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Changes in Concanavalin A-Reactive Proteins in Neurological Disorders

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Changes of glycosylation of cerebrospinal fluid proteins such as α_2-macroglobulin, and prostaglandin D synthase were studied by lectin blotting, using concanavalin A, in multiple sclerosis (n = 42) and neuropathies (n = 20) in comparison to neurological controls (n = 22). The concanavalin A-reactivity of α_2-macroglobulin, which was increased in the neuropathies but not in multiple sclerosis compared to controls, correlated with the total concanavalin A-reactivity in controls and neuropathies but not in multiple sclerosis, indicating that the protein could be abnormally glycosylated in the latter disease. Although the concentration and the concanavalin A-reactivity of prostaglandin D synthase were not significantly different in the three groups, the two parameters correlated only in neuropathies but not in controls or multiple sclerosis, probably due to the high heterogeneity of the protein. These changes deserve to be studied in further detail in view of their potential clinical applications. J. Clin. Lab. Anal. 13:158–165, 1999. © 1999 Wiley-Liss, Inc.

Key words: multiple sclerosis; prostaglandin D synthase; α_2-macroglobulin; α_1-antitrypsin; β_2-microglobulin; glycosylation

INTRODUCTION

The etiopathogenesis of most chronic inflammatory disorders such as rheumatoid arthritis and systemic lupus erythematosus is still unknown and we speculate that processes capable of affecting the antigenicity of endogenous proteins such as glycosylation, may play a pathological role (1–8). In particular, it was demonstrated that both acute (e.g., α_1-antitrypsin [α_1-at]) (5) and non-acute phase proteins (e.g., α_2-macroglobulin [α_2-MG]) (6) may be abnormally glycosylated in these diseases and that their immunoreactivity may be consequently modified (5).

We consider these observations relevant both therapeutically and diagnostically for autoimmune diseases: it was shown that the abnormal glycosylation of serum proteins may be prevented by bindarit (4), a drug devoid of any activity on cyclo-oxygenase or experimental models of acute inflammation, but effective on the secondary phase of adjuvant arthritis in the rat (9), an experimental model of rheumatoid arthritis; and the evaluation of protein microheterogeneity due to glycosylation may be useful for the differential diagnosis (2,10) or the evaluation of the activity (11–12) of certain rheumatic diseases.

The microheterogeneity, i.e., differences of the carbohydrate moieties, of cerebrospinal fluid proteins such as transferrin (6,13–16) prostaglandin D synthase (PGD-S) or β-trace protein (17–21), and other proteins (22) is a well-known phenomenon. However, although alterations of self-antigens were speculated to be involved in the etiopathogenesis of multiple sclerosis (MS) (23), only a few studies were performed to examine the possible abnormalities of these proteins in this disease (16,24–26).

The aim of this work was to study the glycosylation pattern, using the lectin concanavalin A (Con A) as a probe, of selected cerebrospinal fluid (CSF) proteins in MS and different motor and sensory neuropathies (N) in comparison with that occurring in control subjects (CTRL), corresponding to patients with disc hernias and related conditions.

MATERIALS AND METHODS

Human Cerebrospinal Fluid and Serum Samples

Cerebrospinal fluid (CSF), collected by lumbar puncture, and serum samples were obtained from patients with definite multiple sclerosis (MS, n = 42), different motor and sensory

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neuropathies (N, n = 20), and miscellaneous conditions not related to illnesses of the central nervous system such as cervical and lumbar disc hernias (CTRL, n = 22). Samples were stored at –20°C until used.

Biochemicals

Acrylamide, N,N,N′,N′,-tetramethylenediamine (Temed), N,N′,-diallyltartardiamide (DATD), N,N′, methylene-bis(acrylamide) (Bis), glycine, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), ammonium persulfate, and high- and low-molecular weight protein standards were obtained from Bio-Rad (Richmond, CA). Protein A-peroxidase conjugate, 4-chloro-1-naphthol, bovine serum albumin (BSA, Cohn's fraction V), Nonidet P-40 (NP-40, octylphenoxypoly (ethoxyethanol)), sodium phosphate, sodium chloride, sodium hydroxide, and polyoxyethylene-sorbitan monolaurate (Tween-20) were obtained from Sigma Chemical Co. (St Louis, MO). Tris and phosphoric acid (85%) were from Aldrich Chemical Co. (Milwaukee, WI). Coomassie blue R-250 were from United States Biochemical Corp. (Cleveland, OH). Nitrocellulose paper (0.45 µm pore size) was from Schleicher & Schuell, Inc. (Keene, NH).

