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# Expression of neutral glycosphingolipids and gangliosides in human skeletal and heart muscle determined by indirect immunofluorescence staining

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The expression of neutral glycosphingolipids and gangliosides has been studied in human skeletal and heart muscle using indirect immunofluorescence microscopy. Transversal and longitudinal cryosections were immunostained with specific monoclonal and polyclonal antibodies against the neutral glycosphingolipids lactosylceramide, globoside, Forssman glycosphingolipid, gangliotetraosylceramide, lacto-*N-neo*tetraosylceramide and against the gangliosides $G_{M3}$(Neu5Ac) and $G_{M1}$(Neu5Ac). To confirm the lipid nature of positive staining, control sections were treated with methanol and chloroform:methanol (1:1) before immunostaining. These controls were found to be either negative or strongly reduced in fluorescence intensity, suggesting that lipid bound oligosaccharides were detected. In human skeletal muscle, lactosylceramide was found to be the main neutral glycosphinogolipid. Globoside was moderately expressed, lacto-*N-neo*tetraosylceramide and gangliotetraosylceramide were minimally expressed and Forssman glycosphingolipid was not detected in human skeletal muscle. The intensities of the immunohistological stains of $G_{M3}$ and $G_{M1}$ correlated to the fact that $G_{M3}$ is the major ganglioside in skeletal muscle whereas $G_{M1}$ is expressed only weakly. In human heart muscle globoside was the major neutral glycosphingolipid. Lactosylceramide and lacto-*N-neo*tetraosylceramide were moderately expressed, gangliotetraosylceramide was weakly expressed and the Forssman glycosphingolipid was not expressed at all in cardiac muscle. $G_{M3}$ and $G_{M1}$ were detected with almost identical intensity. All glycosphingolipids were present in plasma membranes as well as at the intracellular level.

*Keywords*: gangliosides; neutral GSLs; human skeletal muscle; human heart muscle; immunohistochemistry

*Abbreviations used*: BSA, bovine serum albumin; DAPI, 4′,6-diamidine-2-phenylindole-dihydrochloride; DTAF, fluorescein isothiocyanate derivative; GSL(s), glycosphingolipid(s); Neu5Ac, *N*-acetylneuraminic acid [50]; PBS, phosphate buffered saline. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations [51] and the nomenclature of Svennerholm [52]. Lactosylceramide or LacCer, Galβ1-4Glcβ1-1Cer; gangliotriaosylceramide or GgOse$_3$Cer, GalNAcβ1-4Galβ1-4Glcβ1-1Cer; globotriaosylceramide or GbOse$_3$Cer, Galα1-4Galβ1-4Glcβ1-1Cer; gangliotetraosylceramide or GgOse$_4$Cer, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; globotetraosylceramide or GbOse$_4$Cer, GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer; lacto-*N-neo*tetraosylceramide or nLcOse$_4$Cer, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; Forssman GSL or GbOse$_5$Cer, GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer; $G_{M3}$, II$^3$Neu5Ac-LacCer; $G_{M2}$, II$^3$Neu5Ac-GgOse$_3$Cer; $G_{M1}$, II$^3$Neu5Ac-GgOse$_4$Cer; $G_{D3}$ II$^3$(Neu5Ac)$_2$-LacCer; $G_{D2}$, II$^3$(Neu5Ac)$_2$-GgOse$_3$Cer; $G_{D1a}$, IV$^3$Neu5Ac, II$^3$Neu5Ac-GgOse$_4$Cer; $G_{D1b}$, II$^3$(Neu5Ac)$_2$-GgOse$_4$Cer.

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## Introduction

Glycosphingolipids (GSLs) are ubiquitous membrane components which exhibit a huge structural variety. They 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 characterized by the presence of one or more sialic acids [1, 2]. Although they are most abundant in neural tissues, gangliosides have been detected as minor constituents of plasma membranes of all vertebrate tissues and species, where they are assembled in cell- and species-specific patterns [3]. They act as receptors for toxins and bacteria [4], viruses [5] and other ligands and are believed to be involved in cell-cell recognition phenomena. Gangliosides have been implicated in a number of biological processes including the regulation of cell growth, differentiation and morphogenesis. The modulation of membrane permeability and fluidity, and the determination of antigenic sites as well as influencing signal transduction through GSL specific interactions with plasma membrane proteins (for reviews see [6–9] and references therein) are well known functional attributes.

