Příloha 1:

Revidovaná McDonaldova diagnostická kritéria roztroušené sklerózy z r. 2005 (16)

<table>
<tr><td colspan="2">Klinický obraz</td><td rowspan="2">Doplňující údaje potřebné k diagnóze roztroušené sklerózy</td></tr>
<tr><td>Ataky</td><td>Objektivní známky léze (lézí)</td></tr>
<tr><td>2 nebo více</td><td>2 nebo více</td><td>žádné, klinická symptomatika je dostačující</td></tr>
<tr><td>2 nebo více</td><td>1</td><td>• diseminace v prostoru podle MR mozku nebo
• 2 či více lézí na MR mozku kompatibilní s RS a pozitivní MMM nebo
• další klinická ataka z jiné lokalizace</td></tr>
<tr><td>1</td><td>2 nebo více</td><td>• diseminace v čase na MR mozku nebo
• druhá klinická ataka</td></tr>
<tr><td>1 (mono-symptomatická)</td><td>1</td><td>• diseminace v prostoru podle MR mozku nebo
• 2 nebo více lézí na MR mozku kompatibilní s RS a pozitivní MMM a
• diseminace v čase podle MR mozku nebo
• další klinická ataka z jiné lokalizace</td></tr>
<tr><td>0 (progrese od začátku)</td><td>1</td><td>progrese nemoci po 1 rok (retrospektivně či prospektivně) a nejméně 2 ze 3 následujících:
• pozitivní MR mozku (9 nebo více T2 vážených lézí nebo 4 či více T2 vážených lézí s pozitivními VEP)
• pozitivní MR míchy (2 nebo více fokálních T2 lézí)
• pozitivní MMM</td></tr>
<tr><td colspan="3">Co znamená pozitivní nález na MRI? [(175, 176)]</td></tr>
<tr><td colspan="3">Musí být splněna 3 ze čtyř následujících kritérií:
• 1 Gd-enhancující léze mozku či míchy nebo 9 T2 hyperintenzních mozkových a/nebo míšních lézí v případě, že není žádná Gd-enhancující
• 1 nebo více infratentoriálních mozkových nebo míšních lézí
• 1 nebo více juxtakortikálních lézí
• 3 nebo více periventrikulárních lézí</td></tr>
<tr><td colspan="3">Co znamená diseminace v čase:</td></tr>
<tr><td colspan="3">• Gd-enhancující léze detekovaná na MRI nejméně 3 měsíce po začátku prvních klinických příznaků, a to v jiné lokalizaci než léze, která způsobila první ataku nebo
• nová T2 vážená léze detekovaná MRI (ve srovnání s prvním MRI) nejméně 30 dní po začátku prvních klinických příznaků</td></tr>
</table>

Co je pozitivní MMM:
• oligoklonální IgG pásy v MMM (nepřítomné v séru) **nebo** zvýšený IgG index
Co je pozitivní VEP:
• prodloužený, ale dobře zachovaný tvar vlny

Příloha 2: Revidovaná El Escorialská kritéria doporučující využití elektrofyziologických testů pro diagnostiku ALS (88)

1 podmínky pro diagnostiku ALS

- ***A. Přítomnost***
  1. degenerace dolního motoneuronu (LMN) – klinická, elektrofyziologická, neuropatologická
  2. degenerace horního motoneuronu (UMN) – klinická
  3. progresivní rozvoj příznaků v postižené oblasti nebo jejich šíření do dalších oblastí – trvání onemocnění, fyzické a elektrofyziologické testy
- ***B. Nepřítomnost***
  1. dalších chorobných procesů, které by mohly vysvětlit degenerace LMN či UMN – elektrofyziologická a patologických nálezů
  2. dalších onemocnění, která mohou být vysvětlena na základě zobrazovacích metod – klinická, elektrofyziologická

