materials and methods

Muscle

Human *quadriceps femoris* and human cardiac muscle were obtained at autopsy 12 h post mortem of two blood group A males aged 39 and 66 years approved by the Institutional Ethical Committee of the School of Medicine Zagreb. The cause of death was a car accident. There were no clinical records of muscle or heart disease and the muscles appeared macroscopically normal.

Isolation of GSLs from skeletal and heart muscle

GSLs were extracted from 21 g of fresh skeletal and 17 g heart muscle with chloroform : methanol (2:1, 1:1, and 1:2, by volume). Chloroform and methanol of analytical grade (Merck, Darmstadt, Germany) were distilled before use. Gangliosides and neutral GSLs were isolated according to standard procedures [4], and separated by anion exchange chromatography on DEAE Sepharose (Pharmacia Fine Chemicals, Freiburg, Germany) as described by Müthing *et al.* [27]. Gangliosides were further purified by adsorption chromatography on Iatrobeads 6RS-8060 (Macherey-Nagel, Düren, Germany) [28]. Whole gangliosides were eluted with chloroform : methanol (1:2, by volume). The neutral GSL-containing fraction was applied to a Silica gel 60 column and whole neutral GSLs were eluted with chloroform : methanol (40:60, by volume). The final purification was performed by Florisil chromatography as their peracetylated derivatives [29].

Reference GSLs

A reference neutral GSL fraction with LacCer and $nLcOse_4Cer$ as main components was prepared from human granulocytes as described by Müthing and Neumann [30]. Neutral GSL fractions containing globoside and Forssman GSL were isolated from human and sheep red blood cells, respectively, by standard procedures as described above. A reference fraction containing LacCer, $GgOse_3Cer$ and $GgOse_4Cer$ as major neutral GSLs was prepared from the murine lymphoreticular tumour cell line MDAY-D2 (unpublished data).

A preparation of human brain gangliosides, comprising G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}, was purchased from Supelco Inc. (Bellefonte, PA, USA). A ganglioside mixture composed of G_{M3}, $IV^3Neu5Ac\text{-}nLcOse_4Cer$, $IV^6Neu5Ac\text{-}nLcOse_4Cer$ and $VI^3Neu5Ac\text{-}nLcOse_6Cer$ was prepared from human granulocytes as described by Müthing *et al.* [31]. The isolation and characterization of G_{M1b}-type gangliosides from murine MDAY-D2 tumour cells have been described [32]. G_{M2} and G_{D3} gangliosides were purchased from Dr Pallmann GmbH (Munich, Germany).

Thin-layer chromatography

Silica gel 60 precoated glass-backed high performance thin layer chromatography plates (HPTLC plates, size 10 cm × 10 cm; E. Merck, Darmstadt, Germany) were used. Neutral GSLs were separated in solvent 1, chloroform : methanol : water (120:70:17, by volume). Gangliosides were chromatographed in solvent 2, chloroform : methanol : water (120:85:20, by volume) containing 2 mM $CaCl_2$. Neutral GSLs were visualized with orcinol [33] and gangliosides with resorcinol [34].

For quantitative estimation, orcinol and resorcinol as well as immunostained chromatograms (see below) were scanned with a Desaga CD60 scanner (Desaga, Heidelberg, Germany)

equipped with an IBM compatible personal computer and densitometric software. GSL amounts, corresponding to identical wet weight quantities of each muscle tissue, were applied and bands were measured in reflectance mode at 550 nm (orcinol), 580 nm (resorcinol) and 630 nm (indolylphosphate) with a light beam slit of 0.1 mm × 2 mm.

Antibodies

All polyclonal anti-GSL antibodies were produced according to the method of Kasai *et al.* [35]. HPLC purified LacCer, $GbOse_3Cer$, $GbOse_4Cer$, $GgOse_4Cer$ and $nLcOse_4Cer$ were used for immunization. The anti-LacCer antibody strongly binds to lactosylceramide (Galβ1-4Glc-R) and in considerable quantity to $nLcOse_4Cer$ (Galβ1-4GlcNAc-R) due to some homology of the disaccharide termini. The specificity of the anti-$nLcOse_4Cer$ antibody has been recently described [30]. The anti-$GbOse_3Cer$ antibody will be characterized in this study. The anti-globoside antibody strongly reacted with $GbOse_4Cer$ (GalNAcβ1-3Gal-R) and only trace cross-reactivity was observed towards $GgOse_4Cer$ (Galβ1-3GalNAc-R). The applicability of this antibody has been recently reported [17]. Rat IgG2c monoclonal anti-Forssman GSL antibody was a kind gift of Dr U. Bethke [36]. Mouse monoclonal IgM anti-$GgOse_3Cer$ antibody [37] was produced with hybridoma clone 2D4 (TIB 185), obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA). The highly specific rabbit antibody against $GgOse_4Cer$ has been characterized as earlier reported [38, 39]. All employed anti-neutral GSL antibodies are listed in Table 1.

The anti-G_{M3}(Neu5Ac) antibody was used as recently described [17] and the exact specificity has been explained in a recent paper [40]. The characteristics of the new anti-G_{M2}(Neu5Ac) antibody will be reported in this publication. Anti-G_{D3} monoclonal mouse IgG3 antibody [41] was produced with hybridoma clone R_{24} (ATCC, HB 8445). Anti-ganglioside antibodies are listed in Table 2.

