Original Article

Synergy of Serum and Cerebrospinal Fluid Antibodies Against Axonal Cytoskeletal Proteins in Patients with Different Neurological Diseases

(anti-neurofilament antibodies / anti-tubulin antibodies / cerebrospinal fluid / intrathecal synthesis / multiple sclerosis / neurodegenerative diseases)

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Abstract. Autoantibodies against different axonal cytoskeletal proteins [the light (NFL) and medium (NFM) subunit of neurofilament and tubulin (TUB)] in serum and cerebrospinal fluid may be generated in response to the release of cytoskeleton from damaged neurons. We studied the relationships among these autoantibodies. Paired cerebrospinal fluid (CSF) and serum samples were obtained from 47 multiple sclerosis (MS) patients, 14 patients with neurodegenerative diseases, 21 patients with various neurological diseases and 16 normal control subjects. Levels of antibodies against NFL, NFM and TUB were related to each other in CSF in all groups, whereas close association of anti-cytoskeletal antibodies in serum was found in the MS group only. A concordant spectrum of anti-cytoskeletal antibodies is present in serum of MS patients, unlike in other neurological patients. The synergy between the spectrum of anti-cytoskeletal antibodies in serum and CSF might be one of the immunological features typical for the MS patients.

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Abbreviations: CD – diseased controls, CDEG – controls with neurodegenerative disorders, CN – normal controls, CSF – cerebrospinal fluid, Ig – immunoglobulin, IT – intrathecal synthesis, i.e. locally in the CNS compartment, LP – lumbar puncture, MS – multiple sclerosis, NFL – light subunit of neurofilament, NFM – medium subunit of neurofilament, TUB – tubulin.

Introduction

The neuronal cytoskeleton is composed of neurofilaments and microtubules. Three types of neurofilament proteins are designated as a light (NFL), a medium (NFM) and a heavy (NFH) subunit according to their molecular weight. These three different proteins form a heteropolymer triplet structure. NFL serves as the backbone to which NFM and NFH are attached. Neurofilaments are found mainly inside the axons of neurons (Al-Chalabi and Miller, 2003; Petzold, 2005). Microtubules composed of proteins α- and β-tubulins (TUB) are the other important components of cellular cytoskeleton. The structures of cytoskeleton may be released from the damaged neurons into the extracellular space. The interaction of cytoskeletal proteins with immunocompetent cells can result in the synthesis of autoantibodies (Zaffaroni, 2003; Petzold, 2005).

Various autoantibodies to cytoskeletal proteins have been reported in several neurological diseases (Kurki et al., 1986; Sadiq et al., 1991; Couratier et al., 1998; Salih et al., 1998; Terryberry et al., 1998; Bornstein et al., 2001; Silber et al., 2002; Eikelenboom et al., 2003; Ehling et al., 2004; Skoda et al., 2006; Bartos et al., 2007a; b). The elevated intrathecal synthesis of anti-NFL was described in patients with progressive disease course of multiple sclerosis (MS) (Silber et al., 2002). The close relationship between the levels of intrathecal anti-NFL immunoglobulin G (IgG) antibodies and the markers of cerebral atrophy was demonstrated in MS patients

(Eikelenboom et al., 2003). Ehling et al. (Ehling et al., 2004) found elevated serum antibodies to NFL in the primary progressive form of MS. In our previous studies we have observed an elevated intrathecal production of antibodies to NFM in MS patients (Bartos et al., 2007a) but not in case of the antibodies to NFL (Bartos et al., 2007b). Serum anti-neurofilament antibodies were also elevated in patients following stroke (Bornstein et al., 2001) and were detected more frequently in patients with amyotrophic lateral sclerosis (Couratier et al., 1998). Other anti-cytoskeletal antibodies were reported to be induced by neuronal damage. Several clinical conditions (e.g. brain trauma, neurodegenerative diseases) are associated with the elevated production of anti-tubulin antibodies (Terryberry et al., 1998; Skoda et al., 2006).