Although muscles together make up approximately 40% of total body weight, there have been only a few studies of the GSL expression in muscle, probably because of their low concentrations compared with that in neural tissue [10–12]. In particular the amount of gangliosides is relatively low in extraneural tissues, but the lowest amounts so far have been found in myocardium and in skeletal muscle [13]. For this reason relatively little is known about the localization of GSLs in mammalian skeletal and heart muscle, especially in humans. The concept that GSLs occur predominantly in the outer leaflet of the plasma membrane persists despite an increasing body of biochemical and histochemical data giving evidence for the presence of considerable intracellular quantities of these compounds [14–19]. Immunohistochemistry should be an adequate method for detecting not only the presence of weakly expressed compounds but also for discovering their localization in tissue structures. Therefore in this study we have investigated the expression of GSLs in both types of human muscle by indirect immunofluorescence microscopy using specific monoclonal and polyclonal antibodies. To our knowledge, this is the first report of the immunohistochemical analysis of neutral GSLs and gangliosides in human skeletal and cardiac muscle. Preliminary results have been reported [20].

## Materials and methods

*Muscle*

Human *quadriceps femoris*, *rectus abdominalis* and human cardiac muscle were obtained at autopsy 12–15 h post mortem of two males aged 39 and 66 years and two females aged 42 and 52 years. The cause of death was either a car accident or a suicide. There were no clinical records of muscle or heart disease and the muscles appeared macroscopically normal.

*Antibodies*

All polyclonal anti-GSL antibodies were produced according to the method of Kasai *et al.* [21]. HPLC purified LacCer, $GbOse_4Cer$, $GgOse_4Cer$ and $nLcOse_4Cer$ were used for immunization. The highly specific rabbit antibody against $GgOse_4Cer$ has been characterized as earlier reported [22, 23], and the specificity of the anti-$nLcOse_4Cer$ antibody has been recently described [24]. The anti-globoside antibody strongly reacted with $GbOse_4Cer$ (GalNAc$\beta$1-3Gal-R) and only trace cross-reactivity was observed towards $GgOse_4Cer$(Gal$\beta$1-3GalNAc-R). All other neutral GSLs tested, LacCer, $GbOse_3Cer$, $GgOse_3Cer$, $nLcOse_4Cer$ and Forssman GSL were found to be completely negative. The applicability of this antibody has been recently reported [25]. The anti-LacCer antibody strongly binds to lactosylceramide (Gal$\beta$1-4Glc-R) and in considerable quantity to $nLcOse_4Cer$ (Gal$\beta$1-4GlcNAc-R) due to the homology of the disaccharide termini. The antibody did not cross-react at all with $GbOse_3Cer$, $GgOse_3Cer$, $GbOse_4Cer$ and Forssman GSL, when up to 5 µg was tested on HPTLC plates. When high quantities (> 5 µg) of $GgOse_4Cer$ were applied to a TLC plate, trace cross-reactivity was observed. The anti-$G_{M3}$(Neu5Ac) antibody was used as recently described [25] and the exact specificity is explained in a very recent paper [26]. The anti-$G_{M1}$(Neu5Ac) antibody showed identical binding property compared with $G_{M1}$-specific cholera toxin B subunit (choleragenoid) as described [27]. Mouse monoclonal IgM anti-$nLcOse_4Cer$ antibody was produced with the clone 1B2-1B7 (TIB 189, American Type Culture Collection, USA) [28]. Rat IgG2c monoclonal anti-Forssman GSL antibody [29] was a kind gift of Dr U. Bethke (Biotest Pharma GmbH, Germany). $G_{M3}$(Neu5Ac) and $G_{M1}$(Neu5Ac), used for immunization, were from Dr Pallmann GmbH (Germany). All employed anti-GSL antibodies are listed in Table 1.

*Section preparation from skeletal muscle*

Muscle tissue was cut into small blocks, frozen in propane chilled with liquid nitrogen and stored at −70°C. Frozen tissue was cut into 8 µm sections with a cryomicrotome (Kryostat 1720, Leitz, Germany). Cryosections were mounted on gelatinized microscope glass slides. Air dried cryosections were fixed for 4 min in 2.5% glutaraldehyde in phosphate buffered saline (PBS). After rinsing four times in cold PBS for 10 min each, the sections were incubated three times for 4 min each with freshly prepared 0.1% $NaBH_4$ in PBS. The sections were then rinsed and unspecific binding of antibodies was blocked with 1% bovine serum albumin (BSA), 0.02% $NaN_3$ in PBS for 30 min.