Table 1. Neutral GSL specific monoclonal and polyclonal antibodies employed for TLC immunostaining.

GSL antigen	*Structure*	*Detection*	*Dilution*
LacCer	Galβ1-4Glcβ1-1Cer	Chicken antiserum	1:500
$GbOse_3Cer$	Galα1-4Galβ1-4Glcβ1-1Cer	Chicken antiserum	1:500
$GbOse_4Cer$	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	Chicken antiserum	1:1000
Forssman GSL	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	Rat IgG2c, mab[a]	1:20[b]
$nLcOse_4Cer$	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	Chicken antiserum	1:1000
$GgOse_3Cer$	GalNAcβ1-4Galβ1-4Glcβ1-1Cer	2D4, mouse IgM, mab[a]	1:20[b]
$GgOse_4Cer$	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer	Rabbit antiserum	1:1000

[a]mab, monoclonal antibody.
[b]diluted hybridoma supernatant.

Table 2. GSL specific monoclonal and polyclonal antibodies and choleragenoid employed for TLC immunostaining of gangliosides.

Ganglioside	*Structure*	*Detection*	*Dilution*
G_{M3}	II^3Neu5Ac-LacCer	Chicken antiserum	1:1000
G_{D3}	$II^3(Neu5Ac)_2$-LacCer	R_{24}, mouse IgG3, mab[a]	1:20[b]
G_{M2}	II^3Neu5Ac-$GgOse_3Cer$	Chicken antiserum	1:2000
G_{M1}	II^3Neu5Ac-$GgOse_4Cer$	Choleragenoid	250 ng ml^{-1}
G_{M1b}	IV^3Neu5Ac-$GgOse_4Cer$[c]	Rabbit antiserum	1:1000
G_{D1a}	IV^3Neu5Ac,II^3Neu5Ac-$GgOse_4Cer$[d]	Choleragenoid	250 ng ml^{-1}
G_{D1b}	$II^3(Neu5Ac)_2$-$GgOse_4Cer$[d]	Choleragenoid	250 ng ml^{-1}
$G_{D1\alpha}$	IV^3Neu5Ac,III^6Neu5Ac-$GgOse_4Cer$[c]	Rabbit antiserum	1:1000
G_{T1b}	IV^3Neu5Ac,$II^3(Neu5Ac)_2$-$GgOse_4Cer$[d]	Choleragenoid	250 ng ml^{-1}
IV^3nLc4	IV^3Neu5Ac-$nLcOse_4Cer$[e]	Chicken antiserum	1:2000
IV^6nLc4	IV^6Neu5Ac-$nLcOse_4Cer$[e]	Chicken antiserum	1:2000
VI^3nLc6	VI^3Neu5Ac-$nLcOse_6Cer$[e]	Chicken antiserum	1:2000

[a]mab, monoclonal antibody.
[b]diluted hybridoma supernatant.
[c]detection with anti-$GgOse_4Cer$ antibody after *V. cholerae* neuraminidase treatment.
[d]detection with choleragenoid after *V. cholerae* neuraminidase treatment.
[e]detection with anti-$nLcOse_4Cer$ antibody after *V. cholerae* neuraminidase treatment.

TLC immunostaining (overlay technique)

The immunostaining procedure was carried out according to Magnani *et al.* [42] with some modifications described by Müthing and Mühlradt [39]. The silica gel was fixed with polyisobutylmethacrylate (Plexigum P28; Röhm, Darmstadt, Germany). For blocking of nonspecific binding, the plates were soaked for 15 min in solution A (phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin). The plates were then overlayed for 1 h with anti-GSL antibodies, diluted in solution A. Unbound antibodies were removed by washing the plate five times with solution B (PBS, 0.05% Tween 21). Rabbit anti-chicken IgG, goat anti-rabbit IgG, goat anti-mouse IgM, goat anti-rat IgG, all affinity chromatography-purified and labelled with alkaline phosphatase, were purchased from Dianova (Hamburg, Germany) and diluted 1:1000 in solution A. After 1 h incubation, the plates were washed as described above, followed by twofold rinsing with 0.1 M glycine buffer, pH 10.4, supplemented with 1 mM $ZnCl_2$ and 1 mM $MgCl_2$, to remove phosphate. Bound antibodies were visualized with 0.05% (w/v) 5-bromo-4-chloro-3-indolylphosphate (Biomol, Hamburg, Germany) dissolved in the same buffer.

Detection of G_{M1a}-type gangliosides

The HPTLC binding assay using the cholera toxin B subunit (choleragenoid) for specific detection of G_{M1a} has been developed by Magnani *et al.* [43] and was used according to the modifications described by Müthing *et al.* [44]. Briefly, gangliosides were chromatographed and the silica gel fixed plate was overlayed with choleragenoid (Sigma, No. C-7771, Deisenhofen, Germany; 250 ng ml^{-1}) in solution A (see above) for 2 h. Goat anti-choleragenoid antiserum (Calbiochem, No. 227040, Frankfurt a. M., Germany; 1:2000 in solution A) and alkaline phosphatase conjugated rabbit anti-goat IgG antiserum (Dianova; 1:2000 in solution A) were used for immunostaining. Bound antibodies were visualized as described above. To reveal the presence of G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b}, the plates were preincubated with 5 mU ml^{-1} *V. cholerae* neuraminidase (Behring, Marburg, Germany) for 2 h at 37°C before combined choleragenoid-immunostaining. This technique has been originally developed by Wu and Ledeen [45].