2 Diagnostika – kategorie

- klinicky jistá ALS – klinicky nebo elektrofyziologicky dokázána přítomnost postižení LMN i UMN v bulbární oblasti a nejméně dvou oblastí současně, nebo postižení LMN a UMN ve třech oblastech.
- klinicky pravděpodobná ALS - známky postižení LMN a UMN ve dvou oblastech
- pravděpodobná, laboratorně podpořená ALS – klinické známky postižení UMN a LMN pouze jedné oblasti a EMG důkaz akutních denervačních změn ve dvou nebo více svalech dvou nebo více končetin
- klinicky možná ALS – známky postižení LMN a UMN společně pouze v jedné oblasti nebo postižení UMN ve dvou či více oblastech nebo známky postižení LMN rostrálně k UMN

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Cerebrospinal fluid antibodies to tubulin are elevated in the patients with multiple sclerosis

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Keywords:
anti-tubulin antibodies, cerebrospinal fluid, ELISA, multiple sclerosis, tubulin

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Background and purpose: The aim of this study was to compare the levels of anti-tubulin antibodies (anti-TU) in cerebrospinal fluid (CSF) and serum using bovine tubulin as the antigen in one enzyme-linked immunosorbent assay (ELISA) method (anti-TUb antibodies) and a synthetic neuron-specific octapeptide of tubulin in a second ELISA method (anti-TUs antibodies). *Methods:* Paired CSF and serum samples were obtained from 34 multiple sclerosis (MS) patients, 13 patients with various other neurological diseases (control diseases) and 17 normal control patients (CN). *Results:* CSF levels of anti-TUs and anti-TUb antibodies were significantly lower in the CN group when compared to those in the MS group. On the contrary, serum levels of anti-TU antibodies did not differ among groups. The intrathecal synthesis of anti-TUs antibodies in comparison with anti-TUb was significantly increased in all groups. Significant correlations between anti-TUb and anti-TUs antibodies were observed in the CSF of all three groups. However, with regard to serum, a similar relationship was only found in the MS group. *Conclusions:* The estimation of anti-TU in CSF can contribute to the overall assessment of axonal damage; on the contrary serum anti-tubulin antibodies were not useful for differential purposes in MS. The antibodies to the neuron-specific portion of tubulin seemed to be synthesised predominantly intrathecally.

Introduction

Tubulin is the basic component of microtubules found in tubular structures of the cytoskeleton and comprises as much as 20% of the cellular protein in brain [1]. It is formed by a heterodimer of structurally similar α- and β-tubulins having a highly homologous primary structure and molecular weights of about 50 kDa. The structure of both tubulin subunits is formed by three domains. The C-terminal region, exposed on the outside surface of the microtubule, represents the major source of variation between the α- and β-tubulin isotypes [2]. Eukaryotic cells express multiple isotypes of both tubulin subunits which are encoded by a multigene family [2]. All cells synthesize α- and β-tubulins, but some isoforms are preferentially expressed in the brain. Seven β-tubulin isotype classes, with different tissue occurrences, have been observed in vertebrates [3,4]. Class III β-tubulin is considered to be a cytoskeletal protein with neuronal specificity [5]. It is the most abundant isoform in cells of neuronal origin [6,7]. Its determination is used as a marker of normal and neoplastic nerve tissues [7].

It is known that components of the cytoskeleton, such as tubulin or neurofilaments, may be released from damaged neurons into the extracellular space. These otherwise hidden tissue proteins may then act as extracellular antigens [8]. The presence of anti-cytoskeletal antibodies in serum and cerebrospinal fluid (CSF) has been reported in several neurological diseases [8–16]. Silber *et al*. [17] found statistically not significant elevated intrathecal levels of anti-tubulin antibodies in patients with multiple sclerosis (MS) and Terryberry *et al*. [18] observed higher frequencies of anti-tubulin antibodies in sera and CSF in MS patients in comparison with controls.

The auto-antibodies may even influence cellular processes. It has been shown that antibodies to neuron-specific β-tubulin can interfere with normal tubulin polymerization–depolymerization dynamics [19].

