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Immunohistological analyses of neutral glycosphingolipids and gangliosides in normal mouse skeletal muscle and in mice with neuromuscular diseases

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The expression of neutral glycosphingolipids (GSLs) and gangliosides was investigated in cryosections of normal mouse skeletal muscle and in muscle of mice with neuromuscular diseases using indirect immunofluorescence microscopy. Transversal and longitudinal sections were immunostained with specific polyclonal antibodies against lactosylceramide, lacto-*N*-neotetraosylceramide, globoside, G_{M3}(Neu5Ac), G_{M3}(Neu5Gc) and G_{M1}(Neu5Ac) as well as monoclonal anti-Forssman GSL antibody. In normal CBA/J mouse muscle (control) the main immunohistochemically detected ganglioside was G_{M3}(Neu5Ac) followed by moderately expressed G_{M3}(Neu5Gc) and G_{M1}. The neutral GSLs lactosylceramide and globoside were stained with almost identical, high fluorescence intensity. Low amounts of lacto-*N*-neotetraosylceramide and trace quantities of Forssman GSL were immunostained. All GSLs were detected in the sarcolemma, but also in considerable amounts at the intracellular level. Mice with neuromuscular diseases were the A2G-adr mouse mutant (a model for human recessive myotonia of Becker type), the BL6-wr mutant (a model for motor neuron disease) and the BL10-mdx mouse mutant (a model for human Duchenne muscular dystrophy). No changes in GSL expression were found in the A2G-adr mouse, while muscle of the BL6-wr mouse showed increased intensity of immunofluorescence in stainings with anti-lactosylceramide and anti-G_{M3}(Neu5Ac) antibodies. Muscle of BL10-mdx mice showed the most prominent changes in GSL expression with reduced fluorescence intensity for all antibodies. Major differences were not observed in the intensities of GSLs, but there were significant differences in the patterns of distribution on plasma membrane and at the subcellular level. The exact nature and pathogenesis of these changes should be elucidated since such investigations could furnish advances in understanding the functional role of neutral GSLs and gangliosides in normal as well as in diseased muscle.

Keywords: gangliosides, neutral glycosphingolipids, antibodies, immunohistochemistry, mouse mutants, A2G-adr, BL6-wr, BL10-mdx

Abbreviations: BSA, bovine serum albumin; DAPI, 4', 6-diamidine-2-phenylindole-dihydrochloride; DTAF, dichlorotriazinylamino-fluorescein; GSL(s), glycosphingolipid(s); Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid [53]; PBS, phosphate buffered saline. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations [54] and the nomenclature of Svennerholm [55]. Lactosylceramide or LacCer, Galβ1-4Glcβ1-1Cer; gangliotriaosylceramide or $GgOse_3Cer$, GalNAcβ1-4Galβ1-4Glcβ1-1Cer; globotriaosylceramide or $GbOse_3Cer$, Galα1-4Galβ1-4Glcβ1-1Cer; gangliotetraosylceramide or $GgOse_4Cer$, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; globotetraosylceramide or $GbOse_4Cer$, GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer; lacto-*N*-neotetraosylceramide or $nLcOse_4Cer$, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; Forssman GSL or $GbOse_5Cer$, GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer; G_{M3}, II^{3}Neu5Ac-LacCer; G_{M1}, II^{3}Neu5Ac-$GgOse_4Cer$.

