Human muscles - Fresh muscle biopsy samples from patients with ALS, FSH, and normal (scoliosis) were obtained from the Departments of Neurosurgery and Orthopedic Surgery, Baylor College of Medicine.

Isolation of Glycolipids - Muscle biopsy samples (0.5 - 1.5 g) were used for the isolation of glycolipids. The procedures for isolation of gangliosides and neutral GSLs have been previously described [8,10,11].

TLC and Quantification of Glycolipids - TLC of gangliosides was performed on precoated silica gel plates (E. Merck) with chloroform-methanol-water (55:45:10, v/v) containing 0.02% $CaCl_2.2H_2O$ or chloroform-methanol-2.5 N NH_4OH (60:40:9, v/v). The ganglioside bands were visualized with a resorcinol spray [12]. TLC of neutral glycolipids were performed with chloroform-methanol-water (60:35:8, v/v) and the neutral glycolipid bands were visualized with an α-naphthol sulfuric acid spray [13]. The yield of gangliosides in each preparation was determined by GLC analysis of the lipid-bound sialic acid [14].

Enzymes and Antibodies - Neuraminidase from Vibrio cholerae was obtained from CalBiochem-Behring, La Jolla, CA and neuraminidase from Arthrobacter ureafaciens from Boeringer Mannheim Biochemicals, Indianapolis, IN. Purified rabbit antibody to CTH, LT, As G_{M2}, Glob, PG, As G_{M1}, Forss, G_{M2}, G_{M1} and G_{D3} have been prepared in this laboratory [15-19]. Affinity purified goat anti-rabbit Ig antibodies was purchased from Tago Inc., Burlingame, CA and radioiodinated with ^{125}NaI using standard chloramine T technique.

Immunostaining on Thin Layer Plate - The immunostaining procedure of Brockhaus et al [20] was used with some modifications [10]. The GSL mixtures were separated on an almunium-backed HPTLC plate (E. Merck) in chloroform-methanol-water (60:35:8, v/v). Then the plate was overlaid with a panel of purified anti-GSL IgG antibodies (10-50 μg/ml). After overnight incubation at 4°C, the plate was layered with ^{125}I-labeled goat anti-rabbit IgG (10^6 cpm/ml) for 3 hours, exposed to X-ray film for 24 hours, developed, and the GSLs were identified.

RESULTS

The total ganglioside content of muscles from normal, ALS and FSH individuals is summarized in Table 1. An increase of approximately 1.8 fold in the total ganglioside content was seen in ALS patients compared to normal

Table 1

Total ganglioside concentration in normal and degenerated human muscles[a]

	μg lipid-bound sialic acid/50 mg wet muscle[b]
Normal (7)	1.01 ± 0.10
ALS (14)	1.8 ± 0.06
FSH muscular dystrophy (2)	1.93 ± 0.14

[a]Determined by gas-liquid chromatography. Number of muscles studied are indicated in parentheses.

[b]Values are given as mean ± SD.

muscles (scoliosis). In the FSH muscles, the increase was approximately 1.9 fold. TLC of gangliosides isolated from muscles of normal (scoliosis), ALS and FSH is shown in Figure 1. Identification of the major gangliosides was as follows:

G_{M3} and G_{D3} - The identification of G_{M3} and G_{D3} was based on TLC mobilities of the native gangliosides (Figure 1) and their Vibrio cholerae neuraminidase product, lactosylceramide (Figure 2).

G_{M2} - This ganglioside from muscles displayed 2 bands on TLC: one co-migrating with brain G_{M2} and other migrating slightly faster than brain G_{M2} (Figure 1). The appearance of these G_{M2} bands in muscles was due to the heterogeneity in their ceramide moiety. Vibrio cholerae neuraminidase failed to cleave the sialic acid residue of both gangliosides, suggesting that the sialic acid residue was present on the internal sugar [21] (see Figure 2). However,