Detection of G_{M1b}-type gangliosides

After chromatography the HPTLC plate was incubated with 2.5 mU ml^{-1} *V. cholerae* neuraminidase (2 h, 37 °C) followed by incubation with a rabbit anti-$GgOse_4Cer$ antiserum as previously described [39]. Alkaline phosphatase-conjugated second goat anti-rabbit antibody was used to visualize bound first antibodies as described above.

Detection of neolacto-series gangliosides

Neuraminidase treatment of neolacto-series gangliosides with α2-3 substituted sialic acid is necessary before immunostaining with anti-$nLcOse_4Cer$ antibody, whereas α2-6 sialylated neolacto-type gangliosides can be detected without enzyme treatment, since sialylation in position 6 of the terminal galactose does not hinder recognition [30].

Briefly, gangliosides were chromatographed and the silica gel fixed plate was incubated with 2.5 mU ml^{-1} *V. cholerae* neuraminidase for 2 h at 37 °C. The immunostaining procedure with chicken anti-$nLcOse_4Cer$ antibody was performed as described above. Positive controls, buffer only without enzyme, obtained by anti-$nLcOse_4Cer$ stain indicate the presence of α2-6 sialylated neolacto-gangliosides; additionally appearing immunostained bands after neuraminidase treatment indicate α2-3 sialylated neolacto-type gangliosides.

Results

In this paper we describe the comparative TLC immunostaining investigation of GSLs from human skeletal and heart muscle. GSL preparations from various sources, containing muscle relevant GSLs, were used as references. A panel of specific polyclonal and monoclonal antibodies as well as the ganglioside G_{M1}-specific choleragenoid, previously proved to be successful for TLC and histochemical immunostaining, were used in this study.

Orcinol stain of human skeletal muscle, heart muscle and reference neutral GSLs

The globo-series neutral GSLs $GbOse_3Cer$ and $GbOse_4Cer$ (globoside) are well known constituents of the neutral GSL fraction from human erythrocytes (Fig. 1, lane a), and the Forssman GSL (GalNAcα1-3$GbOse_4Cer$) is the dominant

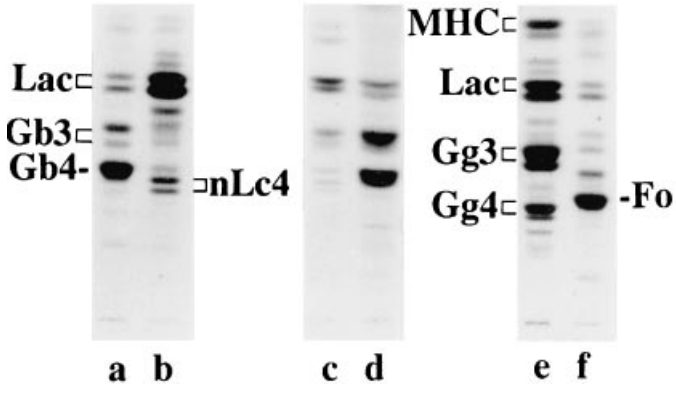

Figure 1. Orcinol stain of the neutral GSL fractions from human erythrocytes (lane a, 8 μg), human granulocytes (lane b, 20 μg), human skeletal muscle (lane c), human heart muscle (lane d), murine lymphoreticular tumour cell line MDAY-D2 (lane e, 20 μg) and sheep erythrocytes (lane f, 10 μg). Each muscle GSL probe (lanes c and d) corresponds to 0.4 g wet weight of tissue. GSLs were chromatographed in solvent 1. Lac, lactosylceramide; Gb3, $GbOse_3Cer$; Gb4, $GbOse_4Cer$; nLc4, $nLcOse_4Cer$; MHC, monohexosylceramide; Gg3, $GgOse_3Cer$; Gg4, $GgOse_4Cer$; Fo, Forssman GSL.

glycosphingolipid of sheep erythrocytes (Fig. 1, lane f). Lactosylceramide is the major compound in the neutral GSL fraction of human granulocytes, which also comprises $nLcOse_4Cer$ (paragloboside; Fig. 1, lane b). Ganglio-series $GgOse_3Cer$ and $GgOse_4Cer$ are strongly expressed in murine lymphoreticular MDAY-D2 cells as demonstrated in Fig. 1 (lane e). These neutral GSL preparations served as reference structures for comparative TLC immunostaining analysis (see below).

The orcinol stains of neutral GSLs of human skeletal and heart muscle are shown in Fig. 1 (lanes c and d, respectively). Equal quantities of GSLs corresponding to 0.4 g muscle wet weight of each probe were applied to enable quantitative comparison of both types of muscle. As deduced from orcinol stained TLC of Fig. 1 (lanes c and d), considerable quantitative differences were detected. Whereas lactosylceramide is the main neutral GSL in skeletal muscle, tri- and tetrahexosylceramides are the dominant ones in myocardium. The presence of globo- and neolacto-series and the absence of ganglio-series neutral GSLs as well as their different quantitative expression were verified by use of specific anti-GSL antibodies (listed in Table 1) as demonstrated in the following paragraphs.