General Methods

CSF total protein content, serum, and CSF albumin and immunoglobulin levels were determined by nephelometry using a Beckman Array 360 System. Leukocyte counts, in CSF samples diluted 1:1 with the Turk solution (3% acetic acid), were performed using a Zeiss microscope equipped with a Fuchs-Rosenthal cell counting chamber. The barrier index (BI) and the IgG index were calculated using the formulas reported in (27–28):

$$\text{Barrier index (BI)} = [\text{albumin}]_{CSF}/[\text{albumin}]_{serum} \cdot 1000$$

$$\text{IgG index (IgGI)} = [\text{IgG}]_{CSF}/[\text{IgG}]_{serum} : [\text{albumin}]_{CSF} / [\text{albumin}]_{serum}$$

Detection of Oligoclonal Bands

Oligoclonal bands were detected in CSF samples by isoelectrofocusing on polyacrylamide gels using Ampholine PAGplates® from Pharmacia-LKB (Uppssala, Sweden). Briefly, volumes of 25 µL of serum, diluted 1:10 with saline, and CSF, concentrated about 60-fold by minicon-CS15 (Amicon, Beverly, MA) equipped with a membrane with a cut-off of 15 Kd, were loaded onto a 5% *T*-3% *C* polyacrylamide gel, presenting a pH gradient of 3.5–9.5, and the electrophoresis was performed for 1–3 hr at 1,500 V, 50 mAmps, 30 W, using 1 M phosphoric acid as anodic solution and 1 M sodium hydroxide as catodic solution. Following the electrophoresis, proteins were stained with Coomassie blue R.

Quantitative Protein Analysis by Concanavalin A and Immunoblotting

Aliquots of serum (0.3 µL) and CSF (30 µL) were fractionated by electrophoresis on a 7.5–12.5% *T* polyacrylamide gels (PAGE) in the presence of SDS (29) and the proteins were electrophoretically transferred to nitrocellulose paper (30–31) and stained with concanavalin A (1,32) or with a monospecific antiserum (33).

Densitometric analysis was performed using the scanner Hewlett Packard IIC, interfaced with a personal computer equipped with the image analysis software Winbasic 4® from Advanced American Biotechnology, (Fullerton, CA). In all analysis a quality control CSF or serum sample (QC) was run in triplicate to calculate the relative band intensity of a band (defined as the area under the densitometric peak (AUC) divided by the average AUC of QC) and to calculate the intra- and the interassay coefficients of variation (CVs).

Analysis of α_1-Antitrypsin and β_2-Microglobulin by ELISA

The immunoreactivity of α_1-antitripsin (α_1-AT) in all CSF samples was measured by ELISA, using a monoclonal antibody directed against an epitope variable upon glycosylation (2,3,5). The concentration of β_2-microglobulin in all samples was determined by a kit provided by ELIAS (Freiburg, Germany).

Statistical Analysis

Parametric (ANOVA) and nonparametric tests (Kruskal-Wallis) and linear regression analysis were performed using the software package Sigma-Stat® (Jandel), version 2.0 for Windows 95®.

TABLE 1. Biochemical Analyses of Serum and CSF Samples From Patients With Neurological Disorders[a]

	CTRL (n = 22)	N (n = 20)	SM (n = 42)
Serum			
Albumin (mg/100 mL)	4088 ± 1001	3950 ± 756	3784 ± 564
IgG (mg/100 mL)	885 ± 378	908 ± 356	1022 ± 210
IgA (mg/100 mL)	214 ± 86	197 ± 122	189 ± 79
IgM (mg/100 mL)	153 ± 89	154 ± 95	133 ± 97
CSF			
Total protein (mg/100 mL)	28.9 ± 6.3	53.5 ± 38.2*	38.6 ± 20.6
Albumin (mg/100 mL)	16.8 ± 5.7	31.6 ± 18.5*	23.6 ± 13.6
IgG (mg/100 mL)	1.9 ± 0.7	5.4 ± 5.6*	5.9 ± 4.5*
Leukocytes (cells/mm^3)	1.9 ± 1.3	2.6 ± 2.6	4.9 ± 5.4*
Oligoclonal bands (%)	0	10	90

[a]Data are mean ± S.D.

[b]Occurrence of oligoclonal bands as detected by isolelectrofocusing on polyacrylamide gels.