*Immunostaining procedure*

After blocking, sections were washed three times for 10 min with 0.05% Tween 21 in PBS and then incubated for 2 h

**Table 1.** GSL specific monoclonal and polyclonal antibodies employed for immunohistological analyses.

| *GSL antigen* | *Structure* | *Antibody* |
|---|---|---|
| LacCer | Galβ1-4Glcβ1-1Cer | chicken antiserum |
| $nLcOse_4Cer$ | Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer | chicken antiserum |
| $nLcOse_4Cer$ | Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer | mouse IgM, mAb[a] |
| $GbOse_4Cer$ | GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer | chicken antiserum |
| $GgOse_4Cer$ | Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer | rabbit antiserum |
| Forssman GSL | GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer | rat IgG2c, mAb[a] |
| $G_{M3}$(Neu5Ac) | $II^3$Neu5Ac-LacCer | chicken antiserum |
| $G_{M1}$(Neu5Ac) | $II^3$Neu5Ac-$GgOse_4$Cer | rabbit antiserum |

[a] mAb, monoclonal antibody (hybridoma supernatant).

with 50 μl of anti-GSL antibodies, diluted 1:15 in 1% BSA, 0.02% $NaN_3$ in PBS. After incubation, sections were rinsed three times each with PBS and stained for 1 h with a 1:40 dilution of fluorescein-conjugated second antibodies in 1% BSA, 0.02% $NaN_3$ in PBS. DTAF-conjugated affinity chromatography-purified rabbit anti-chicken IgG, donkey anti-rat IgG and mouse anti-rabbit IgG antisera were from Dianova (Germany). Control slides were stained only with these second antibodies. After washing in PBS, nuclear DNA of the muscle cells was stained with 4′,6-diamidine-2-phenylindole-dihydrochloride (DAPI, Boehringer, Germany). 50 μl of a 1:1000 diluted stock solution (0.01% DAPI in PBS) were used for staining per section ($10^{-5}$% final concentration). The sections were then washed again and embedded with Mowiol (Hoechst, Germany). 40% Mowiol in glycerol (w/w) was diluted with Tris/HCl buffer (pH 8.5) to a final 20% and this was used for embedding. Aliquots were frozen and stored at −20°C.

### *Immunofluorescence microscopy*

Bound DTAF labelled antibodies as well as stained nuclei were evaluated under a fluorescence microscope (Axioskop, Zeiss, Germany) equipped with a camera (MC 63 A, Zeiss). Filter sets used were adequate to the maxima of absorption/emission of DTAF (495 nm/528 nm) and of DAPI (368 nm/488 nm). The sections were photographed on a professional black and white negative film (Kodak TMAX 400 ASA, Eastman Kodak Company, USA).

### *Lipid extraction from cryosections*

To confirm the lipid nature of the positive staining with the anti-GSL antibodies, air dried sections were treated with methanol and chloroform:methanol (1:1, by vol), for 10 min each [30]. After lipid extraction, the sections were air dried, rinsed with PBS for 10 min and stained by the same procedure as described above.

### *Neuraminidase treatment*

Sections were prepared as described above and before blocking with BSA were exposed to 50 μl of acylneuraminyl hydrolase from Vibrio cholerae (Behring, Germany) at a concentration of 0.2 U $ml^{-1}$ in 50 mmol $l^{-1}$ sodium acetate and 9 mmol $l^{-1}$ calcium chloride buffer solution at pH 5.5 for 1 h at 37°C. Control sections were incubated with the buffer only. Delipidated control sections (methanol and chloroform:methanol treated) were handled in the same manner. After enzyme treatment, the sections were washed with PBS, blocked with 1% BSA and immunostained as described above.

## Results

We have investigated the expression of GSLs of human skeletal and heart muscle from four individuals using indirect immunofluorescence microscopy. Monoclonal antibodies against Forssman GSL and $nLcOse_4Cer$ as well as polyclonal anti-LacCer, anti-$nLcOse_4Cer$, anti-$GbOse_4Cer$, anti-$GgOse_4Cer$, anti-$G_{M3}$(Neu5Ac) and anti-$G_{M1}$(Neu5Ac) were used for examinations of transversal and longitudinal muscle cryosections. All employed anti-GSL antibodies together with the antigenic GSL structures are listed in Table 1. All secondary antibodies were DTAF-labelled. DAPI stain for nuclear DNA was applied on all sections as a second stain after immunostaining with specific anti-GSL antibodies. The DAPI stain does not interfere with the DTAF label due to their different maxima of absorption/emission and therefore this double staining procedure is practicable. The advantage is that in all the sections regardless of the anti-GSL antibody used we could detect the nuclei on the same section and that way confirm the localization of sarcolemma.