The aim of this study was to evaluate anti-tubulin antibodies (anti-TU) in the serum and CSF in patients with MS and in patients with other neurological diseases. We wanted to determine if various populations of anti-tubulin IgG antibodies could be differentiated using two distinct, yet similar antigens – whole (intact)

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natural tubulin and synthetic tubulin fragment possesses neuronal specificity and if the relationship between neuron-specific and whole-spectrum anti-tubulin antibodies exists. The other goal was to determine if the anti-tubulin responses differed between CSF and serum, and also to estimate the proportion of neuron-specific antibodies (anti-TUs) relative to the whole spectrum of anti-tubulin antibodies (anti-TUb) as well as their intrathecal synthesis. An additional aim was to evaluate the significance of anti-TU antibodies for clinical purposes, especially in patients with MS.

Patients and methods

Subjects

Paired CSF and serum samples were obtained from 34 MS patients, 13 patients with various other neurological diseases (control diseases, CD) and 17 normal control patients (CN). Five patients were examined twice. The clinical data about patients and the treatment for patients with MS are presented in Table 1.

The diagnosis and course of MS at the time of lumbar puncture (LP) were determined using established criteria [20,21]. Fifteen patients were classified as having relapsing–remitting MS, eight patients had secondary progressive MS and six patients had primary progressive MS. Five patients had a clinically isolated syndrome (CIS) as the first manifestation of MS [22]. The disability score for all MS patients was evaluated using the expanded disability status scale (EDSS) [23].

The CD group included patients with a variety of conditions (e.g. polyneuropathy, stroke, meningitis, and hemidysesthesia). The CN patients presented with conditions such as vertigo, headache (spondylogenic, non-specific, and migraine), psychogenic syndrome and fatigue syndrome. A thorough assessment of these patients did not provide any alternative explanations for their complaints. There was no evidence of any structural, hemorrhagic or inflammatory etiology in the CN patients.

All subjects gave written informed consents regarding study participation. The Ethics Committee of the Third Faculty of Medicine, Charles University, Prague approved the study. Specimens were stored at −20°C until analyzed.

Table 1 Clinical characteristics of subjects

Diagnostic group	MS	CD	CN
Number of patients	34	13	17
Female sex n (%)	19 (56)	12 (92)	15 (88)
Age at LP (years)	39 (31–50)	46 (38–53)	36 (26–45)
Disease duration until LP (years)	5.0 (3–13)	n.a.	n.a.
EDSS at LP therapy	3.5 (1.5–5.0)	n.a.	n.a.
none	11	n.a.	n.a.
IS alone	12	n.a.	n.a.
IS + IM	11	n.a.	n.a.
IgG index[a]	0.81 (0.6–1.13)	0.52 (0.47–0.58)	0.51 (0.49–0.60)

Data are expressed as number or median (25th–75th percentile).
MS, multiple sclerosis; CD, diseased controls; CN, normal controls; EDSS, Expanded disability status scale; LP, lumbar puncture; IS, immunosuppressive therapy (steroids or azathioprin or both); IM, immunomodulatory therapy (interferon-beta or glatiramer acetate); n.a., not applicable.
[a]The IgG index indicating intrathecal production of total IgG was calculated as the CSF/serum ratio of concentration of IgG related to the albumin CSF/serum ratio. The pathological value is above 0.7.

Methods

Anti-tubulin antibodies were determined by ELISA methods. Two antigens were used: (i) whole (intact) natural tubulin protein, purified from bovine brains, and (ii) a synthetic tubulin fragment. The octapeptide fragment possesses neuronal specificity and corresponds to the C-end amino acid sequence of class III β-tubulin. The natural protein could serve as an antigen which could reveal the entire spectrum of anti-tubulin antibodies, regardless of neuronal specificity. It was assumed that neuron-specific antibodies (exactly neuron-specific octapeptide antibodies) could be differentiated from antibodies formed against the whole tubulin antigen.

ELISA determination of anti-tubulin IgG antibodies using bovine tubulin (anti-TUb)

An adapted ELISA method was used for the determination of anti-tubulin IgG antibodies [17].