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Introduction

Neutral glycosphingolipids (GSLs) as well as gangliosides are located primarily in the outer leaflet of plasma membranes with the hydrophobic ceramide inserted in the lipid bilayer, whereas hydrophilic carbohydrate and sialic acids protrude into the extracellular space [1]. GSLs have been detected primarily as constituents of plasma membranes of all vertebrate tissues and species, but an increasing body of biochemical and histochemical data indicates the presence of considerable intracellular quantities of these compounds [2–5]. Gangliosides, for example, are implicated in cell type-specific adhesion processes as ligands for selectins and in a number of biological processes including the regulation of cell growth, differentiation and morphogenesis as well as oncogenesis (for reviews see [6–10] and references therein). They can act as biomodulators and their modulatory effects on protein phosphorylation were studied in skeletal muscle where several protein phosphorylation cascades have been well characterized [11, 12]. Although GSLs play a role in myogenesis and muscle cell differentiation [13] as well as in neuromuscular recognition [14], although it has been shown that the composition of muscle gangliosides changes after denervation [15], and although GSLs have been used as therapeutic agents in several neuromuscular disorders with varied success [16–24], little is known about their expression and distribution in both normal [25] and especially diseased muscle. We wanted to determine the expression of these compounds in normal mouse skeletal muscle and in skeletal muscle of several mouse mutants that are thought to be a good model for human diseases [26]. Therefore, we have investigated muscle GSL expression in normal CBA/J mouse (control), A2G-adr mouse mutant (a model for human recessive myotonia of Becker type) [27, 28], BL6-wr mouse mutant (a model for motor neuron disease) [29, 30] and in BL10-mdx mouse mutant (a model for human Duchenne muscular dystrophy) [31, 32]. For detection of GSLs in muscle cryosections by indirect immunofluorescence microscopy we used specific monoclonal and polyclonal antibodies. To our knowledge, this is the first report of immunohistochemical analyses of neutral GSLs and gangliosides in normal and diseased mouse skeletal muscle. Preliminary results have been reported [33].

Materials and methods

Animals and muscles

A2G mice carrying the recessive autosomal mutation, arrested development of righting response (adr), were generated by Drs R.L. Watts and D.L. Watts, Guy's Hospital, London. Homozygous adr/adr animals (designated 'ADR') can be recognized from post-natal day 8–9 onward having difficulties in righting themselves when placed supine [34]. Breeder mice of the C57BL/6J wr/+ stock are derived from The Children's Hospital, Harvard University. The phenotypic difference between homozygous wobbler (wr/wr, phenotype WR) mice and normal littermates (WT, genotype wr?/+) was obvious 25–30 days after birth by their reduced body weight, wobbly gait, difficulties in using their front legs and clasped front paws [30]. A mouse mutant has been obtained from a C57BL/10 inbred colony showing spontaneous mutation of X chromosome-linked muscular dystrophy (mdx) [31]. Animals exhibit histological lesions similar to human muscular dystrophy; their clinical symptoms are mild and they are viable and fertile. All inbred mice used in this study were kindly provided by Professor Dr H. Jockusch (Developmental Biology Unit, University of Bielefeld). Fresh skeletal muscles were taken from hindlegs of female 6-weeks-old normal CBA/J mice (control), 6-weeks-old A2G-adr, 9-weeks-old BL6-wr and 9-weeks-old male BL10-mdx mice, always from three different individuals.

Antibodies

All polyclonal anti-GSL antibodies were produced according to the method of Kasai *et al.* [35]. HPLC purified lactosylceramide (LacCer), globotetraosylceramide ($GbOse_4Cer$) and lacto-*N*-neotetraosylceramide ($nLcOse_4Cer$) were used for immunization. The specificity of the anti-$nLcOse_4Cer$ antibody has been recently described [36]. The anti-globoside antibody reacted strongly with $GbOse_4Cer$ (GalNAcβ1-3Gal-R) and use of this antibody has been reported recently [37]. The anti-LacCer antibody binds to lactosylceramide (Galβ1-4Glc-R) and in considerable quantity to $nLcOse_4Cer$ (Galβ1-4GlcNAc-R) due to the homology of the disaccharide termini. The antibody did not cross-react at all with globotriaosylceramide ($GbOse_3Cer$), gangliotriaosylceramide ($GgOse_3Cer$), $GbOse_4Cer$ and Forssman GSL. The anti-G_{M3}(Neu5Ac) antibody was used as described [37] and the specificity has been reported [38]. The anti-G_{M1}(Neu5Ac) antibody showed binding properties identical to G_{M1}-specific cholera toxin B subunit (choleragenoid) [39]. Rat IgG2c monoclonal anti-Forssman GSL antibody [40] was a kind gift of Dr U. Bethke (AmCell, Bergisch-Gladbach, Germany). A highly specific chicken antibody was raised against G_{M3}(Neu5Gc), isolated from a mouse-mouse hybridoma [37, 38]. All anti-GSL antibodies are listed in Table 1. Their applicability for immunostaining of GSL antigens in cryosections of skeletal muscle has been recently reported by Čačić *et al.* [25].