TLC immunostain of LacCer from human skeletal and heart muscle

The presence of LacCer was confirmed in both types of human muscle by immunostaining with anti-LacCer antibody (not shown). LacCer migrates as double band (see Fig. 1, lanes c and d) and is the main neutral GSL in skeletal muscle comprising approximately 45% of total orcinol stain (Fig. 1, lane c), while in heart muscle its relative orcinol positive amount makes up only 15% (Fig. 1, lane d). However, due to different numbers of sugars in di-, tri- and tetraosylceramides, the degree of colour produced by orcinol stain does not indicate molar amounts of single GSLs within the whole mixture. This is true for all comparative orcinol stainings in the following sections.

TLC immunostain of globo-series neutral GSLs

For immunostaining analysis of muscle neutral GSLs with specific anti-$GbOse_3Cer$ and anti-$GbOse_4Cer$ antibodies, neutral GSLs from human erythrocytes were used as references as demonstrated in Fig. 2. $GbOse_3Cer$ was immunostained in both skeletal and heart muscle although the quantities in skeletal muscle (Fig. 2A, lane b) were only 25% of those found in heart muscle (Fig. 2A, lane c). Total amounts of trihexosylceramide have been determined as 9.3 and 37.6 nmol g^{-1} wet weight for human skeletal [18] and heart muscle [20], respectively. Thus, our relative quantitation is in excellent agreement with data from two different groups. The anti-$GbOse_3Cer$ antibody (Fig. 2A) specifically binds to $GbOse_3Cer$ and did not cross react with any of the other major GSLs. However, a weak positive binding with

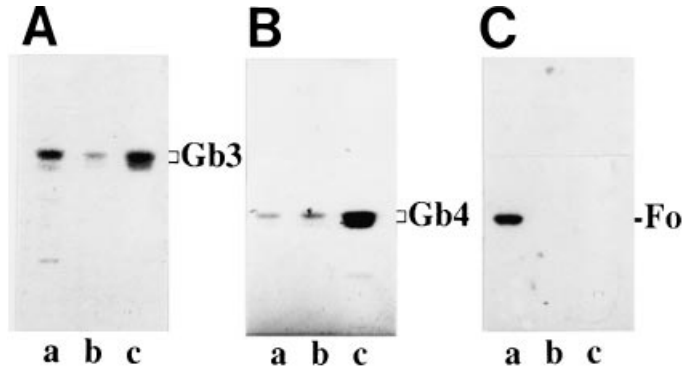

Figure 2. TLC immunostain of the neutral GSL fractions from human skeletal and heart muscle with anti-$GbOse_3Cer$ (A), anti-$GbOse_4Cer$ (B) and anti-Forssman GSL antibody (C). Lanes a, 4 μg (A) and 1 μg (B) neutral GSLs from human erythrocytes and 1 μg neutral GSLs from sheep erythrocytes (C); lanes b, neutral GSLs from human skeletal muscle corresponding to 0.1 g (A and B) and 0.4 g (C) of wet weight tissue; lanes c, neutral GSLs from human heart muscle corresponding to 0.1 g (A and B) and 0.4 g (C) of wet weight tissue. GSLs were chromatographed in solvent 1. Gb3, $GbOse_3Cer$; Gb4, $GbOse_4Cer$; Fo, Forssman GSL.

an unknown minor compound was observed near the origin (Fig. 2A, lane a). Similarly, $GbOse_4Cer$ was also detected with its specific antibody in both types of muscles (Fig. 2B). The content of globoside, being the main neutral GSL in myocardium (see Fig. 1, lane d), was found to be four times lower by immunostaining in skeletal muscle (Fig. 2B, lanes b and c), where this compound comprises only *ca.* 7% of orcinol positive bands (Fig. 1, lane c). The Forssman GSL was not found in either skeletal (Fig. 2C, lane b) or cardiac muscle (Fig. 2C, lane c). It was strongly immunostained in the neutral GSL fraction of sheep erythrocytes that served as the reference mixture (Fig. 2C, lane a) where it is the main neutral GSL.

TLC immunostain of neolacto-series neutral GSLs

Neutral GSLs from skeletal and cardiac muscle were cochromatographed with neutral GSLs from human granulocytes (Fig. 3A) and immunostained with anti-$nLcOse_4Cer$ antibody. Two positive $nLcOse_4Cer$ bands, migrating to similar positions as did two granulocyte $nLcOse_4Cer$ bands, were also detected in human skeletal (Fig. 3A, lane b) and in human heart muscle (Fig. 3A, lane c). In the case of granulocytes neutral GSLs, double bands are known to be due to the substitution with C_{24}- and C_{16}-fatty acids in the ceramide portion (upper and lower bands, respectively, in Fig. 3A, lane a). The quantities in skeletal muscle (Fig. 3, lane b) were 1.8-fold larger than in the heart muscle (Fig. 3, lane c). Small quantities of $nLcOse_6Cer$ are known to be expressed in human granulocytes and were also stained in the neutral GSL fraction of skeletal muscle with the anti-$nLcOse_4Cer$ antibody (Fig. 3A, lane b). Only a trace of