We used tubulin purified from bovine brain (purity >99%, determined by SDS-PAGE electrophoresis) (Cytoskeleton, Denver, CO, USA) as the antigen for coating the wells. For the analysis, serum samples were diluted 1:400 in 1% BSA–PBS and CSF was assayed undiluted. The absorbance of each well was read at 450 and 620 nm using a microplate reader (Labsystems, Helsinki, Finland). For comparative purposes, the same pool of human sera was used as the standard in all analytical series. Serial dilutions of stock pooled serum were performed on each plate. A standard curve was generated from the absorbance data. The highest standard concentration was defined to be 100 arbitrary units. This allowed the absorbance to be transformed into arbitrary units, in which the CSF and serum concentrations of auto-antibodies were expressed. Serum concentrations were multiplied by the dilution factor. The inter-assay and the intra-assay variation for anti-tubulin IgG was 7.3% (n = 5) and 5.9% (n = 5), respectively.

ELISA determination of anti-tubulin IgG antibodies using neuron-specific tubulin octapeptide (anti-TUs)
A commercial ELISA kit (Human anti-β III TUBULIN IgG Vidia, Prague, Czech Republic) was used for the determination of neuron-specific anti-tubulin antibodies. The wells of microplates were coated with synthetic ESESQGPK octapeptide, which corresponded to the C-end amino acid sequence of beta-tubulin class III. A cysteine spacer served for the attachment of the N-terminal of the oligopeptide. Serum samples were diluted 1:100 whilst the CSF samples were used undiluted. ELISA was carried out using the standard protocol. The results were expressed as an index (In) calculated according to the formula: absorbance of sample/cut-off absorbance. The cut-off value was defined by the manufacturer of the ELISA set as the average absorbance of serum samples from blood donors + 2 SD ($n = 30$). The values of serum anti-TU cut-off were compared with those for CSF in group of CN patients. There were not significant differences. The mean inter-assay and intra-assay variation for anti-tubulin IgG was 3.1% ($n = 16$) and 3.0% ($n = 8$), respectively.

Determination of albumin and total IgG in serum and CSF
The concentrations of albumin and total IgG in serum and CSF were assayed using immunonephelometry.

Calculations for determination of intrathecal IgG synthesis and antibodies to neuron-specific tubulin octapeptide/antibodies to bovine tubulin quotient (anti-TUs/anti-TUb quotient)
The intrathecal synthesis of anti-tubulin IgG antibodies was estimated using the following formula for antibody specificity index (ASI): (CSF anti-TUb or anti-TUs IgG/serum anti-TUb or anti-TUs IgG)/(CSF IgG total/serum IgG total).

Presumably, the proportion of neuron-specific antibodies to all antibodies against tubulin was expressed as the quotient of antibodies to neuron-specific tubulin octapeptide/antibodies to bovine tubulin (anti-TUs/anti-TUb quotient). This quotient was calculated for serum (serum quotient) and for CSF (CSF quotient) using anti-tubulin levels expressed as absorbance according to the formula: serum quotient = [(serum anti-TUs $\times$ 100)/(serum anti-TUb antibodies $\times$ 400)]; CSF quotient = CSF anti-TUs/CSF anti-TUb antibodies.

Statistics

Relationships between anti-tubulin antibodies were evaluated using the Spearman's correlation coefficient. Differences amongst all groups were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney *U*-test. The Wilcoxon pair test was used for the statistical analysis of repeated measures and for the comparison of serum and CSF quotient and ASI. The significance level for all tests was $P < 0.05$.

Results

Relationships between levels of bovine tubulin antibodies to neuron-specific tubulin octapeptide antibodies in CSF and serum

In the CSF, levels of antibodies to bovine tubulin were significantly related to those against neuron-specific tubulin octapeptide (MS: $r = 0.8$, $P < 0.0001$; CD: $r = 0.8$, $P < 0.005$; CN: $r = 0.5$, $P < 0.05$). We also found a weak correlation between both types of anti-TU antibodies in the serum of the MS group (MS: $r = 0.4$, $P < 0.05$; CD: $r = 0.2$, n.s.; CN: $r = 0.4$, n.s.).