For each anti-GSL antibody used, we determined the intensity of fluorescence and the presence of investigated GSL in sarcolemma as well as intracellularly. The intensities of immunofluorescence for each antibody employed for immunohistochemical analysis of human skeletal muscle are listed in Table 2 and for myocardium in Table 3, grading from appearance in the range from − for completely negative stain to + + + + + for immunofluorescence of the highest intensity.

*Immunohistochemical detection of gangliosides in human skeletal muscle*

$G_{M3}$(Neu5Ac) is the major ganglioside in human skeletal muscle [10] and accordingly, anti-$G_{M3}$(Neu5Ac) antibody gave a stain of the highest intensity which was marked with + + + + +. Anti-$G_{M3}$ antibody reacted strongly with the plasma membranes of muscle fibres as we expected, so that sarcolemma was brightly stained expressing regular and even distribution of detected $G_{M3}$ antigen along the membrane. Sections immunostained with this antibody showed also a patterned, strong intracellular fluorescence. The immunofluorescence stains of transversally and longitudinally cut skeletal muscle with anti-$G_{M3}$(Neu5Ac) antibody, in parallel with the corresponding DAPI stain of nuclear DNA of the same field, are shown in Figs 1 and 2, respectively. The detection of $G_{M1}$(Neu5Ac) epitope with anti-$G_{M1}$(Neu5Ac) antibody gave a much lower intensity of immunofluorescence of both sarcolemma and intracellular structures (Fig. 3). The sarcolemma revealed with this antibody appeared thicker and not as a fine, narrow line intensively stained as it was in the cryosections tested with anti-$G_{M3}$ antibody (for comparison see Fig. 1). Also a smaller amount of cytoplasmatic structures was bound by the anti-$G_{M1}$ antibody.

To confirm the lipid nature of these positive stainings, parallel sections were treated with methanol and chloroform:methanol (1:1) before immunostaining. These control stains with anti-$G_{M3}$ and anti-$G_{M1}$ antibodies, though slightly positive, clearly showed a strongly reduced intensity of fluorescence, suggesting that what has been detected are indeed lipid bound oligosaccharides (see Table 2).

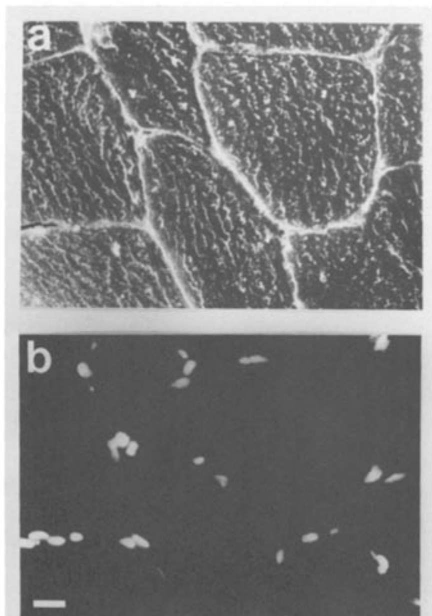


**Figure 1.** Immunofluorescence staining of transversal cryosection from human skeletal muscle with anti-$G_{M3}$(Neu5Ac) antibody. (A) Fluorescence micrograph; (B) DAPI stain of nuclear DNA of the same field. Bar = 10 μm.

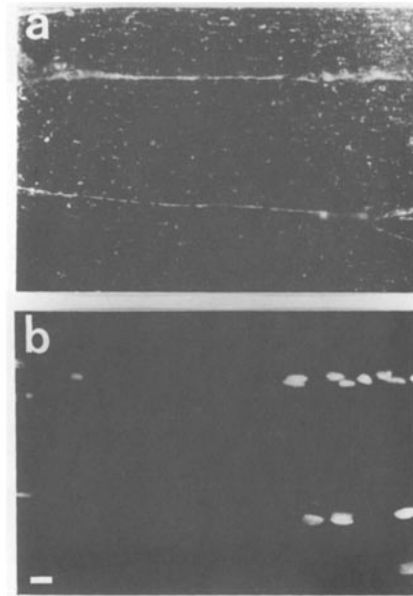


**Figure 2.** Immunofluorescence staining of longitudinal cryosection from human skeletal muscle with anti-$G_{M3}$(Neu5Ac) antibody. (A) Fluorescence micrograph; (B) DAPI stain of nuclear DNA of the same field. Bar = 10 μm.