*$P < 0.05$ by ANOVA, performed with the software package Sigma-Stat (Jandel).

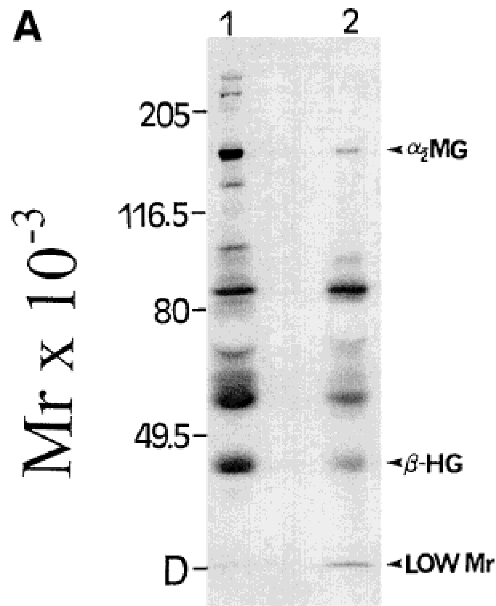
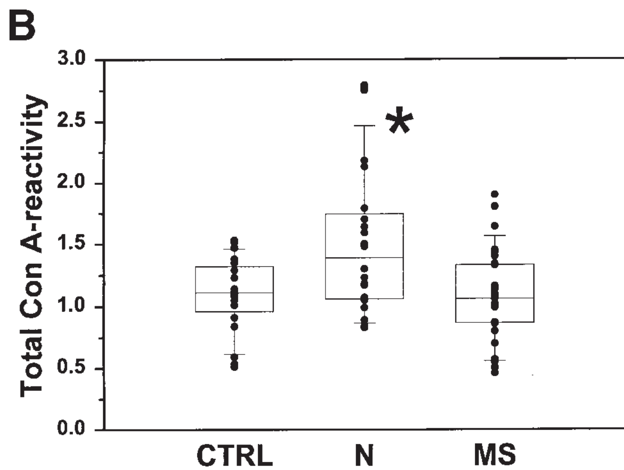
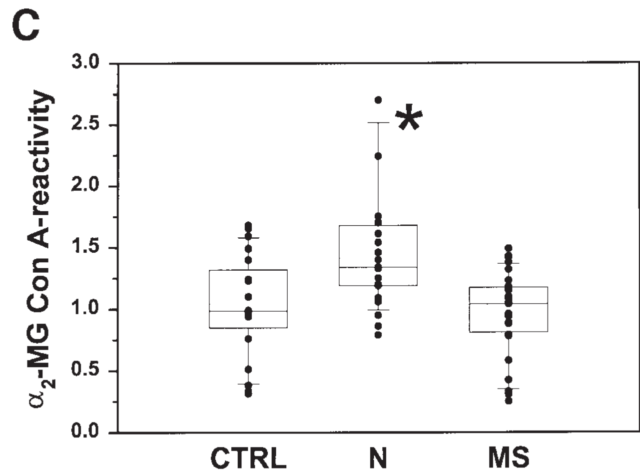
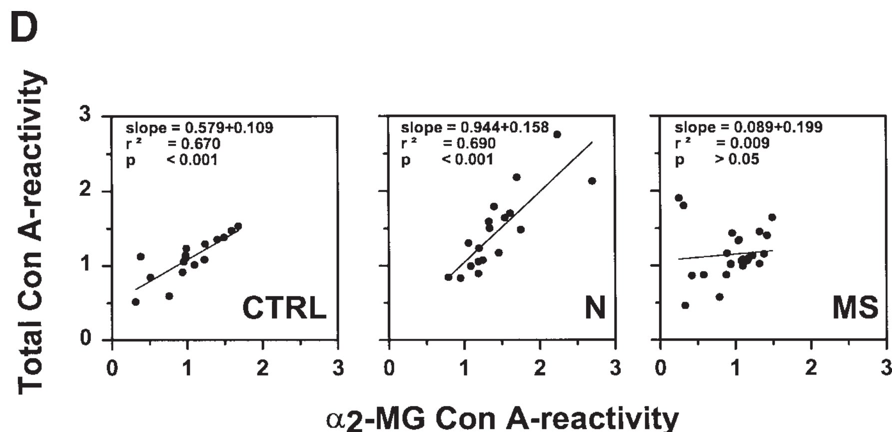