Levels of CSF and serum anti-tubulin antibodies

ELISA with intact bovine tubulin
Cerebrospinal fluid levels of anti-tubulin antibodies in the MS group were significantly higher than in the CN group ($P < 0.001$). Anti-tubulin antibody levels in the CSF were also higher in the CD group than in the CN group ($P < 0.05$). CSF antibody levels did not differ between the MS and CD groups (Fig. 1a). No differences in serum anti-tubulin antibodies were found amongst the three groups (Fig. 1b).

ELISA with neuron-specific tubulin octapeptide
Cerebrospinal fluid anti-tubulin levels were higher in the MS group than in the CN group ($P < 0.005$). The differences between the CD and CN groups, and the MS group and the CD group were not significant (Fig. 2a). Serum levels of anti-tubulin antibodies did not differ amongst the three groups (Fig. 2b).

Follow-up investigation of CSF and serum anti-tubulin antibodies

The levels of both the serum anti-tubulin antibodies and the CSF anti-tubulin antibodies remained similar over time (Table 2).

Intrathecal synthesis of anti-tubulin antibodies

The intrathecal synthesis of anti-TU antibodies to neuron-specific tubulin octapeptide expressed as ASI was significantly higher than the intrathecal synthesis of anti-TU antibodies to bovine tubulin in all groups

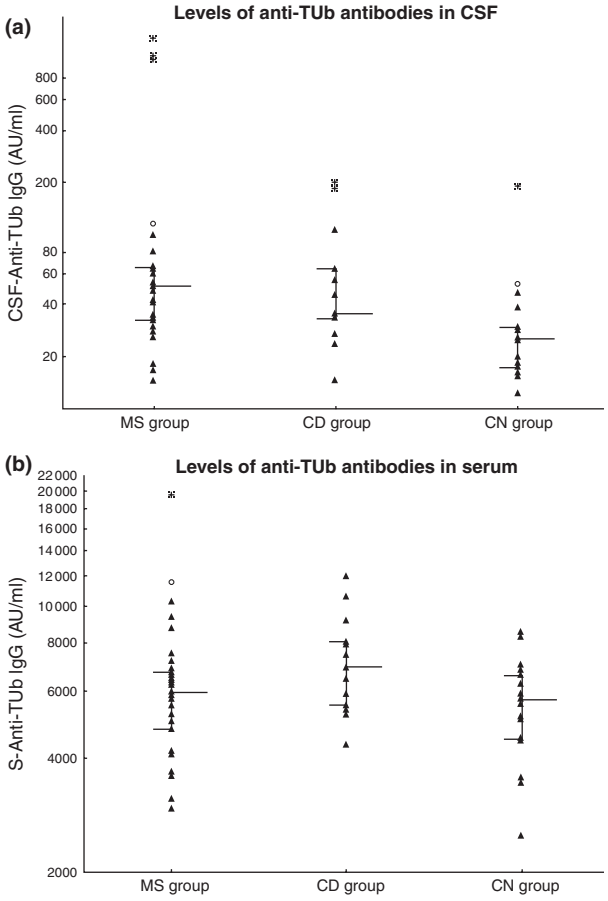

Figure 1 Levels of cerebrospinal fluid (CSF) and serum anti-tubulin antibodies (anti-TUb) using the bovine tubulin antigen in patients and controls. (a) The levels of CSF-anti-TUb antibodies in multiple sclerosis (MS) and control diseases (CD) patients were higher than those found in the normal control (CN) group (MS and CN $P < 0.001$, CD and CN $P < 0.05$). The levels of CSF anti-TUb in the MS and the CD groups did not differ. (b) No differences in serum anti-tubulin antibodies were found among groups. The symbol]– represents the median and the 25th and 75th percentile. The adjacent scatter plot represents the individual values of patients.