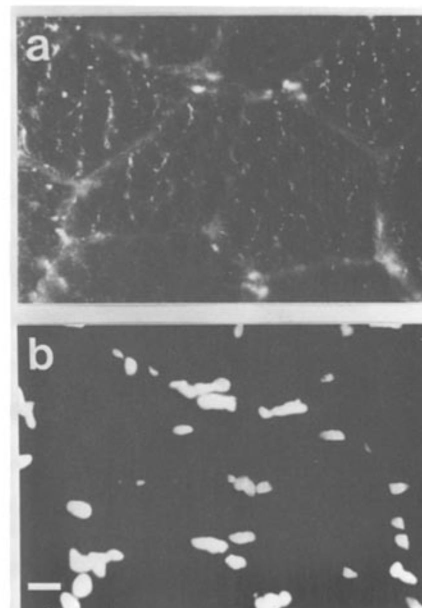


**Figure 3.** Localization of ganglioside $G_{M1}$-epitope via anti-$G_{M1}$ antibody immunoreactivity in a transversal cryosection of human skeletal muscle. (A) Fluorescence micrograph; (B) DAPI stain of nuclear DNA of the same field. Bar = 20 μm.

*Immunohistochemical detection of neutral GSLs in human skeletal muscle*

In our investigation LacCer was found to be the main neutral GSL because the immunostain with anti-LacCer

**Table 2.** Synopsis of the immunohistological analyses of cryosections of human skeletal muscle with GSL specific antibodies.

| *Primary anti-GSL antibody*[a] | *DTAF-labelled secondary antibody*[b] | *Intensity of fluorescence*[c] | | | |
|---|---|---|---|---|---|
| | | *Sarcolemma* | | *Cytoplasm* | |
| | | *GSL expression* | *Control*[d] | *GSL expression* | *Control*[d] |
| LacCer | rabbit anti-chicken IgG | +++++ | ++ | +++++ | ++ |
| $nLcOse_4Cer$ | rabbit anti-chicken IgG | +++ | + | +++ | + |
| $nLcOse_4Cer$ | goat anti-mouse IgM | – | – | – | – |
| $GbOse_4Cer$ | rabbit anti-chicken IgG | ++++ | + | +++ | + |
| $GgOse_4Cer$ | mouse anti-rabbit IgG | +++ | + | + | + |
| Forssman GSL | donkey anti-rat IgG | – | – | – | – |
| $G_{M3}$(Neu5Ac) | rabbit anti-chicken IgG | +++++ | + | ++++ | + |
| $G_{M1}$(Neu5Ac) | mouse anti-rabbit IgG | +++ | + | ++ | + |

[a] 1:15 dilution of antibodies listed in Table 1.
[b] Antibodies labelled with the more stable fluorescein derivative DTAF were used at a 1:40 dilution.
[c] Grading from appearance in the range of – to +++++.
[d] Treatment of the sections with methanol and chloroform:methanol (1:1).

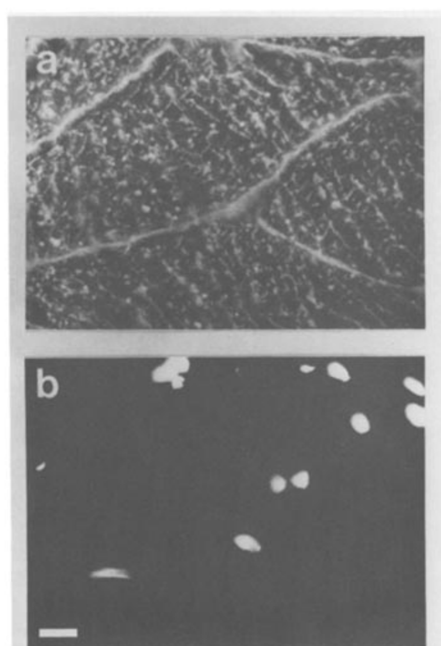


**Figure 4.** Anti-LacCer antibody staining of a cross-cryosection of human skeletal muscle. (A) Fluorescence micrograph; (B) DAPI stain of nuclear DNA of the same field. Bar = 10 μm.

antibody gave the most intensive, bright fluorescence. The localization of LacCer in a transversal section of human skeletal muscle detected with the respective antibody is shown in Fig. 4. Anti-LacCer antibody recognized its antigen present in the sarcolemma and also a large amount of LacCer distributed within the fibres. After lipid extraction of sections with methanol and chloroform:methanol (1:1) before immunostaining with anti-LacCer antibody, the reaction was weak and diffuse with some uneven, faint staining mainly in the central parts of the fibres (not shown, see Table 2).