Fig. 1. Analysis of serum and CSF by Con A-blotting (high M*r* range). **A,** 0.3 μl of serum (lane 1) and 30 μl of cerebrospinal fluid (CSF, lane 2) were fractionated by SDS-PAGE on a 7.5% *T* polyacrylamide gel and the proteins were electrophoretically transferred onto nitrocellulose paper and stained with concanavalin A (Con A). M*r* protein markers (Bio-Rad, cat. N° 161-0309), corresponded to myosin (205,000), β-galactosidase (116,500), bovine serum albumin (80,000), ovalbumin (49,500); the indicated M*r* are the apparent M*r* of the protein marker as modified by the covalent binding of a dye. D, dye front (bromophenol blue); α_2-MG, α_2-macroglobulin; β-HG, β-chain of haptoglobin. **B,** All CSF samples were analyzed as described in A, together with a quality control CSF sample (QC), run triplicate in every gel, and the densitometric analysis was performed using the scanner Hewlett Packard IIC, interfaced with a personal computer equipped with the image analysis software Winbasic 4® from Advanced American Biotechnology, (Fullerton, CA); total Con A-reactivity corresponded to the ratio between the sum of the areas under each detectable densitometric peak (AUC) and the mean AUC of QC. CTRL, neurological controls, n = 22; N, neuropathies, n = 20; MS, definite multiple sclerosis, n = 42. **C,** an analysis, similar to that described in B was performed, but only the electrophoretic band of α_2-MG was considered. **D,** Values reported in B were plotted against those reported in C and a linear regression analysis was performed using the software package Sigma-Stat (Jandel).







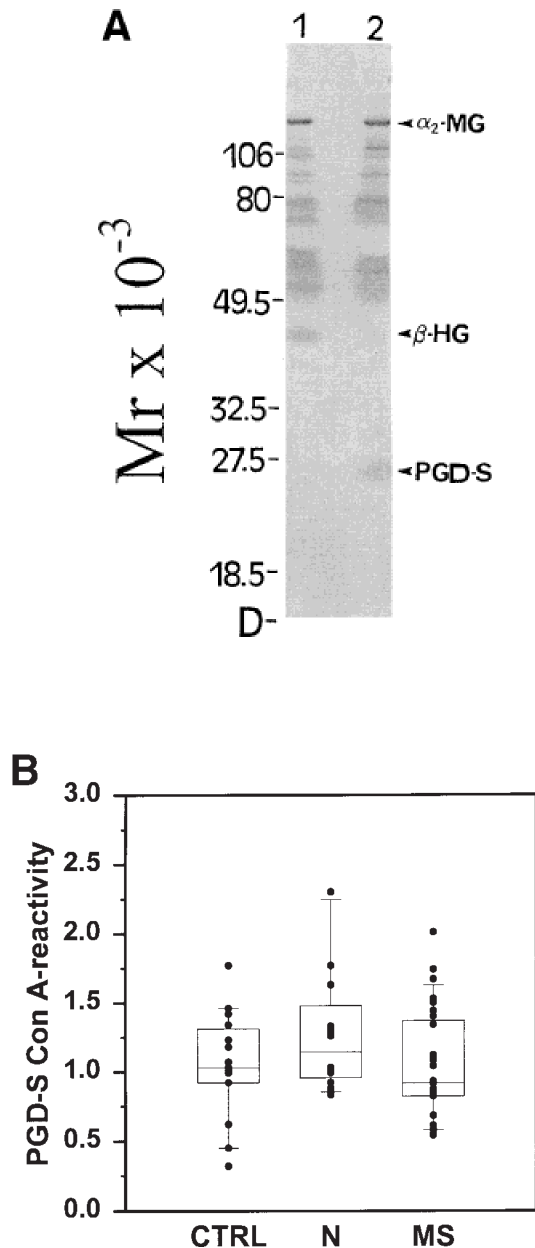


Fig. 2. Analysis of serum and CSF by Con A-blotting (low M*r* range). **A,** 0.3 µL of serum (lane 1) and 30 µL of cerebrospinal fluid (CSF, lane 2) were analyzed as described for Fig. 1, using a higher (12.5% *T*) acrylamide concentration. M*r* protein markers (Bio-Rad, cat. N° 161-0305), corresponded to phosphorylase B (110,000), bovine serum albumin (84,000), ovalbumin (47,000), carbonic anhydrase (33,000), soybean trypsin inhibitor (24,000) and lysozyme (16,000); the indicated M*r* are the apparent M*r* of the protein marker as modified by the covalent binding of a dye. D, dye (bromophenol blue); α_2-MG, α_2-macroglobulin; β-HG, β chain of haptoglobin; PGD-S, prostaglandin D synthase. **B,** the intensity of the Con A-reactive band of PGD-s was quantified and plotted as described in Fig. 1. CTRL, neurological controls, n = 22; N, neuropathies, n = 20; MS, definite multiple sclerosis, n = 42.