Section preparation from skeletal muscle

Muscle tissue was cut into small blocks, frozen in propane chilled with liquid nitrogen and stored at

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

GSL antigen	*Structure*	*Antibody*
LacCer	Galβ1-4Glcβ1-1Cer	Chicken antiserum
$nLcOse_4Cer$	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	Chicken antiserum
$GbOse_4Cer$	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	Chicken antiserum
Forssman GSL	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	Rat IgG2c, mAb[a]
G_{M3}(Neu5Ac)	II^{3}Neu5Ac-LacCer	Chicken antiserum
G_{M3}(Neu5Gc)	II^{3}Neu5Gc-LacCer	Chicken antiserum
G_{M1}(Neu5Ac)	II^{3}Neu5Ac-$GgOse_4Cer$	Rabbit antiserum

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

– 70 °C. The frozen tissue was cut into 8 μm sections with a cryomicrotome (Kryostat 1720, Leitz, Wetzlar, Germany) and mounted on gelatinized microscope glass slides. Air dried cryosections were fixed for 4 min in 2.5% glutaraldehyde in a 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 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 1:40 dilution of dichlorotriazinylamino-fluorescein(DTAF)-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 (Hamburg, 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, Mannheim, Germany). Fifty 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, Frankfurt a. M., Germany). Forty per cent Mowiol in glycerol (w/w) was diluted with a Tris/HCl buffer (pH 8.5) to a final 20% and 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, Oberkochen, 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, Rochester, NY, 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), each for 10 min [41]. After lipid extraction, the sections were air dried, rinsed with PBS for 10 min and stained by the same procedure as described above.

Results

The intensities of immunofluorescence for each antibody employed for immunohistochemical analysis of muscles are listed in Table 2, grading from appearance in the range from – for completely negative stain to + + + + + for the immunofluorescence of the highest intensity. To confirm the lipid nature of these positive stainings, parallel sections were always treated with methanol and chloroform:methanol (1:1) before immunostaining. These control stains were either negative or slightly positive with very reduced intensity of fluorescence, suggesting that what has been detected are indeed lipid bound oligosaccharides.

Immunohistochemical detection of glycosphingolipids in normal CBA/J mouse muscle

The immunofluorescence stain of transversally cut skeletal muscle with anti-G_{M3}(Neu5Ac) antibody in parallel with the corresponding DAPI stain of nuclear DNA of the same field is shown in Fig. 1. Anti-G_{M3}(Neu5Ac) antibody reacted strongly with the plasma membranes of muscle fibres as expected from biochemical analyses [37, 42]. Sections immunostained with this antibody also showed a clustered, strong intracellular fluorescence. The detection of G_{M3}(Neu5Gc)-epitope with anti-G_{M3}(Neu5Gc) antibody gave a lower intensity of immunofluorescence, and the immunostain with anti-G_{M1}(Neu5Ac) was weak and irregular (Table 2). Anti-LacCer and anti-$GbOse_4Cer$ gave fluorescence of the

Table 2. Synopsis of the immunohistochemical sarcolemmal analyses of cryosections from normal and neuromuscular diseased mouse skeletal muscles with GSL specific antibodies.