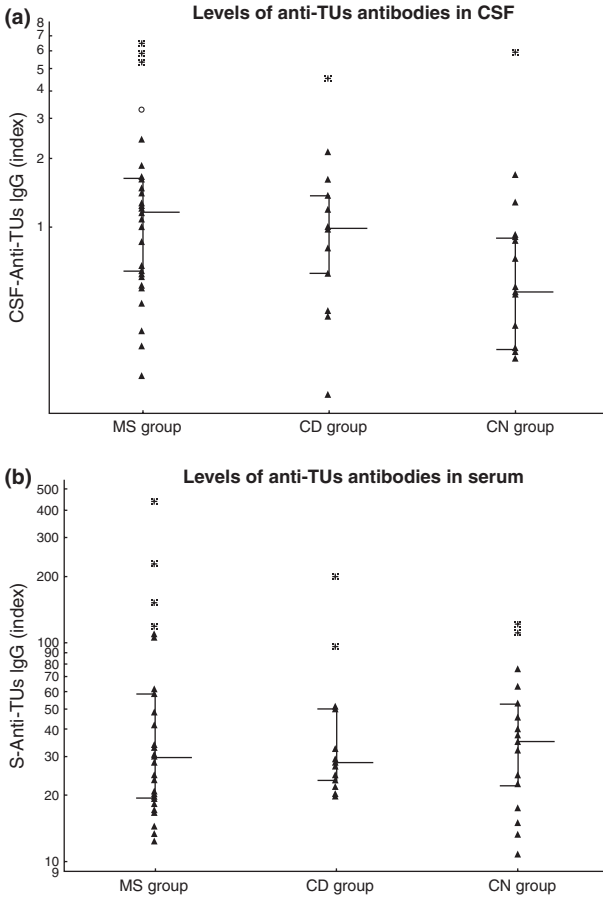

Figure 2 Levels of cerebrospinal fluid (CSF) and serum anti-tubulin antibodies (anti-TUs) using the synthetic neuron-specific octapeptide of tubulin as the antigen in patients and controls. (a) The levels of CSF anti-TUs antibodies were higher in the multiple sclerosis (MS) group than those in the normal control (CN) group ($P < 0.005$). The levels of CSF anti-TUs did not differ between the MS and the control diseases (CD) groups and between the CD and the CN groups. (b) No differences in serum anti-TUs were found among groups. The symbol]– represents the median and the 25th and 75th percentile. The adjacent scatter plot represents the individual values of patients.

(Table 3). No statistically significant differences were found amongst groups for ASI of both anti-TU.

Antibodies to neuron-specific tubulin octapeptide/antibodies to bovine tubulin quotients (anti-TUs/anti-TUb quotients)

A comparison of anti-TUs/anti-TUb quotients in CSF and serum was performed amongst the three groups. We did not find a significant difference amongst serum quotients amongst any combinations of groups. With regard to the CSF quotients, the only significant difference was observed between the MS and CN groups ($P < 0.01$) (Fig. 3a,b). In all patient groups, the CSF quotients were statistically significantly higher than the serum quotients (MS: $P < 0.0001$; CD: $P < 0.005$; CN: $P < 0.001$).

Relationships between anti-tubulin IgG antibodies and total IgG in CSF and serum

Significant correlations between serum anti-TU to bovine tubulin and total serum IgG were observed in all groups. In the CN group, CSF levels of anti-TU antibodies to bovine tubulin were related to the concentration of CSF IgG. We did not find any relationships between anti-tubulin antibodies and total IgG antibodies using the neuron-specific octapeptide in serum/

Table 2 Follow-up of CSF and serum anti-tubulin antibodies in five patients

	Serum, median (25th–75th percentile)		CSF, median (25th–75th percentile)	
	First withdrawal	Second withdrawal	First withdrawal	Second withdrawal
Anti-tubulin antibodies to bovine tubulin (AU/ml)	6204 (5943–6502)	6670 (3734–6772)	61 (59–64)	33 (26–53)
Antitubulin antibodies to neuron-specific octapeptide (In)	23.4 (23.4–28.2)	25.5 (23.4–29.3)	1.2 (1.2–1.3)	0.9 (0.7–1.1)

AU, arbitrary units; In, index calculated according to the formula: absorbance of sample/cut-off absorbance.