RESULTS

Analysis of CSF and Serum Samples

The total protein and the albumin content of cerebrospinal fluid (CSF) was statistically significantly increased ($P < 0.05$ by ANOVA) in the neuropathies (N) compared to neurological controls (CTRL) and definite multiple sclerosis (MS) (Table 1). The CSF level of IgG were increased in both N and MS compared to CTRL (Table 1). The leukocyte counts were significantly increased in MS ($P < 0.05$ by ANOVA) but not in N compared to CTRL (Table 1). As expected, oligoclonal bands were detected in high percentage (90%) in MS but not in N (10%) or CTRL (0%) (Table 1). No differences in serum levels of albumin and the major classes of immunoglobulin were observed (Table 1).

To evaluate the integrity of the ematoencephalic barrier (EEB) and the intrathecal IgG synthesis, the barrier index (BI) and the IgG index (IgGI) were calculated with the formulas reported above; a significant increase of BI was observed in N compared to CTRL, indicating alterations of the EEB, and a significant increase of the IgGI (> 0.7) was observed in 29 MS patients with normal EEB (< 5.5), indicating intrathecal IgG synthesis.

Concanavalin A- and Immunoblotting of CSF and Serum Samples

Serum and CSF samples were analyzed by polyacrylamide analytical gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS), using gels at different concentration, in the range 7.5–12.5% *T* (Fig 1A, 2A): the lowest concentration allowed a good separation of proteins with molecular weight (M*r*) in the range 40,000–200,000 such as α_2-macroglobulin (α_2-MG, an oligomeric protein consisting of 4 subunits of M*r* 180,000 each) and the β chain of haptoglobin (β-HG) (M*r* about 40,000) which were stained with concanavalin A (Con A) (Fig. 1A). In these conditions, small (M*r* < 35,000) Con A-reactive proteins such as prostaglandin D synthase (PGD-S) had the same electrophoretic mobility of the dye front (Fig. 1A). When all CSF samples were analyzed by Con A-blotting and the intensity of the bands was quantified by densitometric scanning, a statistically significant increase ($P < 0.05$ by subsequent ANOVA and Tukey's test) in the total Con A-reactivity was observed in N but not in MS compared to CTRL (Fig. 1B). When the intensity of the band corresponding to the Con A-reactive α_2-MG was compared in the three groups, a significant increase ($P < 0.05$ by subsequent Kruskal-Wallis ANOVA on ranks and Dunn's tests) in N but not MS compared to CTRL. Moreover, a good linear correlation between the total Con A-reactivity and the α_2-MG Con A-reactivity was found for CTRL ($P < 0.001$, $r^2 = 0.670$) and N ($P < 0.001$, $r^2 = 0.690$) but not for MS ($P > 0.05$, $r^2 = 0.009$).