Primary anti-GSL antibody[a]	*Secondary antibody (DTAF-labelled)*[b]	*Intensity of fluorescence*[c]			
		CBA/J	*A2G-adr*	*BL6-wr*	*BL10-mdx*
LacCer	Rabbit anti-chicken IgG	+ + + +	+ + + +	+ + + + +	+ + +
$nLcOse_4Cer$	Rabbit anti-chicken IgG	+ + +	+ + +	+ + +	+ +
$GbOse_4Cer$	Rabbit anti-chicken IgG	+ + + +	+ + + +	+ + + +	+ + +
Forssman GSL	Donkey anti-rat IgG	+	+	+	+
G_{M3}(Neu5Ac)	Rabbit anti-chicken IgG	+ + + +	+ + + +	+ + + + +	+ +
G_{M3}(Neu5Gc)	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 + + + + +.

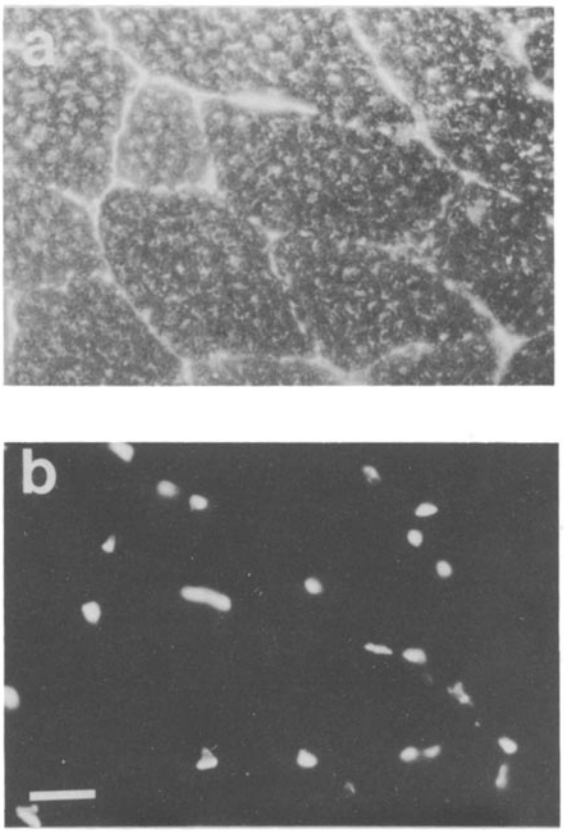

Figure 1. Immunofluorescence staining of transversal cryosection from normal mouse skeletal muscle with anti-G_{M3}(Neu5Ac) antibody. (a) Fluorescence micrograph; (b) DAPI stain of nuclear DNA of the same field. Bar = 25 μm.

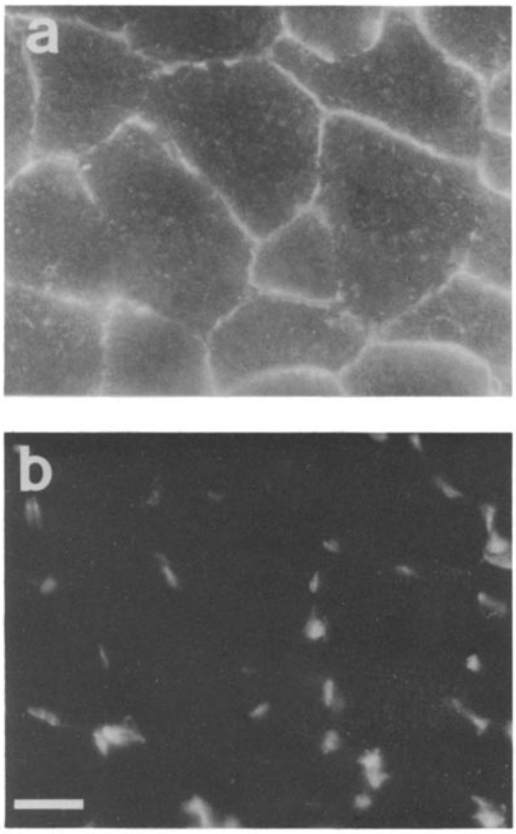

Figure 2. Anti-globoside antibody staining of a cross-cryosection of normal mouse skeletal muscle. (a) Fluorescence micrograph; (b) DAPI stain of nuclear DNA of the same field. Bar = 25 μm.