Table 3 Antibody specificity indices for antibodies to bovine tubulin (anti-TUb) and neuron-specific synthetic octapeptide of tubulin (anti-TUs)

	Antibody specificity index		
Diagnostic group	Anti-TUb, median (25th–75th percentile)	Anti-TUs, median (25th–75th percentile)	*P*
MS group	1.6 (1.3–2.8)	7.7 (2.5–14.3)	< 0.001
CD group	1.8 (1.4–2.7)	6.7 (4.9–13.6)	< 0.005
CN group	1.8 (1.6–2.01)	9.4 (5.1–21.7)	< 0.001

MS, multiple sclerosis; CD, diseased controls; CN, normal controls.

CSF. For all three groups, serum anti-tubulin antibodies, regardless of antigen, were unrelated to those found in the CSF (Table 4).

Relationships between anti-tubulin antibodies and selected demographic variables

The only inverse relationship was found between intrathecal synthesis of anti-TU to bovine tubulin and patient age in the CN group ($r = -0.73$, $P < 0.001$). No other correlations with demographic variables (subject's age, disease duration, and the EDSS score) were found for either anti-TUb or anti-TUs.

Discussion

In this study, we used ELISA to compare anti-tubulin IgG antibodies with two distinct, yet similar antigens: (i) intact bovine tubulin, and (ii) synthetic octapeptide specific for the neuronal tubulin. We found a close relationship between levels of antibodies to whole tubulin and to its synthetic fragment in the CSF of all three groups and in the serum of the MS group.

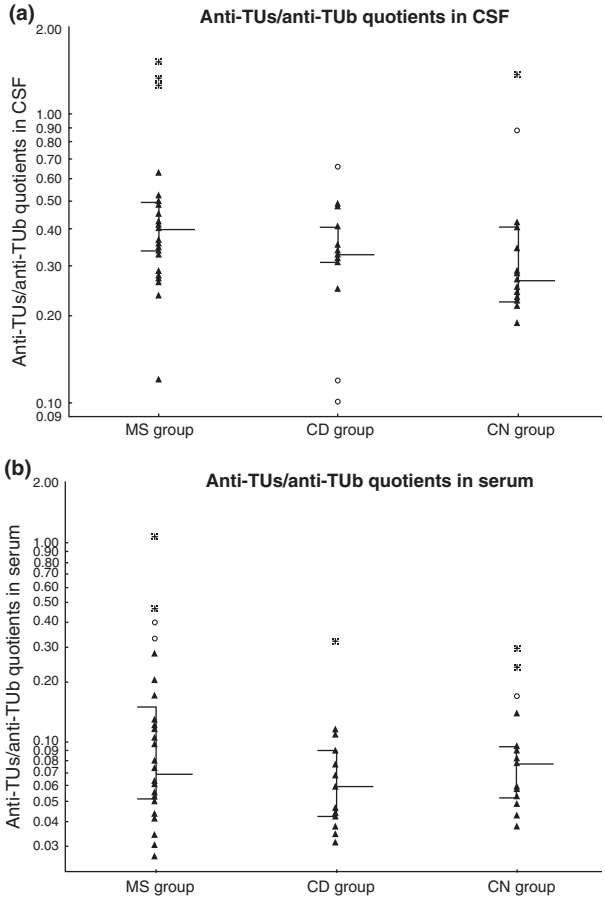

Figure 3 Anti-TUs/anti-TUb quotients expressing the proportion of presumably neuron-specific antibodies related to total antibodies against tubulin. In all patients groups, the cerebrospinal fluid (CSF) quotients were statistically significantly higher than the serum quotient [Multiple sclerosis (MS): $P < 0.0001$, control diseases (CD): $P < 0.005$, normal controls (CN): $P < 0.001$)]. (a) The CSF quotient was higher in the MS group than in the CN group ($P < 0.01$). (b) There were no statistically significant differences among groups relative to the serum quotients. The symbol]– represents the median and the 25th and 75th percentile. The adjacent scatter plot represents the individual values of patients.