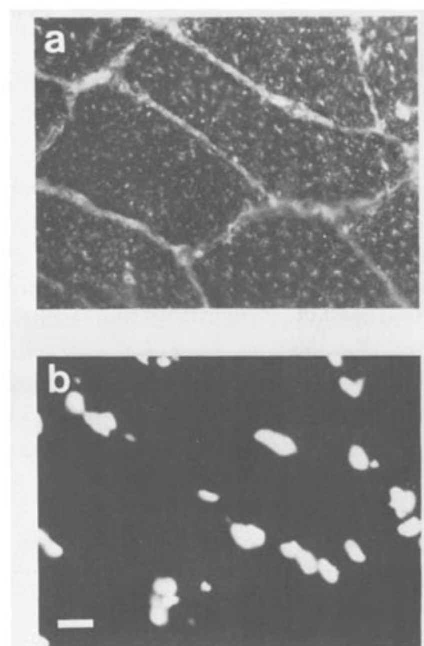


**Figure 5.** Immunofluorescence staining of a transversal cryosection of human skeletal muscle with anti-globoside antibody. (A) Fluorescence micrograph; (B) DAPI stain of nuclear DNA of the same field. Bar = 20 μm.

Globoside is a neutral GSL that was found to be moderately expressed in human skeletal muscle. Anti-globoside antibody strongly stained the sarcolemma showing variable distribution of antigen on the cell surface and a uniform, clustered intracellular stain (Fig. 5). The lipid extraction from cryosection by methanol and chloroform:methanol (1:1) almost completely abolished the staining reaction.

In our investigation the polyclonal anti-$nLcOse_4Cer$

antibody gave a positive stain in skeletal muscle although the monoclonal antibody against the same antigenic structure was negative. However, after neuraminidase treatment the sarcolemma became positively stained, but no intracellular fluorescence was found. The monoclonal anti-$nLcOse_4Cer$ antibody specifically recognizes the Gal$\beta$1-4GlcNAc sequence and does not cross-react with any sialylated *neo*lacto-series gangliosides. After removal of sialic acids by neuraminidase treatment, the *neo*lacto backbone of gangliosides becomes approachable for the monoclonal anti-$nLcOse_4Cer$ antibody. On the other hand, the polyclonal anti-$nLcOse_4Cer$ antibody is well known to bind to $\alpha$2-6 sialylated, but not to $\alpha$2-3 sialylated *neo*lacto core gangliosides without neuraminidase treatment [24]. The control sections treated only with the enzyme buffer without neuraminidase did not differ in any way from the nontreated sections.

$GgOse_4Cer$ is weakly expressed in human skeletal muscle since anti-$GgOse_4Cer$ antibody revealed very low immunofluorescent intensity. We failed to detect the Forssman GSL, because the staining with anti-Forssman GSL monoclonal antibody was always negative.

### *Immunohistochemical detection of gangliosides in human heart muscle*

Both anti-$G_{M3}$(Neu5Ac) and anti-$G_{M1}$(Neu5Ac) antibodies bound to their corresponding antigens localized on the plasma membranes of the cardiac muscle as well as intracellularly. The immunoreactivity was of high density along with a homogeneous distribution on the heart plasma membranes but with uneven, irregular intracellular stain. The intensities of anti-$G_{M3}$ and anti-$G_{M1}$ antibodies mediated immunofluorescence were almost identical, although a little brighter in cryosections exposed to anti-$G_{M3}$ antibody (see Table 3).

### *Immunohistochemical detection of neutral GSLs in human heart muscle*

From all tested anti-neutral GSLs antibodies the anti-$GbOse_4Cer$ antibody gave the stain of highest intensity, implying that globoside is the main myocardial neutral GSL in humans. The immunofluorescence stain of human heart muscle with anti-$GbOse_4Cer$ is shown in Fig. 6. The anti-$GbOse_4Cer$ antibody reacted strongly with the plasma membranes of cardiac cells and, although the cytoplasm was positively stained, no regular pattern of antibody binding could be observed as opposed to the immunostained sections of skeletal muscle with anti-globoside antibody (see Fig. 5). In myocardium the cytoplasmal antibody derived fluorescence showed homogeneous globoside distribution indicated by a more uniform binding of this antibody.

Anti-LacCer and anti-$nLcOse_4Cer$ antibodies also gave positive staining, almost identical in strength, but of a lesser degree compared with the globoside expression.

Anti-$GgOse_4Cer$ antibody gave a very low immunofluorescent intensity and the incubation with the anti-Forssman GSL monoclonal antibody was negative.

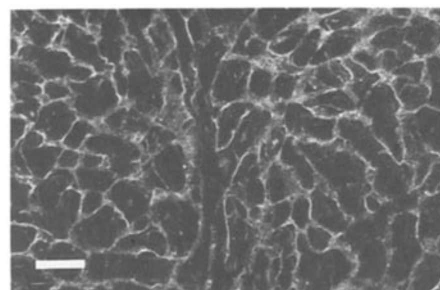

**Figure 6.** Immunofluorescence staining of a transversal cryosection from human heart muscle with anti-globoside antibody. Bar = 20 μm.