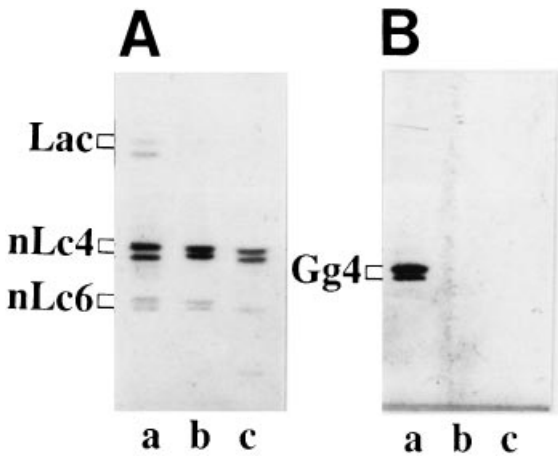

Figure 3. TLC immunostain of the neutral GSL fractions from human skeletal and heart muscle with the anti-nLcOse$_4$Cer antibody (A) and anti-GgOse$_4$Cer antibody (B). Lanes a, 5 μg neutral GSLs from human granulocytes (A) and 2 μg neutral GSLs from murine lymphoreticular tumour cell line MDAY-D2 (B) (references); lanes b, neutral GSLs from human skeletal muscle corresponding to 0.4 g (A and B) of wet weight tissue; lanes c, neutral GSLs from human heart muscle corresponding to 0.4 g (A and B) of wet weight tissue. GSLs were chromatographed in solvent 1. Lac, lactosylceramide; nLc4, nLcOse$_4$Cer; nLc6, nLcOse$_6$Cer; Gg4, GgOse$_4$Cer.

a band migrating to lower band nLcOse$_6$Cer of human granulocytes was detected in the heart muscle.

Ganglio-series neutral GSLs

Neutral GSLs of the ganglio-series (GgOse$_3$Cer and GgOse$_4$Cer) were not found in either muscle type. Immunostaining with anti-GgOse$_4$Cer polyclonal antibody is shown in Fig. 3B, where two positive bands were revealed only in an extract of murine lymphoreticular tumour cell line MDAY-D2 (Fig. 3B, lane a). Both muscle fractions were found to be completely negative (Fig. 3B, lanes b and c).

Gangliosides from human skeletal and heart muscle

The main ganglioside detected by resorcinol stain in both types of muscle was G_{M3} (sialyllactosylceramide) comprising almost two-thirds of total skeletal and approximately one-half of total heart gangliosides (Fig. 4A, lanes b and c, respectively). The two G_{M3} bands of skeletal muscle (Fig. 4A, lane b) migrated similar to reference G_{M3} from human granulocytes ganglioside fraction (Fig. 4A, lane a). Double bands of gangliosides from human granulocytes are derived from C_{24}- and C_{16}-fatty acid substitution in their ceramides, and somewhat different fatty acid composition is responsible for the heterogeneity of G_{M3} in human skeletal muscle [18]. Compared to reference gangliosides from human granulocytes, cross reactivity with IV^{3}Neu5Ac-nLcOse$_4$Cer can be excluded. In human heart muscle, G_{M3} appears dominantly as a single upper band (Fig. 4A, lane c), probably due to dominance of long chain fatty acids [20].

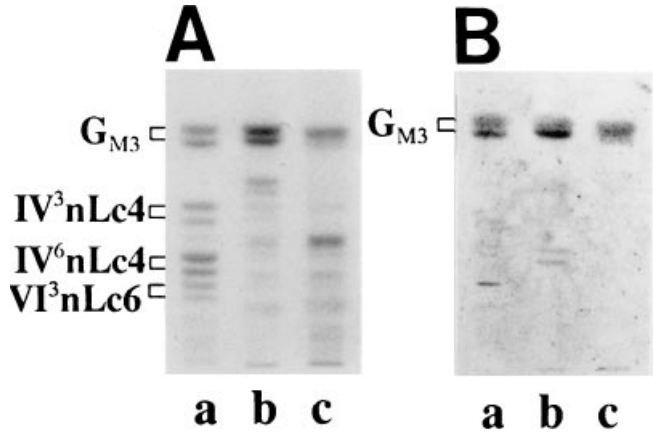

Figure 4. Resorcinol stain (A) and TLC immunostain (B) of gangliosides from human skeletal and heart muscle with the anti-G_{M3}(Neu5Ac) antibody. Lanes a, 7μg gangliosides from human granulocytes (reference); lanes b, gangliosides from human skeletal muscle corresponding to 0.2 g of wet weight tissue; lanes c, gangliosides from human heart muscle corresponding to 0.2 g of wet weight tissue. Gangliosides were chromatographed in solvent 2. nLc4, nLcOse$_4$Cer; nLc6, nLcOse$_6$Cer.

The presence of ganglio- and neolacto-series gangliosides and the absence of G_{M1b}-type gangliosides as well as their different quantitative expression were verified by use of specific anti-ganglioside antibodies (listed in Table 2) as demonstrated in the following sections.