So far the correlation between anti-cytoskeletal antibodies has been explored only marginally (Salih et al., 1998; Silber et al., 2002). In the present study we focused on antibodies to NFL, NFM and TUB in the patients with different neurological diseases. We compared the cerebrospinal fluid (CSF) and serum (S) levels as well as the intrathecal synthesis (IT) of anti-NFL IgG, anti-NFM IgG and anti-TUB IgG in the group of patients with multiple sclerosis and in those with other neurological diseases.

Material and Methods

Paired CSF and serum samples were obtained from 47 multiple sclerosis (MS) patients (34 women and 13 men, mean age 39 ± 11.2 years), 14 patients with neurodegenerative diseases (CDEG) (8 women and 6 men, mean age 57.6 ± 14.2 years), 21 patients with various unrelated neurological diseases (control diseased group – CD) (18 women and 3 men, mean age 47.8 ± 16.8 years) and 16 normal control subjects (CN) (14 women and 2 men, mean age 36.9 ± 10.4 years).

The diagnosis and the course of MS at the time of lumbar puncture (LP) were determined using established criteria (Poser et al., 1983; Lublin and Reingold, 1996). Twenty-nine patients had the relapsing-remitting form, 13 patients had the secondary progressive course and 5 patients had the primary progressive form of MS. The median of disease duration at the time of LP was 5.6 years in MS patients (range 0.58–41 years). The Expanded Disability Status Scale (EDSS) used for the assessment of the level of disability at LP and the Multiple Sclerosis Severity Score (MSSS) used for determination of disease severity were 3.0 (0–6.5) and 5.6 (0.2–9.7) [median (range)], respectively (Kurtzke, 1983; Roxburgh et al., 2005). The therapy in 32 patients included immunosuppressive therapy alone or combination of immunosuppressive therapy with immunomodulatory agents; others had received no treatment prior to their LP.

The patients in the group with neurodegenerative disorders (CDEG) included these diagnoses: amyotrophic lateral sclerosis (N = 10), frontotemporal dementia (N = 1), multiple system atrophy (N = 1), progressive supraventricular palsy (N = 1) and undetermined neurodegeneration (N = 1). The diseased control (CD) group consisted of patients with miscellaneous diseases (e.g. polyneuropathy, meningitis, and stroke). Normal control (CN) subjects presented mostly with vertigo or headache (vertebrogenic, migraine) or with neurotic or fatigue syndromes, but their detailed assessment revealed no abnormalities.

All subjects gave written informed consents to participating in the study. The Ethics Committee of the Third Faculty of Medicine of Charles University in Prague approved the study. Specimens were stored at –20 °C until analysis. Biochemists performing assays were blinded to the diagnosis.

Cerebrospinal fluid and serum IgG anti-neurocytoskeletal autoantibodies were analysed by enzyme-linked immunosorbent assay (ELISA) using modified methods according to Silber et al. (2002). The anti-NFL and anti-NFM were examined in the patients of all groups. The antibodies to tubulin were not measured in CDEG patients and in several patients of other groups because of the lack of biological material.

The 68-kD light bovine neurofilament (Progen, Heidelberg, Germany), 160-kD bovine neurofilament subunit (Progen, Heidelberg, Germany) or bovine tubulin (Cytoskeleton, Denver, CO) were used as antigens for coating the wells. Bovine neurofilaments and tubulin were purified from bovine spinal cord and bovine brain, respectively. We used serial dilutions of the stock pooled human serum as a standard in all of the analytical series and the absorbances of the standards were used for the construction of a standard curve. The highest standard concentration was defined to be 100 arbitrary concentration units (AU). The results of autoantibody concentrations were expressed in arbitrary units (AU). The inter-assay and intra-assay variations for all ELISA methods did not exceed 10 %.

The CSF and serum albumin were determined by immunonephelometry. To assess intrathecal synthesis of anti-NFL, anti-NFM and anti-TUB IgG antibodies, the anti-NFL/NFM/TUB index was calculated: (CSF anti-neurocytoskeletal IgG/serum anti-neurocytoskeletal IgG)/(CSF albumin/serum albumin).

The data were checked for their distribution by kurtosis and skew test and nonparametric statistics was used because of the non-normal distribution. Relationships between variables were studied using the Spearman's correlation coefficient. The significance level for all tests was 0.05. Analyses were made with Statistica, version 7 (StatSoft, Tulsa, OK).