highest intensity. The staining of normal mouse skeletal muscle with anti-globoside antibody is shown in Fig. 2. We detected a fine, regular and even distribution of globoside in the sarcolemma as well as smaller amounts of intracellularly localized globoside. Anti-$nLcOse_4Cer$ gave a positive staining of moderate intensity while anti-Forssman GSL antibody staining was of minimal fluorescence.

Immunohistochemical detection of glycosphingolipids in A2G-adr mouse mutant muscle

In this mouse mutant the results obtained with immunostaining were very similar to the results from normal mouse skeletal muscle (Table 2). Anti-G_{M3}(Neu5Ac) antibody gave a very luminous immunofluorescent stain and the sarcolemma showed irregular and uneven distribution of G_{M3}(Neu5Ac) with sites of accumulated fluorescence giving a 'thicker' membrane (Fig. 3). Anti-G_{M3}(Neu5Gc) and anti-G_{M1}(Neu5Ac) antibody stainings were of moderate intensity. All anti-neutral GSL antibodies that we used gave a positive staining in this mouse mutant graded with the same intensity as for normal mouse muscle. Sarcolemma stained with anti-$GbOse_4Cer$ antibody appeared more spacious than in the case of control muscle and more intracellularly located fluorescence was detected. The same result was obtained with anti-LacCer staining: 'thicker' membranes and considerable amounts of intracellular LacCer. Anti-$nLcOse_4Cer$ was mildly positive while anti-Forssman GSL gave weak immunofluorescence. All muscle fibres were smaller in diameter than in normal mouse muscle, but no fibre degeneration was observed.

Immunohistochemical detection of glycosphingolipids in BL6-wr mouse mutant muscle

The intensity of fluorescence for all anti-GSL antibodies was either identical to normal mouse muscle, or increased as was the case with anti-G_{M3}(Neu5Ac) (Fig. 4) and anti-LacCer (Fig. 5). Intracellular stain was very similar to that in the normal mouse but the sarcolemma showed uneven distribution of immunofluorescence. The average diameter of the individual muscle fibre was slightly smaller than in the normal mouse muscle. There was no evidence of on-going degeneration in the muscle such as central nuclei, increased endomysial connective tissue or lymphocyte infiltration.

Immunohistochemical detection of glycosphingolipids in BL10-mdx mouse mutant muscle

All anti-GSL antibodies gave significantly reduced immunofluorescence intensity in cryosections from this mouse mutant (Table 2). Immunostaining was weak and identical in intensity with anti-G_{M3}(Neu5Ac) and anti-G_{M3}(Neu5Gc). The localization of anti-G_{M3}(Neu5Ac) antibody immunoreactivity is shown in Fig. 6. Muscle fibres show variations in size due to the enlargement of