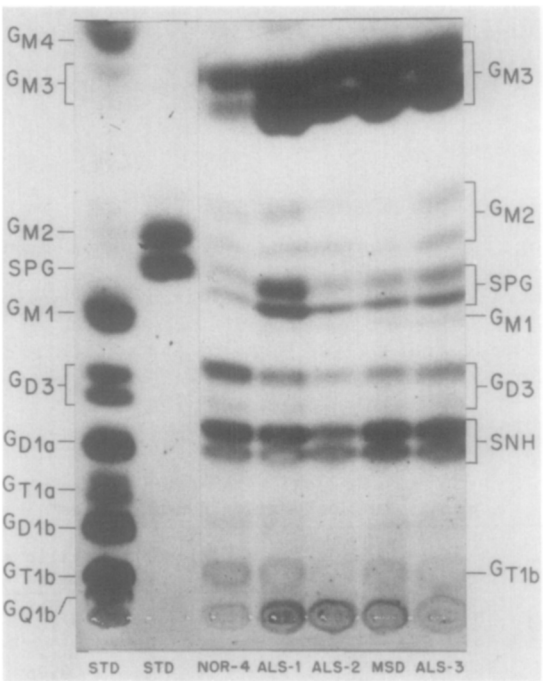

Figure 1. Thin-layer chromatogram of human muscle gangliosides. The solvent was chloroform-methanol-water 55:45:10 containing 0.02% $CaCl_2.2H_2O$ (w/v) and gangliosides were detected with resorcinol reagent. Lanes STDs contained standard ganglioside mixtures. Lane NOR-4 contained gangliosides from normal, ALS-1, ALS-2 and ALS-3 from ALS and MSD from FSH muscles. Each lane contained gangliosides from 200 mg of wet muscles. The gangliosides indicated in the right panel have been identified.

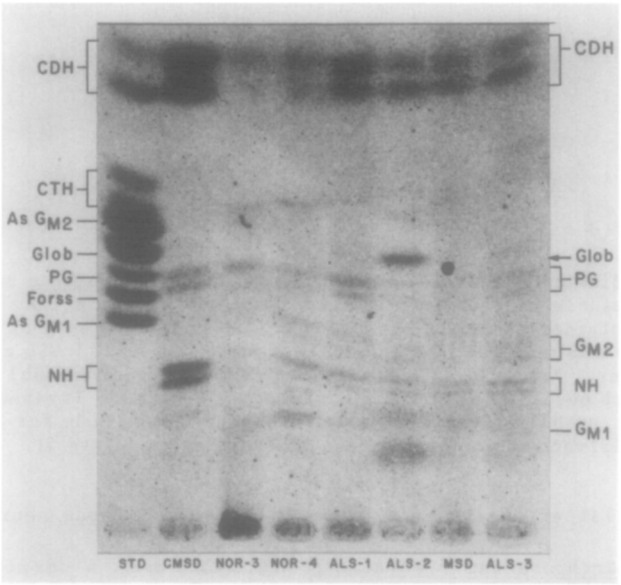

Figure 2. Thin-layer chromatogram of products produced from muscle gangliosides by Vibrio cholerae neuraminidase. The solvent was chloroform-methanol-water 60:35:8 (v/v) and glycolipids were detected with α-naphthol reagent. Gangliosides G_{M2} and G_{M1} were resorcinol positive. Lane CMSD contained glycolipids produced from chicken muscular dystrophic gangliosides. Each lane (human) contained glycolipids produced from 100 mg of wet muscles. The glycolipids indicated in the right panel have been identified. The arrow on the right panel indicates globotetraosylceramide generated from ALS muscles. Other abbreviations are as in Figure 1.

sialic acid from both these bands was lost following treatment with Arthrobacter ureafaciens in the presence of a bile salt [22]. The TLC migration of the desilylated bands (thus obtained by the above treatment) was in the region of brain asialo G_{M2}. Immunostaining with mouse monoclonal anti-asialo G_{M2} (kindly provided by Dr. S. Hakomori) confirmed the identification of these bands as As G_{M2}. The G_{M2} bands were also confirmed by immunostaining with affinity purfied anti-G_{M2} antibody.

(2-3)SPG - This ganglioside from normal and diseased muscles displayed 2 bands on TLC that co-migrated with the bands of chicken muscle (2-3)SPG (data not shown). On treatment with Vibrio cholerae neuraminidase, these two gangliosides produced 2 asialo glycolipids that co-migrated with the 2 bands of chicken muscle PG (Figure 2). These two bands were identified by immunostaining with affinity purified rabbit anti-PG (Figure 3).

G_{M1} - This ganglioside was present as a minor component in both normal and diseased muscles (Figure 1). It co-migrated on TLC with brain G_{M1} and was

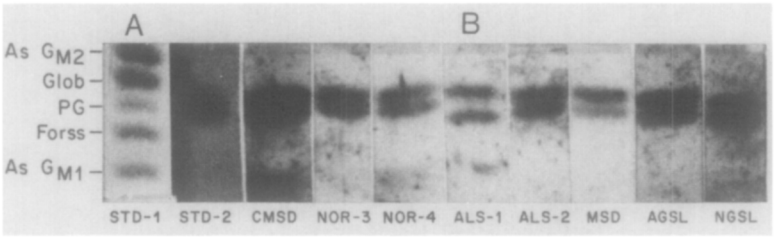

Figure 3. Binding of anti-PG antibody to the *Vibrio cholerae* neuraminidase products of msucle gangliosides (3 μg lipid-bound sialic acid). Lanes STD-1 and STD-2 contained standard GSL mixture; AGSL contained 4 μg of neutral GSL mixture from ALS muscles; NGSL contained 4 μg of neutral GSL mixture from normal muscles. Panel A, detection with α-naphthol spray; Panel B, GSLs was overlaid with purified anti-PG IgG antibody (10 μg/ml), layered with ^{125}I-labeled goat rabbit Ig (10^6 cpm/ml), exposed to X-ray film for 24 hours and developed. Solvent and other abbreviations are as in Figure 2.