Results

The main results are summarized in Tables 1–3. Correlations between anti-NFL IgG and anti-NFM IgG levels in CSF were statistically significant in all groups. Levels of anti-NFL and anti-NFM IgG antibodies in serum correlate significantly only in the MS and less closely in the CD group. Intrathecal (IT) synthesis of anti-NFL IgG antibodies was significantly related to intrathecal synthesis of anti-NFM IgG antibodies in each group (Table 1).

Table 1. Relationships between levels of anti-NFL IgG and anti-NFM IgG antibodies in patients and controls

Patient groups	Serum		CSF		IT	
	r	P	r	P	r	P
MS (N = 47)	0.7	< 0.0001	0.9	< 0.0001	0.8	< 0.0001
CDEG (N = 14)	0.1	n.s.	0.8	< 0.0005	0.8	< 0.005
CD (N = 21)	0.5	< 0.05	0.7	< 0.001	0.7	< 0.001
CN (N = 16)	0.5	n.s.	0.8	< 0.0001	0.8	< 0.0001

N = number of subjects; r = Spearman's correlation coefficient; n.s. = not significant; P = value of significance

Table 2. Relationships between levels of anti-NFL IgG and anti-tubulin IgG antibodies in patients and controls

Patient groups	Serum		CSF		IT	
	r	P	r	P	r	P
MS (N = 39)	0.6	< 0.0001	0.6	< 0.0005	0.5	< 0.005
CD (N = 13)	0.3	n.s.	0.4	n.s.	0.21	n.s.
CN (N = 15)	0.2	n.s.	0.7	< 0.005	0.60	< 0.05

Abbreviations are explained in Table 1.

Table 3. Relationships between levels of anti-NFM IgG and anti-TUB IgG antibodies in patients and controls

Patient groups	Serum		CSF		IT	
	r	P	r	P	r	P
MS (N = 39)	0.6	< 0.0005	0.6	< 0.0001	0.6	< 0.0001
CD (N = 13)	0.1	n.s.	0.6	< 0.05	0	n.s.
CN (N = 15)	0	n.s.	0.7	< 0.005	0.7	< 0.001

Abbreviations are explained in Table 1.

Anti-TUB antibody responses were significantly associated with the levels of both anti-NFL and NFM antibodies in CSF in the MS, CD and the CN groups except for no correlation with anti-NFL antibodies in the CD group. Anti-TUB antibodies significantly corresponded to anti-NFL and anti-NFM antibodies in serum only in MS patients. IT synthesis of antibodies to tubulin positively correlated with IT synthesis of both anti-neurofilament antibodies in the MS and CN groups (Tables 2 and 3).

Anti-cytoskeletal antibodies were not related to clinical parameters of the disease. The only exceptions were correlations between intrathecal synthesis of anti-NFM or anti-NFL with EDSS and anti-NFL with MSSS (Bartos et al., 2007a; b; Svarcova et al., 2008).

Levels of autoantibodies in different patient groups and follow-up data on the level of antibodies can be found in our previous studies (Bartos et al., 2007a; b; Svarcova et al., 2008).

Discussion

In the present study we found significant relationships between selected anti-cytoskeletal antibodies in the serum and cerebrospinal fluid. In MS patients, all examined anti-cytoskeletal antibodies correlated with each other in the CSF and in the serum as well as those calculated as IT synthesis. We also found significant correlations between anti-NFL and anti-NFM antibodies in CSF and intrathecally in the patients with neurodegenerative disorders and in the heterogenic group of patients with various neurological diseases. The close association was found not only in the diseased groups, but also in the normal controls. It seems that the close relationship between anti-NFL and anti-NFM is a general phenomenon. The highest correlation between anti-NFM and anti-NFL found in the MS group may suggest that the release of neurofilament subunits during axonal damage and the immune response to both of the neurofilament subunits proceed in a similar way. The possibility of the cross-reactivity may contribute to the high correlation between the anti-NF antibodies. A certain structural similarity exists between subunits of neurofilaments. The central α-helical rod domains of both NFL and NFM contain highly conserved motifs (Al-Chalabi and Miller, 2003; Petzold, 2005). The antibodies to certain common epitopes may explain the cross-reactivity between anti-NFL and anti-NFM. Cross-reactivity experiments of IgG antibodies with different neurofilament polypeptides demonstrated the evidence of the cross-reaction only in some serum samples (Salih et al., 1998).