Fig. 2A shows a typical Con A-blotting analysis of CSF

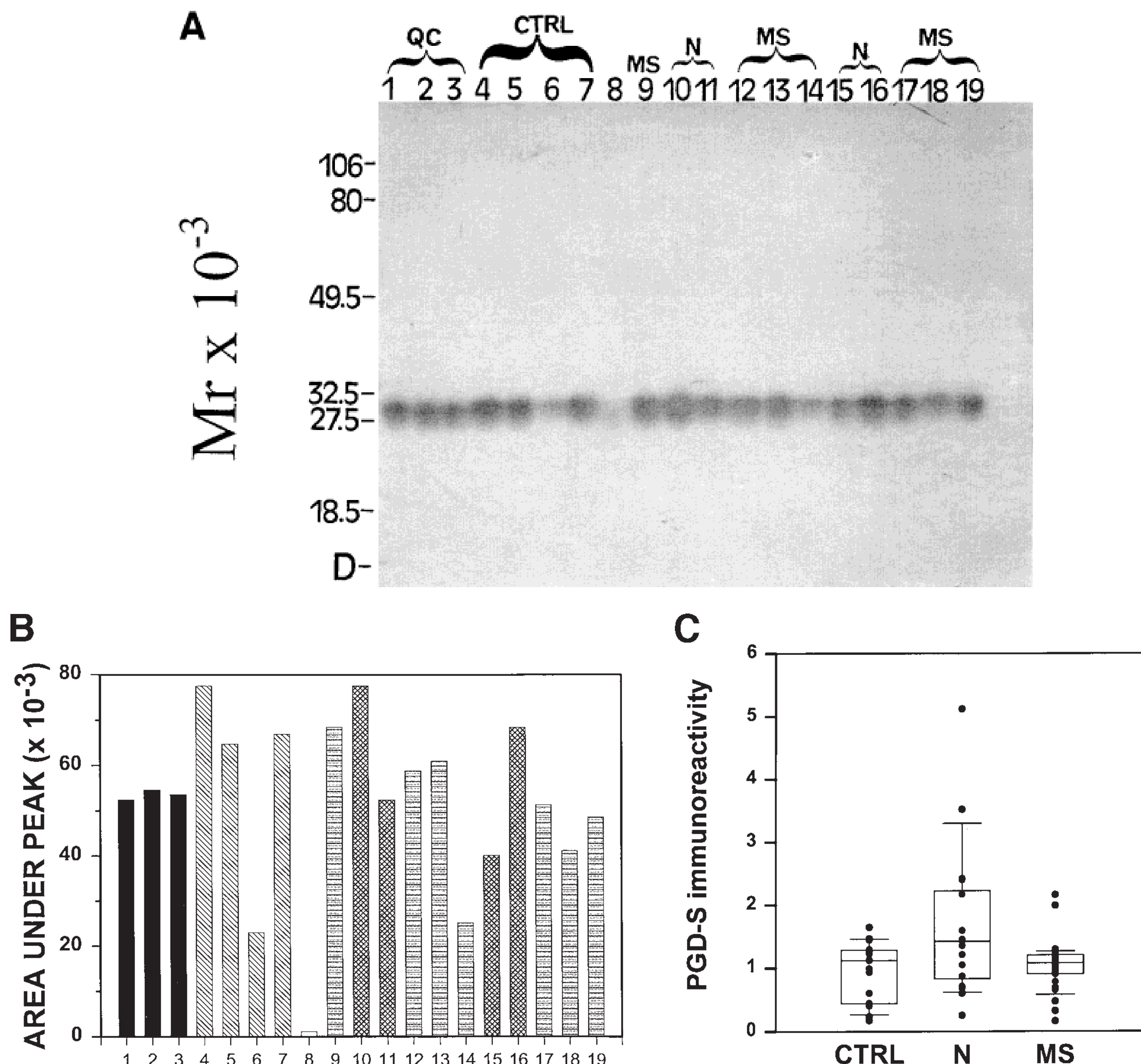


Fig. 3. CSF levels of prostaglandin D-synthase by immunoblotting. **A,** All CSF samples were analyzed as described in Fig. 2 and the immunostaining was performed using a monospecific antibody raised against purified prostaglandin D synthase (PGD-S). Lane 8 was not loaded to verify the entity of the sample cross-over during electrophoresis. QC, quality control CSF sample; CTRL, neurological controls; N, neuropathies; MS, definite multiple sclerosis. **B,** The intensity of the PGDS-bands shown in A, expressed as area under the densitometric peak (AUC), was plotted to show the high variability which was not due to the method, as demonstrated by the low variability of the QC samples. **C,** The PGD-S immunoreactivity, corresponding to the relative intensity of the PGD-S bands, calculated by dividing the AUC of each band by the average AUC of QC samples, was plotted for the three groups: CTRL, neurological controls, n=22, ; N, neuropathies, n=20; MS, definite multiple sclerosis, n=42.

(30 μL) and serum (0.3 μl) using a 12.5% *T* polyacrylamide gel. In these conditions, a prominent band with apparent M*r* of 30,000, which corresponded to PGD-S, as confirmed by immunological analysis (Fig. 3A), was seen in CSF but not in the serum sample (Fig. 2A). When all CSF samples were analyzed this way and the intensity of the PGD-S was quantified by densitometric scanning, no significant changes were observed in the three groups CTRL, N, and MS (Fig. 2B).