**Table 3.** Synopsis of the immunohistological analyses of cryosections of human myocardium with GSL specific antibodies.

| *Primary anti-GSL antibody*[a] | *DTAF-labelled secondary antibody*[b] | *Intensity of fluorescence*[c] | | | |
|---|---|---|---|---|---|
| | | *Sarcolemma* | | *Cytoplasm* | |
| | | *GSL expression* | *Control*[d] | *GSL expression* | *Control*[d] |
| LacCer | rabbit anti-chicken IgG | +++ | + | +++ | + |
| $nLcOse_4Cer$ | rabbit anti-chicken IgG | +++ | + | +++ | + |
| $nLcOse_4Cer$ | goat anti-mouse IgM | − | − | − | − |
| $GbOse_4Cer$ | rabbit anti-chicken IgG | +++++ | + | ++++ | + |
| $GgOse_4Cer$ | mouse anti-rabbit IgG | ++ | + | ++ | + |
| Forssman GSL | donkey anti-rat IgG | − | − | − | − |
| $G_{M3}$(Neu5Ac) | rabbit anti-chicken IgG | +++++ | + | ++++ | + |
| $G_{M1}$(Neu5Ac) | mouse anti-rabbit IgG | ++++ | + | +++ | + |

[a] 1:15 dilution of antibodies listed in Table 1.
[b] Antibodies labelled with the more stable fluorescein derivative DTAF were used at a 1:40 dilution.
[c] Grading from appearance in the range of − to +++++.
[d] Treatment of the sections with methanol and chloroform:methanol (1:1).

*Comparative analyses of GSL expression for different individuals*

We have analysed the muscle tissue from four adult individuals, two males and two females. Two different striated muscles were chosen for investigation: *quadriceps femoris* and *rectus abdominalis* muscle. Heart muscle was always taken from the inferior left ventricle. Between the four investigated individuals there were only minor and negligible differences in the expression of GSLs and the intensity and distribution of immunofluorescence were comparable for all of them. The results presented in Tables 2 and 3 are applicable to all four individuals.

**Discussion**

There have been relatively few studies of GSLs in muscles probably because of their low concentrations compared with neural tissue. The first study of qualitative and quantitative patterns of gangliosides in several extraneural tissues of rat, rabbit and pig was performed by Puro and coworkers [13] and they found the lowest ganglioside concentrations of all to be present in cardiac and skeletal muscles of all three examined species. Since then several studies of GSLs in skeletal muscles have been performed in numerous animal species, mainly in chicken [12, 31–33], mouse [25, 34], rabbit [35–38] and pig [39, 40]. In all investigations carried out so far remarkable species variations in the ganglioside patterns of skeletal muscles have been found, although mostly in the minor gangliosides [11]. The pattern of neutral GSLs also showed interspecies difference [25]. The GSLs of human skeletal muscle were first examined by Svennerholm *et al.* in 1972 [10] and since then there have been only a few studies of skeletal muscles in man [11, 31, 41] and even less of human heart muscle [42, 43].

Despite the well established interspecies differences, the major ganglioside component in skeletal muscles of all so far examined vertebrate animals was found to be $G_{M3}$ [11, 31–41]. As was expected from these data, the anti-$G_{M3}$(Neu5Ac) staining of skeletal muscle cryosections gave the highest intensity of stain in our investigation. This is in agreement with the findings that $G_{M3}$(Neu5Ac) also predominates in human skeletal muscle, constituting almost 2/3 of the total gangliosides [10, 11]. The less abundant sialic acid carrying GSLs of human skeletal muscle are $G_{M2}$, $G_{D1a}$ and a not fully characterized monosialosyltetraglycosylceramide [10, 11, 31, 44]. $G_{M1}$ is present in very small amounts, so the detection with anti-$G_{M1}$(Neu5Ac) antibody gave a much lower intensity of immunofluorescence.

The total content of gangliosides in human skeletal muscle is very small, 52 nmol $g^{-1}$ wet weight, but the total content of neutral GSLs is even smaller, approximately 40 nmol $g^{-1}$ wet weight [10]. In all of the previous studies concerning neutral GSL expression in human skeletal muscle, lactosylceramide was found to be predominant, followed by $GbOse_3Cer$, $GbOse_4Cer$, monohexosylceramides which were found to consist mainly of galactosylceramide, and $nLcOse_4Cer$ [10, 31, 41]. Our investigation confirmed LacCer being the main neutral GSL in skeletal muscle, globoside being only moderately and $nLcOse_4Cer$ and $GgOse_4Cer$ weakly expressed. The absence of Forssman GSL was characteristic for human skeletal muscle.