In contrast to CSF antibodies, the levels of anti-cytoskeletal antibodies in the serum correlated only in the group consisting of MS patients and not in the other groups. It is intriguing that antibodies against neuron-specific and otherwise hidden neurocytoskeletal antigens are present in the serum. We hypothesize that this is possible by the transfer of cytoskeletal fragments from the CNS compartment to the periphery. Cytoskeletal and myelin debris, released by neurons, are removed by macrophages which may be able to reach the periphery, e.g. the cervical lymph nodes (Fabriek et al., 2005). Anti-NFM antibodies could reflect not only antigen release, but may also be triggered by the process of molecular mimicry. According to this concept antibodies

are induced from exposure to exogenous agents, possibly virus-derived peptides. Such antibodies developed within the systemic compartment may cross-react with neuronal antigens (Prat and Antel, 2005).

In the present study we observed that a concordant spectrum of antibodies against cytoskeletal proteins is present in the serum of MS patients, unlike in other neurological patients. This is probably caused by the continuous breakdown of neurons and axons associated with dysregulated immunity in MS. Anti-neurocytoskeletal antibody synergy in the serum may reflect the response of dysregulated immune system to the release of neurocytoskeletal components to extracellular space and even to the serum. Myelin debris was found in the cervical lymph nodes (Fabriek et al., 2005). It may be a similar principle to MRZ reaction (simultaneous occurrence of antibodies to measles, rubella and varicella zoster (Reiber et al., 1998). This new finding should be verified in other MS patients and control groups. The synergy between the spectrum of anti-cytoskeletal antibodies in the serum and CSF might be one of the immunological features specific for the MS patients.

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References

Al-Chalabi, A., Miller, C. C. (2003) Neurofilaments and neurological disease. *Bioessays* **25**, 346-55.

Bartos, A., Fialova, L., Soukupova, J., Kukal, J., Malbohan, I., Pitha, J. (2007a) Elevated intrathecal antibodies against the medium neurofilament subunit in multiple sclerosis. *J. Neurol.* **254**, 20-25.

Bartos, A., Fialova, L., Soukupova, J., Kukal, J., Malbohan, I., Pitha, J. (2007b) Antibodies against light neurofilaments in multiple sclerosis patients. *Acta Neurol. Scand.* **116**, 100-107.

Bornstein, N. M., Aronovich, B., Korczyn, A. D., Shavit, S., Michaelson, D. M., Chapman, J. (2001) Antibodies to brain antigens following stroke. *Neurology* **56**, 529-530.

Couratier, P., Yi, F. H., Preud'homme, J. L., Clavelou, P., White, A., Sindou, P., Vallat, J. M., Jauberteau, M. O. (1998) Serum autoantibodies to neurofilament proteins in sporadic amyotrophic lateral sclerosis. *J. Neurol. Sci.* **154**, 137-145.

Ehling, R., Lutterotti, A., Wanschitz, J., Khalil, M., Gneiss, C., Deisenhammer, F., Reindl, M., Berger, T. (2004) Increased frequencies of serum antibodies to neurofilament light in patients with primary chronic progressive multiple sclerosis. *Mult. Scler.* **10**, 601-606.

Eikelenboom, M. J., Petzold, A., Lazeron, R. H., Silber, E., Sharief, M., Thompson, E. J., Barkhof, F., Giovannoni, G., Polman, C. H., Uitdehaag, B. M. (2003) Multiple sclerosis: Neurofilament light chain antibodies are correlated to cerebral atrophy. *Neurology* **60**, 219-223.