TLC immunostain of human skeletal and heart muscle ganglioside fraction with anti-G_{M3}, anti-G_{D3} and anti-G_{M2} antibody

Resorcinol detection of G_{M3} in muscle probes was confirmed by TLC immunostaining with anti-G_{M3}(Neu5Ac) antibody (Fig. 4B). A faint positive doublet of unknown structure was detected in the middle of the chromatogram between G_{M3} and the origin (Fig. 4B, lane b). In all cases, upper bands of G_{M3}(Neu5Ac), carrying long-chain fatty acids, were less intensively stained by the polyclonal antibody compared to lower bands. However, it is assumed, that the plasticisers favour oligosaccharide exposure towards the hydrophilic environment. Furthermore, enhanced binding ability of G_{M3} with long-chain fatty acids using the monoclonal M2590 antibody has been reported [46]. Control of Neu5Gc substitution of G_{M3} in our study was performed by TLC immunostaining with a highly specific chicken anti-G_{M3}(Neu5Gc) antibody, which exclusively binds to G_{M3}(Neu5Gc) and does not bind to G_{M3}(Neu5Ac). Not even trace amounts of G_{M3}(Neu5Gc) were detected in human skeletal and cardiac muscle (not shown).

Monoclonal R_{24} mouse anti-G_{D3} antibody was used for detection of this disialoganglioside, but no positive bands were detected in either skeletal or heart muscle tissue (not shown). TLC immunostaining with anti-G_{M2} antibody gave an interesting result, detecting this ganglioside only in skeletal, but not in human heart muscle (Fig. 5B, lanes b and c,

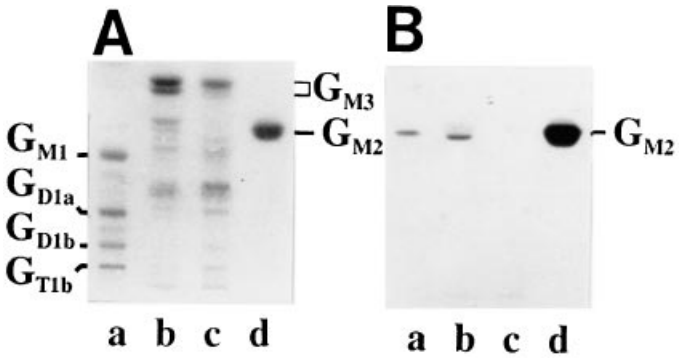

Figure 5. Resorcinol stain (A) and TLC immunostain (B) of gangliosides from human skeletal and heart muscle with the anti-G_{M2} antibody. Lanes a, 4 μg of human brain gangliosides (reference); lanes b, gangliosides from human skeletal muscle corresponding to 0.2 g of wet weight tissue; lanes c, gangliosides from human heart muscle corresponding to 0.2 g of wet weight tissue; lanes d, 2.5 μg of G_{M2} (reference). Gangliosides were chromatographed in solvent 2.

respectively). However, only traces of G_{M2} were detected by this highly specific and sensitive antibody. The antibody did not bind either to precursor G_{M3} or to elongated G_{M1} and G_{M1}-core gangliosides (Fig. 5).

Detection of G_{M1}-core gangliosides in human skeletal and heart muscle with combined choleragenoid-neuraminidase-antibody reaction

Detection of ganglio-series gangliosides of the G_{M1}-type with combined choleragenoid, neuraminidase and anti-choleragenoid antibody detection revealed the presence of these compounds in both muscle types with a very different pattern of distribution. G_{M1} can be visualized on a HPTLC plate with choleragenoid, and gangliosides G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b} can be detected by conversion with *V. cholerae* neuraminidase to G_{M1} before treatment with choleragenoid. The suitability of this specific overlay assay is demonstrated in Fig. 6, using a reference ganglioside mixture from human brain as positive and human granulocytes as negative control. G_{M1} of human brain (Fig. 6A, lane a), human skeletal muscle (Fig. 6A, lane b) and human heart muscle (Fig. 6A, lane c) was detected by choleragenoid. The higher sialylated gangliosides with G_{M1}-backbone were detected after neuraminidase treatment (Fig. 6B, lanes a–c) revealing a different distribution of these compounds in skeletal and cardiac muscle. The presence of the whole group of G_{M1}-type gangliosides is much larger in heart than in skeletal muscle. Only small quantities of G_{M1} and G_{D1a} together with traces of G_{D1b} and G_{T1b} could be detected in skeletal muscle (Fig. 6B, lane b), whereas much larger amounts of these gangliosides with G_{M1}-backbone are found in heart muscle (Fig. 6B, lane c). The amounts of G_{M1} and G_{D1a} are 1.5-fold higher while the quantities of G_{D1b} and G_{T1b} are even eight- to nine-fold larger in cardiac than in skeletal muscle.

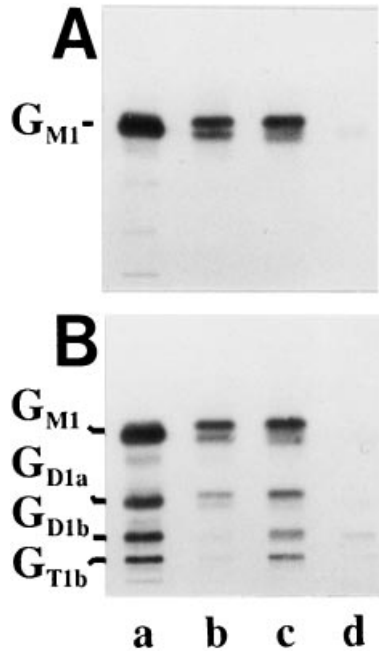

Figure 6. Detection of gangliotetraose-type gangliosides in skeletal and heart muscle by means of a combined neuraminidase and choleragenoid-immunostaining assay. Gangliosides were chromatographed in solvent 2, and then stained with choleragenoid without (A) and with previous *V. cholerae* neuraminidase treatment (B). Lanes a, 0.08 μg of human brain gangliosides (reference); lanes b, gangliosides from human skeletal muscle corresponding to 0.004 g of wet weight tissue; lanes c, gangliosides from human heart muscle corresponding to 0.004 g of wet weight tissue; lanes d, 0.1 μg of human granulocytes gangliosides (reference).