When the levels of PGD-S were evaluated in all CSF samples by subsequent immunoblotting (Fig. 3A) and densitometric analysis (Fig. 3B), a high individual variability was noted (Fig. 3B) but no significant overall changes were observed in the three groups (Fig. 3C). However, a good linear correlation ($P < 0.001$, $r^2 = 0.662$) between the PGD-S levels (Fig. 3C) and its Con A-reactivity (Fig. 2B) was observed in N (Fig. 4) but not in MS and CTRL. The analysis in triplicate

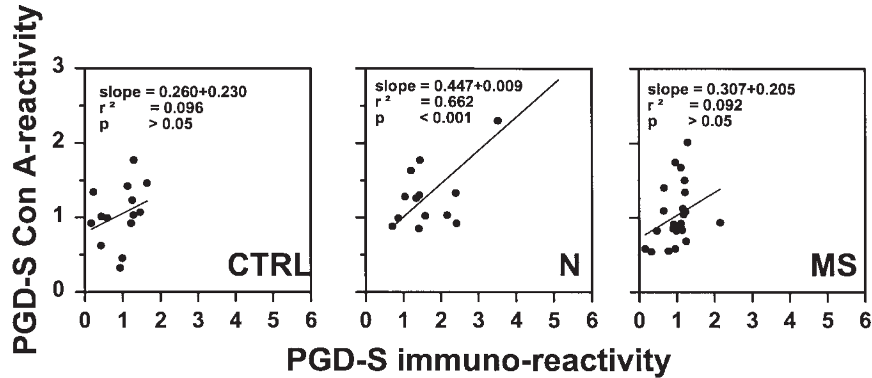


Fig. 4. Relationship between the Con A-reactivity and the concentration of prostaglandin D synthase. Data reported in Fig. 2B were plotted against those of Fig. 3C; a good linear correlation was found in the neuropathies (N, n = 22) but not in control subjects (CTRL, n = 20) and patients with definite multiple sclerosis (MS, n = 42)

of quality control CSF sample (QC) (Fig. 3B) in all experiments allowed the calculation of the relative band intensity and of intra- and interassay coefficients of variations (CVs) which were about 2 and 5% respectively.

Analysis of α_1-Antytrypsin and β_2-Microglobulin by ELISA

No significant changes were observed for the levels of α_1-antitrypsin (Fig. 5) and β_2-microglobulin (Fig. 6), measured by enzyme-linked immunosorbent assay (ELISA) in all CSF samples.

DISCUSSION

Changes of glycosylation, evaluated by the affinity for the lectin concanavalin A (con A) of selected cerebrospinal fluid (CSF) proteins, were observed in patients with definite multiple sclerosis (MS) and with different sensory or motor neuropathies (N), in comparison with subjects with conditions not related to disease of the central nervous system, such as disc hernias (CTRL).

Preliminary biochemical analyses of the serum and CSF samples in MS and N in comparison with CTRL (Table 1,2) yielded results in good agreement with the literature (34).

The Con A-reactivity of CSF proteins was significantly increased in N but not in MS compared to CTRL (Fig. 1B), and we speculated that α_2-macroglobulin (α_2-MG) may be partly responsible for this phenomenon, as indicated by the increase in its Con A-reactivity in N but not in MS compared to CTRL (Fig. 1C) and to the good correlation between the total and the α_2-MG Con A-reactivity (Fig. 1D). This observation is in line with the previously reported microheterogeneity of α_2-MG in

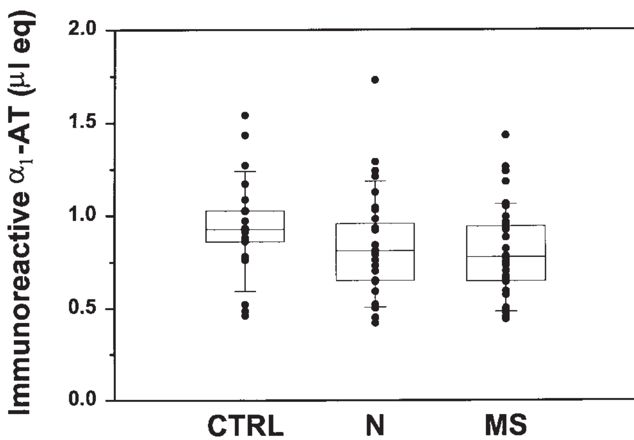


Fig. 5. Immunoreactivity of α_1-antitrypsin measured by ELISA, using a monoclonal antibody sensitive to glycosylation changes. The immunoreactivity of α_1-antitrypsin (α_1-AT) was measured in all CSF samples by ELISA using a monoclonal antibody recognizing an epitope subject to changes upon a specific type of abnormal glycosylation (see text). No significant changes were noted in the three groups: CTRL (neurological controls, n = 22), (N, neuropathies, n = 20), MS (definite multiple sclerosis, n = 42).