Qualitatively, human cardiac muscle contains the same gangliosides as those of human skeletal muscle: $G_{M3}$, $G_{M2}$, $G_{M1}$, $G_{D1a}$ and $G_{D1b}$ being the most abundant. However, the respective distribution of gangliosides is quite different. Human myocardium contains a more equalized distribution of $G_{M3}$(31%), $G_{M1}$(22%), $G_{M2}$(18%) and $G_{D3}$(15%) [42], while in skeletal muscle $G_{M3}$(67%) clearly predominates [10]. The immunohistochemical analyses confirm these findings since the immunofluorescence intensities elicited with anti-$G_{M3}$ and anti-$G_{M1}$ antibodies were almost identical in the case of heart muscle (Table 3), unlike the case of high anti-$G_{M3}$ compared with faint anti-$G_{M1}$ stain in skeletal muscle (Table 2). Our results also suggest that human cardiac muscle expresses both gangliosides in identical proportions.

Compared with the simple neutral GSL pattern in skeletal muscle with predominant LacCer and low over-all concentration of about 40 nmol $g^{-1}$ wet weight, the pattern of the neutral GSLs in human heart muscle is much more complex and the total content ranges between 90–160 nmol $g^{-1}$ wet weight [42, 43]. The main neutral GSL in heart muscle is globoside, followed by globotriaosylceramide, lactosylceramide, monohexosylceramide as a mixture of glucosylceramide and galactosylceramide, and lacto-*N-neo*tetraosylceramide [42]. We found moderate expression of LacCer and $nLcOse_4Cer$ and small amounts of $GgOse_4Cer$. As in skeletal muscle, human cardiac muscle also completely lacked Forssman GSL.

Our investigation has shown that, apart from well established interspecies differences in the patterns of GSLs in a particular tissue, striking differences exist for both neutral and acidic compounds in two types of muscle tissue within the same species: skeletal and heart in humans. Heart and skeletal muscle, which have some cytological similarity due to the fact that they are both striated muscle tissue, not only have a different composition of neutral GSLs and gangliosides but they also show immunohistochemical differences in expression of those compounds in plasma membranes as well as at the intracellular level. For many years the prevailing view has been that glycolipids are located primarily in the outer leaflet of the plasma membranes, but recent biochemical and histochemical studies gave more evidence on the presence of these compounds intracellularly [14–19, 45]. It has been suggested that subcellular localization of GSLs varies depending on

the GSL structure and cell type [18]. Our results are in agreement with this statement since we have found that different GSLs give distinct patterns of intracellular expression in each muscle cell type. We have also determined that a single GSL exhibits variable subcellular localization in different cells, even if they are both muscle tissue, although of two different types: skeletal and heart muscle. This could be explained on the basis that certain unique ultrastructural features are necessary for the specific functions that those organs perform.

Recent studies, that involved the possible role of gangliosides in muscle, have shown that they have modulatory effects on protein phosphorylation since it has been found that phosphorylation of phosphorylase b by phosphorylase kinase is stimulated by gangliosides [46]. Although gangliosides can rapidly stimulate the phosphorylation of phosphorylase b *in vitro*, the physiological significance of this ganglioside-related effect on the regulation of glycogen metabolism, that is so important for energy supply in muscle, is unknown. Also the role of several ganglioside-binding proteins, recently identified in the cytosolic fractions of both skeletal and cardiac muscle in guinea pig, remains undiscovered [47]. These findings suggest the existence of specific sorting mechanisms that regulate intracellular shuttling of GSLs.

Since there is a striking difference in GSL expression between skeletal and heart muscle, it is possible that the GSLs present in heart but not in skeletal muscle are somehow involved in specific functions which might be important for the performance of myocardium. The established or presumed effects of GSLs on $\beta$-adrenergic receptor, $Na^+$-transport and several protein kinases (especially $Ca^{2+}$-dependent kinases, see [7] and references therein) are of great importance in the normal function of the heart. For example, $G_{M1}$ of myocardium might be involved in the regulation of calcium permeability or calcium/calmodulin-dependent protein kinases, because it is well known that this ganglioside modulates calcium channels of gastric mucosa [48] as well as $Ca^{2+}$-dependent protein kinase in PC12 cells [49]. It would be of interest to check whether those GSLs found in higher proportions in the heart are implicated in those effects.

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