Overlay tests for detection of ganglio-series gangliosides of the G_{M1b}-type were also performed, giving negative results for G_{M1b} and $G_{D1\alpha}$ in both types of muscles.

TLC immunostain of neolacto-series gangliosides in human skeletal and heart muscle

The selective detection of terminally α2-3 and α2-6 sialylated neolacto-series gangliosides involves immunostaining of separated gangliosides with $nLcOse_4Cer$ antibody after *V. cholerae* neuraminidase treatment or without enzyme treatment. This highly specific polyclonal anti-$nLcOse_4Cer$ antibody binds to the terminal Galβ1-4GlcNAc dissaccharide of $nLcOse_4Cer$ and $nLcOse_6Cer$. The steric hindrance of sialic acid bound at position 3 to the terminal galactose prevents binding of the antibody, whereas sialylation at position 6 to the terminal galactose does not hinder recognition [30]. Neolacto-series gangliosides from human granulocytes composed of G_{M3}(Neu5Ac), IV^3Neu5Ac-$nLcOse_4Cer$, IV^6Neu5Ac-$nLcOse_4Cer$ and VI^3Neu5Ac-$nLcOse_6Cer$ served as references [31]. Each ganglioside migrates as a double band due to substitution with a C_{24}-fatty acid (upper band) and a C_{16}-fatty acid (lower band), as shown on resorcinol staining in Fig. 7A (lane c). The presence of lactosamine-containing gangliosides in human skeletal and

heart muscle was revealed by this sensitive method. Two faint resorcinol positive bands below G_{M3} observed in both muscles (Fig. 7A, lanes a and b) were identified as α2-3 sialylated gangliosides with an $nLcOse_4Cer$ backbone. The overlay assay without neuraminidase treatment showed two positive $IV^6Neu5Ac$-$nLcOse_4Cer$ bands (Fig. 7B, lane c) only in the reference mixture, but no positive α2-6 sialylated $nLcOse_4Cer$ in the muscle probes (Fig. 7B, lanes a and b). After treatment of gangliosides on the chromatogram with neuraminidase, α2-3 sialylated neolacto-series gangliosides became accessible to the antibody as seen in Fig. 7C as newly appearing bands on the level of $IV^3Neu5Ac$-$nLcOse_4Cer$. The intensity of $IV^3Neu5Ac$-$nLcOse_4Cer$ upper bands is almost identical in both muscle types, while the lower band is somewhat less expressed in heart muscle. The positive stain appearing at the position of lower band $IV^6Neu5Ac$-$nLcOse_4Cer$ does not represent this compound (compare with Fig. 7B, lanes a and b) and

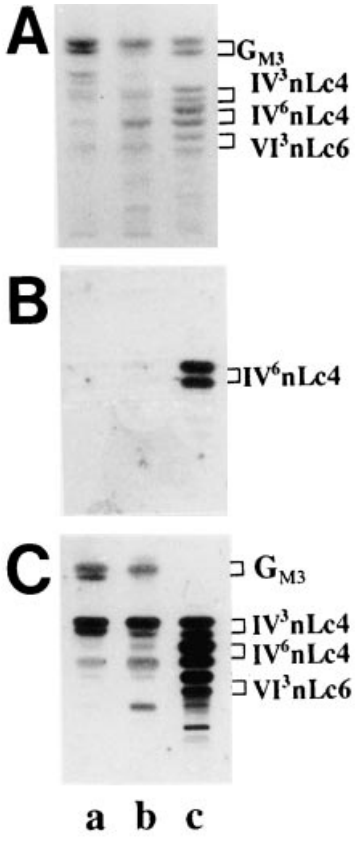

Figure 7. Detection of neolacto-series gangliosides in skeletal and heart muscle. (A) Resorcinol stain, (B) TLC immunostain with the anti-$nLcOse_4Cer$ antibody without *V. cholerae* neuraminidase and (C) after enzyme treatment. Lanes a, gangliosides from human skeletal muscle corresponding to 0.2 g (A) and 0.02 g (B and C) of wet weight tissue; lanes b, gangliosides from human heart muscle corresponding to 0.2 g (A) and 0.02 g (B and C) of wet weight tissue; lanes c, 7 μg (A) and 0.5 μg (B and C) gangliosides from human granulocytes (reference). Gangliosides were chromatographed in solvent 2. nLc4, $nLcOse_4Cer$; nLc6, $nLcOse_6Cer$.

probably shows disialylated $IV^3(Neu5Ac)_2$-$nLcOse_4Cer$. In all ganglioside fractions comprising G_{M3} as the predominant compound visualized with resorcinol (see Fig. 7A), concentration-dependent cross reaction of the anti-$nLcOse_4Cer$ antibody with the Galβ1-4Glc-terminus of desialylated G_{M3} (= LacCer) was observed (Fig. 7C, lanes a and b). This cross reaction with desialylated G_{M3} is not revealed in the human granulocytes ganglioside fraction, because much less GSL material was applied for the immunostain (Fig. 7C, lane c) compared to the resorcinol stain (Fig. 7A) and compared to muscle probes (Fig. 7A–C, lanes a and b).