similar to the latter in its resistance to *Vibrio cholerae* neuraminidase. On treatment with *Arthrobacter ureafaciens* neuraminidase, a desilylated product was obtained which was identified by immunostaining with affinity purified rabbit anti-asialo G_{M1}.

(2-3)SNH - This ganglioside displayed double bands that co-migrated with the SNH bands of chicken muscles and human erythrocytes [9] (data not shown). The principal fatty acid of the upper band was $C_{16:0}$, whereas the lower band contained a predominance of $C_{22:0}$, $C_{24:0}$ and $C_{24:1}$. On treatment with *Vibrio cholerae* neuraminidase, the muscle SNH bands produced 2 asialo glycolipids. They co-migrated with the 2 bands of lacto-N-*nor*-hexaosylceramide obtained from chicken muscle SNH under identical conditions (Figure 2). The 2 SNH bands from chicken muscles and human erythryocytes gave weak positive bands on thin layer plate by immunostaining. This weak cross reactivity of anti-PG with SNH was due to a lactosamine moiety. We took advantage of this to identify the SNH bands in normal and diseased muscles.

G_{T1b} - We found an appreciable amount of G_{T1b} in normal muscles as reported by Svennerholm *et al* [23]. On the other hand, the amount of G_{T1b} was generally reduced in the ALS muscles analyzed (Figure 1). G_{T1b} was identified on the basis of chromatagraphic mobility with brain G_{T1b} (Figure 1). *Vibrio cholerae* neuraminidase was used to cleave external sialic acid residues of G_{T1b} and the hydrolysed product was identified as G_{M1} by immunostaining with affinity purified anti-G_{M1} antibody.

Marker ganglioside in ALS Total gangliosides fractions from ALS, FSH and normal muscles were treated with Vibrio cholerae neuraminidase. When the hydrolyzed products were examined by TLC, a distinct band that co-migrated with globotetraosylceramide (Glob) was seen in ALS-2 [indicated by arrow in Figure 2]. This band was not clearly visible in other ALS samples due to the insensitivity of α-naphthol reagent used for detection [13]. But, however, Glob was detected in all ALS samples when the hydrolyzed products were immunostained with affinity purified anti-Glob antibody (Figure 4). This suggests that the hydrolyzed products of ALS-1 and ALS-3 contained Glob in amounts which were below the detection limit of α-naphthol reagent. Glob was not detected by the highly sensitive immunostaining technique in FSH and normal samples (Figure 4). These results suggest that ALS muscles contained a marker ganglioside which, on hydrolysis, yielded Glob.

DISCUSSION

The total ganlgioside content in muscles from patients with ALS and FSH increased approximately 2 fold compared to unaffected muscles (Table 1). In an earlier study [24], we found a comparable increase in the ganglioside content of degenerating muscles from dystrophic chickens. Increased ganglioside content might be, therefore, associated with various pathological changes of muscles. The ganglioside pattern in muscles from ALS and FSH (Figure 1) showed that the concentrations of G_{M3} and (2-3)SPG were elevated in both diseases. On the other hand, the concentration of G_{T1b} was reduced in ALS but

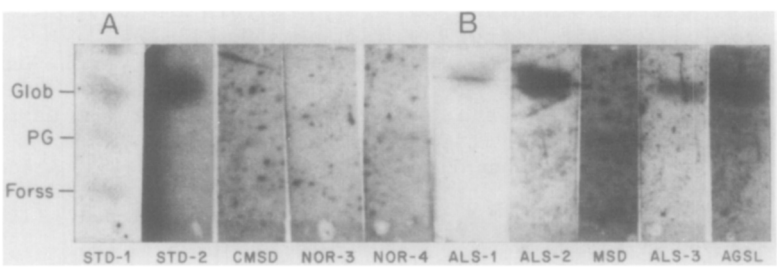

Figure 4. Binding of anti-Glob antibody to the Vibrio cholerae neuraminidase products of muscles gangliosides (3 μg lipid-bound sialic acid). Panel A, detection by α-naphthol spray; Panel B, autoradiogram. The concentration of purified anti-Glob IgG antibody used was 50 μg/ml. Solvent and other details are as in Figure 2.

not in FSH. The most significant result of this study was the presence of a novel ganglioside in ALS muscles. This marker ganglioside was identified by enzymatic hydrolysis and antibody reactivity as sialosylglobotetraosylceramide, NeuAc (α2-3)GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc-Cer.