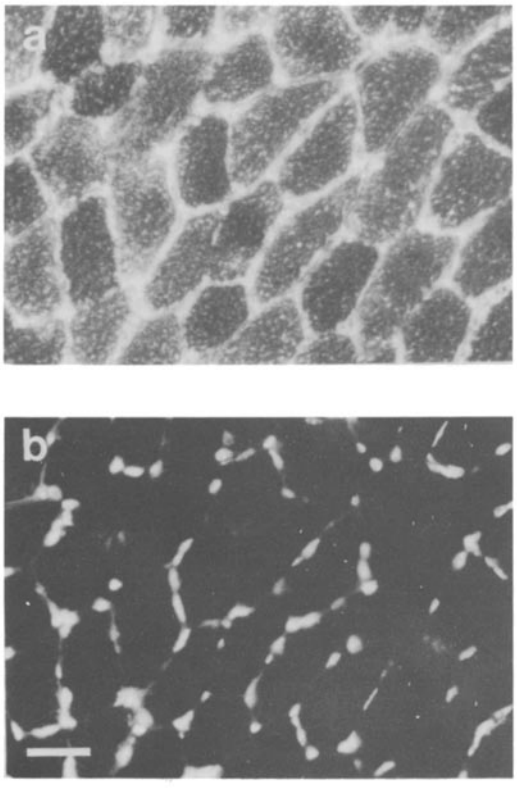

Figure 3. Immunofluorescence staining of transversal cryosection of skeletal muscle from A2G-adr mouse mutant with anti-G_{M3}(Neu5Ac) antibody. (a) Fluorescence micrograph; (b) DAPI stain of nuclear DNA of the same field. Bar = 30 μm.

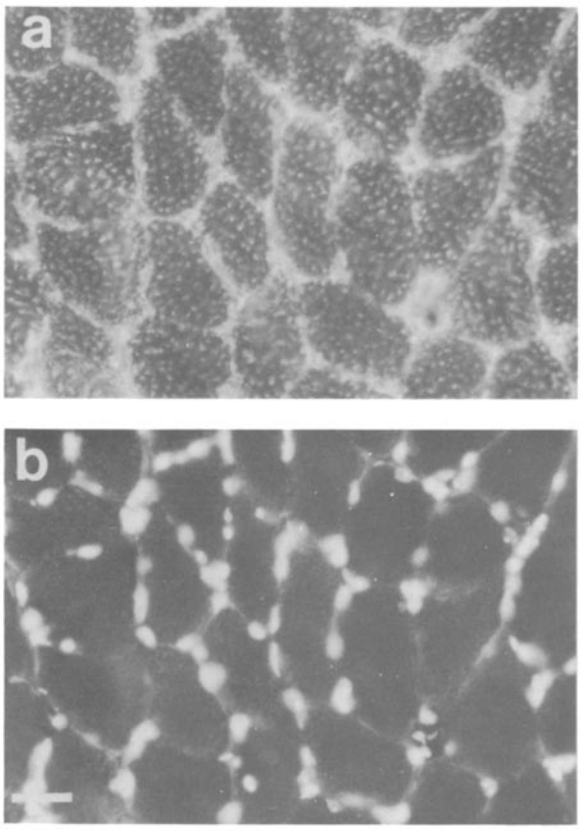

Figure 4. Anti-G_{M3}(Neu5Ac) antibody staining of a cryosection from BL6-wr mouse mutant skeletal muscle. (a) Fluorescence micrograph; (b) DAPI stain of nuclear DNA of the same field. Bar = 20 μm.