Fabriek, B. O., Zwemmer, J. N., Teunissen, C. E., Dijkstra, C. D., Polman, C. H., Laman, J. D., Castelijns, J. A. (2005) In vivo detection of myelin proteins in cervical lymph nodes of MS patients using ultrasound-guided fine-needle aspiration cytology. *J. Neuroimmunol.* **161**, 190-194.

Kurki, P., Helve, T., Dahl, D., Virtanen, I. (1986) Neurofilament antibodies in systemic lupus erythematosus. *J. Rheumatol.* **13**, 69-73.

Kurtzke, J. F. (1983) Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* **33**, 1444-1452.

Lublin, F. D., Reingold, S. C. (1996) Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology* **46**, 907-911.

Petzold, A. (2005) Neurofilament phosphoforms: surrogate markers for axonal injury, degeneration and loss. *J. Neurol. Sci.* **233**, 183-198.

Poser, C. M., Paty, D. W., Scheinberg, L., McDonald, W. I., Davis, F. A., Ebers, G. C., Johnson, K. P., Sibley, W. A., Silberberg, D. H., Tourtellotte, W. W. (1983) New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann. Neurol.* **13**, 227-231.

Prat, A., Antel, J. (2005) Pathogenesis of multiple sclerosis. *Curr. Opin. Neurol.* **18**, 225-230.

Reiber, H., Ungefehr, S., Jacobi, C. (1998) The intrathecal, polyspecific and oligoclonal immune response in multiple sclerosis. *Mult. Scler.* **4**, 111-117.

Roxburgh, R. H., Seaman, S. R., Masterman, T., Hensiek, A. E., Sawcer, S. J., Vukusic, S., Achiti, I., Confavreux, C., Coustans, M., le Page, E., Edan, G., McDonnell, G. V., Hawkins, S., Trojano, M., Liguori, M., Cocco, E., Marrosu, M. G., Tesser, F., Leone, M. A., Weber, A., Zipp, F., Miterski, B., Epplen, J. T., Oturai, A., Soelberg Sørensen, P., Celius, E. G., Téllez Lara, N., Montalban, X., Villoslada, P., Silva, A. M., Marta, M., Leite, I., Dubois, B., Rubio, J., Butzkueven, H., Kilpatrick, T., Mycko, M. P., Selmaj, K. W., Rio, M. E., Sá, M., Salemi, G., Savettieri, G., Hillert, J., Compston, D. A. S. (2005) Multiple sclerosis severity score: using disability and disease duration to rate disease severity. *Neurology* **64**, 1144-1151.

Sadiq, S. A., van den Berg, L. H., Thomas, F. P., Kilidireas, K., Hays, A. P., Latov, N. (1991) Human monoclonal antineurofilament antibody cross-reacts with a neuronal surface protein. *J. Neurosci. Res.* **29**, 319-325.

Salih, A. M., Nixon, N. B., Dawes, P. T., Mattey, D. L. (1998) Prevalence of antibodies to neurofilament polypeptides in patients with rheumatoid arthritis complicated by peripheral neuropathy. *Clin. Exp. Rheumatol.* **16**, 689-694.

Silber, E., Semra, Y. K., Gregson, N. A., Sharief, M. K. (2002) Patients with progressive multiple sclerosis have elevated antibodies to neurofilament subunit. *Neurology* **58**, 1372-1381.

Skoda, D., Kranda, K., Bojar, M., Glosova, L., Baurle, J., Kenney, J., Romportl, D., Pelichovska, M., Cvachovec, K. (2006) Antibody formation against β-tubulin class III in response to brain trauma. *Brain Res. Bull.* **68**, 213-216.

Svarcova, J., Fialova, L., Bartos, A., Steinbachova, M., Malbohan, I. (2008) Cerebrospinal fluid antibodies to tubulin are elevated in the patients with multiple sclerosis. *Eur. J. Neurol.* **15**, 1173-1179.

Terryberry, J. W., Thor, G., Peter, J. B. (1998) Autoantibodies in neurodegenerative diseases: antigen-specific frequencies and intrathecal analysis. *Neurobiol. Aging* **19**, 205-216.

Zaffaroni, M. (2003) Biological indicators of the neurodegenerative phase of multiple sclerosis. *Neurol. Sci.* **24** Suppl 5, S279-282.