Recently Schwarting et al [25] reported a novel ganglioside in human teratocarcinoma cells. Their ganglioside co-migrated on TLC with G_{M2}. Thin layer chromatograms of muscle gangliosides (Figure 1 and data not shown) indicated that the lower band of G_{M2} was elevated in ALS muscles compared to normal and FSH muscles. We believe that the marker ganglioside of ALS co-migrated with the lower band of G_{M2}. Based on the chromatographic behavior and the results of enzymatic hydrolysis and antibody reactivity, we propose that our ganglioside is similar to that described by Schwarting et al [25].

We observed that the concentration of marker ganglioside varied in the muscles examined (results extrapolated from Figure 4 and data not shown). Generally, samples from patients at an advanced stage of disease (e.g. ALS-2) contained a higher concentration of this ganglioside than those from less severely affected patients (e.g. ALS-1 and ALS-3). Studies are in progress to relate the concentration of marker ganglioside to the severity of disease.

REFERENCES

1. Rowland, L.P. (1982) Human Motor Neuron Diseases, pp. 1-13, Raven Press, New York.
2. Stalberg, E. (1982) Human Motor Neuron Disease, pp. 47-59, Raven Press, New York.
3. Ceccarelli, B., Aporti, F. and Finesso, M. (1976) Ganglioside Function: Biochemical and Pharmacological Implications, pp. 275-293, Plenum Press, New York.
4. Obata, K., Oide, M. and Handa, S. (1977) Nature 266, 369-371.
5. Harkomori, S.I. (1981) Ann. Rev. Biochem. 50, 733-784.
6. Bjornskov, E.K., Dekker, N.D., and Morris F.H. (1975) Arch. Neurol. 32: 711-712.
7. Kundu, S.K., Suzuki, A., Sabo, B., McCreary, J., Niver, E., Harman, R. and Marcus, D.M. (1981) J. Immunogenet. 8, 357-365.
8. Kundu, S.K. (1981) Methods Enzymol, Vol. 72, pp. 174-185, Academic Press, New York.
9. Kundu, S.K., Samuelsson, B.E., Pascher, I. and Marcus, D.M. (1983) J. Biol. Chem. In Press.
10. Kundu, S.K., Pleatman, M., Redwine, W.A., Boyd, A.E. and Marcus, D.M. (1983) Biochem. Biophys. Res. Commun. In press.
11. Kundu, S.K. and Suzuki, A. (1981) J. Chromatogr. 224, 249-256.
12. Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604-611.
13. Siakotos, A.N. and Rouser, G. (1965) J. Amer. Oil Chem. Soc. 42, 913-919.

14. Yu, R.K. and Ledeen, R.W. (1970) J. Lipid Res. 11, 506-516.
15. Marcus, D.M. and Schwarting, G.A. (1976) Adv. Immunol. 23, 203-240.
16. Marcus, D.M. (1976) Glycolipid Methodology, pp. 233-245, Amer. Oil Chemists Soc. Press, Champaign, IL.
17. Kundu, S.K. and Roy, S.K. (1979) J. Lipid Res. 20, 825-833.
18. Kundu, S.K., Marcus, D.M. and Veh, R.W. (1980) J. Neurochem. 34, 184-188.
19. Karol, R.A., Kundu, S.K., Suzuki, A. and Marcus, D.M. (1980) Blood Transfus. Immunohemat. 23, 589-598.
20. Brockhaus, M., Magnani, J.L., Herlyn, M.D., Steplewski, Z., Koprowski, H. and Ginsberg, V. (1982) Arch. Biochem. Biophys. 217, 647-651.
21. Li, Y.T., King, M.J. and Li, S.C. (1980) Structure and Function of Gangliosides, pp. 93-104, Plenum Press, New York.
22. Saito, M., Sugano, K. and Nagai, Y. (1979) J. Biol. Chem. 254, 7845-7854.
23. Svennerholm, L., Bruce, A., Mansson, J.E., Rynmark, B.M. and Vanier, M.T. (1972) Biochim. Biophys. Acta 280, 626-636.
24. Kundu, S.K., Misra, L.K. and Luthra, M.G. (1982) FEBS Lett. 150, 359-364.
25. Schwarting, G.E., Carroll, P.G. and DeWolf, W.C. (1983) Biochem. Biophys. Res. Commun. 112, 935-940.
26. Svennerholm, L. (1963) J. Neurochem. 10, 613-623.
27. Ledeen, R.W. and Yu, R.K. (1982) Methods Enzymol. 83, 139-191.