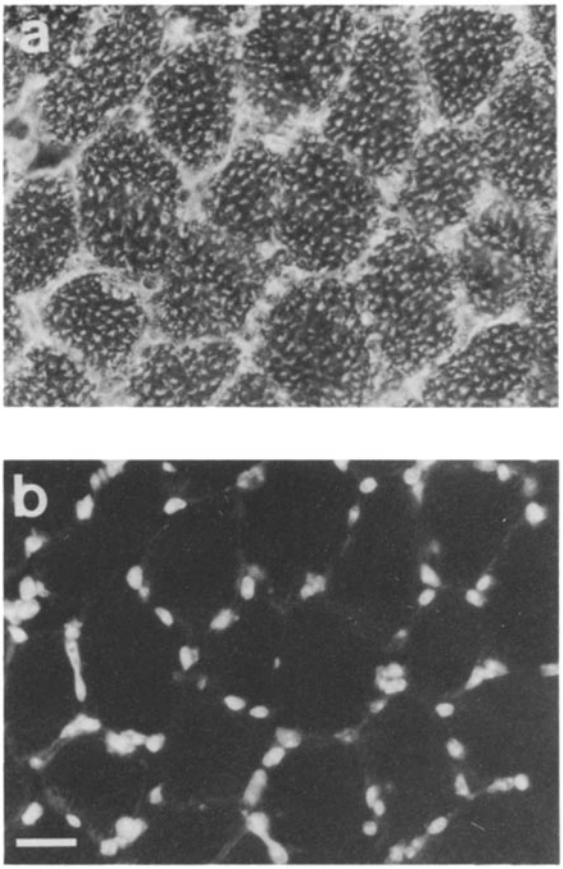

Figure 5. Immunofluorescence staining of transversal cryosection from BL6-wr mouse mutant skeletal muscle with anti-LacCer antibody. (a) Fluorescence micrograph; (b) DAPI stain of nuclear DNA of the same field. Bar = 20 μm.

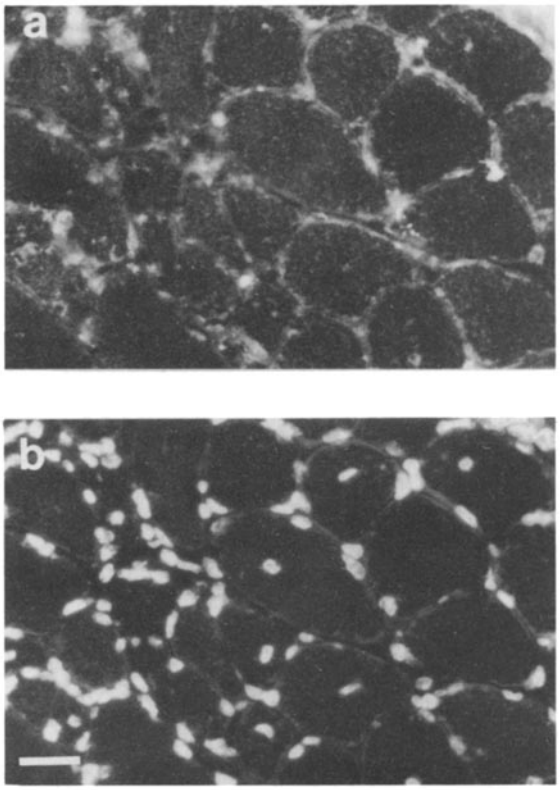

Figure 6. Localization of anti-G_{M3}(Neu5Ac) antibody immunoreactivity in a cryosection of skeletal muscle from BL10-mdx mouse mutant. (a) Fluorescence micrograph; (b) DAPI stain of nuclear DNA of the same field. Bar = 30 μm.

some fibres and atrophy of others. There are several regenerated muscle fibres with central nuclei. Sarcolemmas were stained with anti-G_{M3}(Neu5Ac) antibody, but the stain was weak and discontinuous. The intracellular stain with this antibody was particularly weak. Anti-G_{M1}(Neu5Ac) antibody stained with very low intensity. Anti-LacCer (Fig. 7) and anti-$GbOse_4Cer$ showed an irregular sarcolemmal stain and normal expression at the intracellular level. Anti-$nLcOse_4Cer$ staining was very weak and anti-Forssman GSL antibody gave an even lesser fluorescent intensity.

Discussion

The biochemical analysis of GSL composition of normal mouse skeletal muscle has been reported [37, 42] and these results were confirmed by immunohistochemical analyses in this study.

The A2G-adr mouse is an animal model for human recessive generalized myotonia congenita of the Becker type by the criteria of recessive mode of inheritance, independence of hyperexcitability from neuromuscular transmission and low chloride conductance [27, 34, 43]. The muscular chloride channel is essential for a normal excitability of mature mammalian muscle fibres and its functional destruction by a variety of mutations in the chloride channel gene on mouse chromosome 6 [44] and on human chromosome 7 leads to the disease myotonia [45, 46]. Our investigation of glycosphingolipid expression in muscle of this mutant gave results quite comparable to normal mice. The major changes were slightly 'thicker' membranes indicating an altered lipid environment of the chloride channels and the rearrangement of GSLs in sarcolemma.