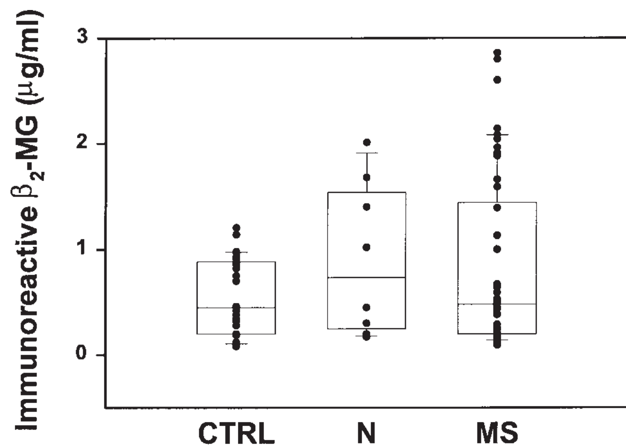


Fig. 6. Determination β_2-microglobulin. β_2-microglobulin (β_2-MG) was measured in all CSF samples by ELISA using a kit from ELIAS (Freiburg, Germany). No significant changes were noted in the three groups: CTRL (neurological controls, n = 22), (N, neuropathies, n = 20), MS (definite multiple sclerosis, n = 42).

TABLE 2. Evaluation of the Integrity of the Ematoencephalic Barrier by the Barrier Index and of the Intrathecal Synthesis of IgG and the IgG Index[a]

	CTRL (n = 22)	N (n = 20)	SM (n = 42)	
Barrier index (BI)[b]	4.9 ± 1.1	8.8 ± 6.5*	6.5 ± 4.5	
			BI < 5.5 (n = 29)	BI > 5.5 (n = 13)
IgG index (IgGI)[c]	0.5 ± 0.1	0.6 ± 0.6	1.1 ± 0.7*	0.7 ± 0.2

[a]Data are mean ± S.E.
[b]Calculated according to the formula: $BI = (C_{CSF\ albumin}/C_{serum\ albumin}) \cdot 10^3$
[c]Calculated according to the formula: $IgGI = C_{CSF\ IgG}/C_{serum\ IgG} : C_{CSF\ albumin}/C_{serum\ albumin}$
*$P < 0.05$ by ANOVA and Tukey's test (Sigma Stat®, Jandel).

neurological diseases (16) and with the alterations of serum α_2-MG, a major acute-phase protein in the rat (35) but not in the human (36), previously observed in patients with different rheumatic diseases (6).

To study low-M*r* proteins, such as prostaglandin D synthase (PGD-S), a very abundant CSF protein (37,38) also known as β-trace (17,18), highly heterogeneous due to structural differences in the carbohydrate moiety (19), we performed a second set of analyses by Con-A blotting, using a higher gel concentration (12.5 vs. 7.5 % *T*) (Fig. 2A); the Con A-reactivity and the concentration of PGD-S appeared highly variable in different individuals (Fig. 3B) but, overall, no significant changes in the three groups were noted (Fig. 2B, 3C), in agreement with previous studies (39,40), which could not find characteristic quantitative changes of this protein in neurological disorders.

However, a good linear correlation between the Con A-reactivity of this protein and its immunoreactivity was found in CTRL and N but not in MS (Fig. 4), indicating that abnormal glycosylation phenomena may occur in the latter disease, in line with other observations (21).

When α_1-antitrypsin was measured by ELISA using a monoclonal antibody directed against an epitope subject to changes upon abnormal glycosylation (2,3,5), no significant changes were observed in the three groups (Fig. 5), indicating that this protein is not abnormally glycosylated in these diseases like it is in other pathological conditions (2,3,5).

The variability of the CSF levels of β_2-microglobulin, measured by ELISA, increased in the sequence CTRL < N < MS but no overall changes were observed (Fig. 6) in agreement with some (41,42) but not all (43–46) previous observations.

In conclusion, we observed specific changes in the glycosylation pattern of selected glycoprotein such as α_2-MG and PGD-S in neurological disorders such as N and MS, which, given the limited utility of several current biochemical analyses (47), deserve to be studied in further detail in view of their possible diagnostic relevance.

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