Discussion

Muscle is known for the lowest ganglioside concentration from all vertebrate tissues studied so far, and probably this is the reason for relatively few studies dealing with muscle glycosphingolipids. In an early comparative analysis on gangliosides in various extraneural tissues, the ganglioside content in cardiac and skeletal muscle of rat, rabbit and pig was found to be one-fiftieth of that in brain and approximately one-third to one-quarter of those in other organs, such as kidney, lung and liver [47]. Further quantification of lipid bound sialic acids of various rabbit tissues gave the lowest concentration in skeletal muscle amounting to 1.4% of that in the brain [48]. G_{M3} and neolacto-series gangliosides are the predominant gangliosides common to heart and skeletal muscle of all avian and mammalian animals investigated so far [16, 17, 47–53]. Ganglio-series [54] as well as globo-series gangliosides [55, 56] have also been reported to be present in chicken skeletal muscle. The GSL expression of human skeletal muscle was first examined by Svennerholm *et al.* [18] and since then there have been only a few more studies of skeletal muscle GSLs in man [16, 19, 21, 57, 58] and even less of human heart muscle [20, 59]. However, very little is known about the biological function of GSLs in muscle tissue [60, 61].

Sensitive enzyme-immunostaining procedures using specific antibodies (overlay technique) have been developed for the detection of GSLs which cannot be detected with classical methods. This approach is a convenient diagnostic tool, particularly if only small samples of human material probes are available, e.g. in the case of cerebrospinal fluid [62, 63] or biopsies of tumour tissues [64]. We show in this paper, using tissue containing the lowest GSL concentration in humans, that reliable GSL composition could be elucidated from small samples of muscle. A panel of specific poly- and monoclonal antibodies was used, and results obtained were in good accordance with formerly performed immunostainings of cryopreserved human skeletal and cardiac muscle sections [21]. Neutral GSLs were identified in TLC runs of muscle aliquots corresponding to 0.1 g wet weight ($GbOse_3Cer$, $GbOse_4Cer$) and 0.4 g wet weight ($nLcOse_4Cer$). The gangliosides G_{M3} and G_{M2} were elucidated in runs which corresponded to 0.2 g wet weight muscle

tissues, whereas only 0.02 g and 0.004 g wet weight aliquots were necessary for identification of terminally sialylated neolacto-type and G_{M1}-core gangliosides (G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}), respectively, in skeletal and heart muscle. Along with the progress in cell culture and recombinant DNA technologies, several groups attempted the transfer of *in vitro* propagated myotube precursor cells (= myoblasts) from autologous as well as heterologous donors to patients suffering from inherited myopathies or from diseases due to special gene defects [65]. The concept of therapy by means of grafts of normal myogenic cells has been proved by myoblast transfer therapy in human Duchenne muscular dystrophy [66, 67]. There are data which provide evidence of the utilization of the high regeneration potential of muscle tissue and makes this muscle cell/gene transfer system an attractive candidate for clinical application [68–72].

From this viewpoint, a series of experiments performed by Leskawa and colleagues, in which they analysed the biosynthesis of GSLs during myogenesis *in vitro* [73, 74] became of special interest. In recent experiments the involvement of membrane GSLs in myoblast/myotube differentiation, i.e. membrane fusion of myoblasts to form large multinucleated myotubes, has been hypothesized, deduced from GSL expression and determination of several glycosyltransferase activities during formation of myotubes [75]. The *in vivo* fusion of implanted myoblasts is essential for successful muscle cell therapy [66, 67]. In this context it is noteworthy to mention that significantly reduced GSL expression in *mdx* mouse skeletal muscle, a model of human Duchenne muscular dystrophy, was observed in a recent immunohistological investigation [76].

In conclusion, TLC based overlay analysis of GSLs from muscle biopsy (recipient) and of myoblast producer (donor) could provide more information concerning the sarcolemmal biomembrane assembly with regards to host *versus* graft compatibility throughout muscle cell gene therapy or rendering insights into the fusion-potential of *in vitro* subconfluent/confluent grown myoblasts [75]. These applications are not speculative, even at this stage of research, but rather practicable in the near future. Pig heart muscle as cardiac xenografts for human therapy are just under investigation [77].

Acknowledgements

This work was financially supported by the Deutsche Forschungsgemeinschaft (DFG) SFB 223 'Pathomechanisms of Cellular Interactions', project C06 and by grants from the Bundesministerium für Forschung und Technologie (German-Croatian cooperation programme, coordinated by Internationales Büro, KFA-Jülich). We thank Dr Johanna Krawczynski and Mrs Gabriele Weiland (KFA-Jülich) for their kind cooperation and administrative help.

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Received 13 February 1996 and accepted as revised 19 April 1996