The BL6-wr mouse with lower motor neuron disease serves as a model for hereditary spinal atrophy such as Werdnig-Hoffmann disease (infantile spinal muscular atrophy) and some cases of amyotrophic lateral sclerosis [29, 30]. Due to the loss of neuronal cells in the spinal cord many muscle fibres become 'denervated' in the classical sense of the word. The autosomal recessive mutation – wr was recently mapped to mouse proximal chromosome 11 [47] and the linkage analysis mapped infantile spinal muscular atrophy to chromosome 5q [48]. In our investigation the average diameter of individual muscle fibres was slightly reduced, but there was no

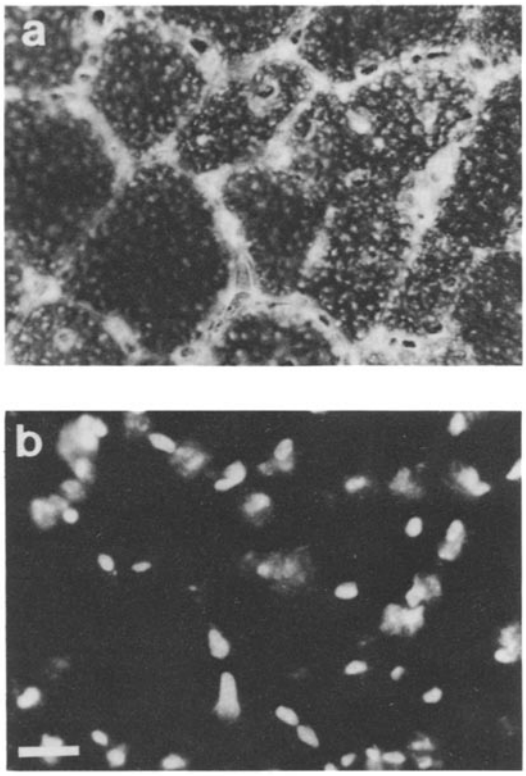

Figure 7. Anti-LacCer antibody staining of a cryosection from BL10-mdx mouse mutant skeletal muscle. (a) Fluorescence micrograph; (b) DAPI stain of nuclear DNA of the same field. Bar = 20 μm.

evidence of on-going degeneration in the muscle. The immunofluorescence intensities for most anti-GSL antibodies were similar to normal mouse muscle. However, anti-G_{M3}(Neu5Ac) and anti-LacCer antibodies gave fluorescence of a slightly higher intensity in the Wobbler skeletal muscle. The general increase in the amount of gangliosides, especially of G_{M3}(Neu5Ac), in denervated muscles has also been reported for cat, rabbit and rat muscle [15]. The exposure of cryptic recognition sites as a result of denervation-induced changes in lectin binding to sarcolemmal glycoproteins has been recently reported [49], but changes in sarcolemmal GSLs have not been investigated so far.

Murine X-linked muscular dystrophy (mdx) and Duchenne muscular dystrophy are genetically homologous and both are characterized by a complete absence of dystrophin [32, 50, 51] together with the loss of > 90% of the associated glycoprotein complex [52]. In the muscles that we have analysed, the immunofluorescence intensity was significantly reduced with all our anti-GSL antibodies indicating lower expression of these compounds in mdx mouse muscle. Muscle membranes were obviously irregular and the intracellular immunoreaction was very weak.

In conclusion, GSL expression in muscle is altered when the muscle is primarily affected with a myopathic disorder such as in the mdx mouse or is secondarily damaged in a neuronopathic lesion such as in Wobbler mouse. Major differences were not observed in the intensities of GSLs, but in the different patterns of distribution on plasma membrane and at the subcellular level. However, the significance of GSLs in the pathology of these diseases remains unclear.

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