Moreover, we observed similar differences amongst groups of subjects in the levels of anti-TU antibodies regardless of antigen. These findings were surprising as protein conformation and tertiary structure are believed to be responsible for antibody binding. The intact tubulin protein, providing numerous antigenic determinants, should have produced a stronger antigen–antibody response than the neuron-specific eight-amino acid piece of tubulin. Despite these assumptions, our data suggest that either the intact tubulin antigen or its eight amino acid fragment can be used interchangeably for ELISA determination of anti-tubulin antibodies in the CSF.

Table 4 Correlations amongst levels of antibodies to anti-TUb, to anti-TUs and total IgG antibodies in CSF and serum

Diagnostic group	Correlations between	Anti-TUb		Anti-TUs	
		r	*P*	*r*	*P*
MS group	S-anti-TU × CSF-anti-TU	0.3	n.s.	0.2	n.s.
	S-anti-TU × S-IgG total	0.5	< 0.005	−0.1	n.s.
	CSF-anti-TU × CSF-IgG total	0.3	n.s.	0.2	n.s.
CD group	S-anti-TU × CSF-anti-TU	0.4	n.s.	−0.03	n.s.
	S-anti-TU × S-IgG total	0.7	< 0.01	0.4	n.s.
	CSF-anti-TU × CSF-IgG total	0.2	n.s.	−0.03	n.s.
CN group	S-anti-TU × CSF-anti-TU	0.3	n.s.	−0.05	n.s.
	S-anti-TU × S-IgG total	0.6	< 0.05	0.4	n.s.
	CSF-anti-TU × CSF-IgG total	0.6	< 0.05	0.4	n.s.

MS, multiple sclerosis; CD, diseased controls; CN, normal controls; S, serum; CSF, cerebrospinal fluid; n.s., not significant; anti-TU, anti-tubulin antibodies; anti-TUb, antibodies to bovine tubulin; anti-TUs, antibodies to the synthetic neuron-specific octapeptide corresponding to the C-end amino acid sequence of β-tubulin class III; *r*, Spearman correlation coefficient.

We also evaluated the clinical relevance of anti-tubulin antibodies. Anti-tubulin antibodies have been explored in several clinical conditions such as brain trauma and neurodegenerative diseases [14,15,18]. In this study, CSF anti-TU antibodies were elevated in patients with MS compared with anti-TU antibody levels of presumably normal subjects. Similarly, Terryberry *et al.* [18] found higher anti-tubulin antibodies in 67% of MS patients in comparison with 4.5% in controls. Silber *et al.* [17] also observed elevated anti-tubulin antibodies in patients with MS but not statistically significant. A brain bovine tubulin as an antigen for ELISA method was used in both studies. We compared two ELISA methods differentiated by antigens. It seems that anti-TU levels to bovine tubulin in CSF may reflect rather axonal damage in general. On the contrary because of statistically significant differences only between MS and CN groups and not between CD and CN anti-TU to neuron-specific octapeptide should be more specific for patients with MS. However, this presumption needs to be confirmed be a larger study. It is possible that a part of tubulin which should induce the synthesis of anti-tubulin antibodies to neuron-specific octapeptide may release more intensively during the process of axonal damage in MS. The relationship between the levels of anti-tubulin antibodies and disability score in MS patients was also described [17]. We were not able to sustain this finding. It is difficult to explain the discrepancy between Silber *et al.* [17] and our study, but the number of patients in previous study was greater and the proportion of patients with regard to various types of MS differed from this study.

Results using serum were clearly different from those using CSF. Serum levels of both types of anti-TU antibodies were similar in all subjects but no relationships between levels of neuron-specific tubulin octapeptide antibodies (anti-TUs) and bovine tubulin antibodies (anti-TUb) with the exception of a weak correlation in the MS group were observed. Levels of antibodies to bovine tubulin in serum corresponded to the total serum IgG levels in all three groups, but similar relationship was not found for antibodies to neuron-specific tubulin octapeptide. We assume that divergent anti-TU antibodies which include those to extraneuronal tubulins as well as to non-specific neuronal epitopes of brain tubulins are present in serum.

The other findings support the conclusion that the spectrum of anti-tubulin antibodies in serum and CSF differs. The evaluation of ASI demonstrated that the synthesis of antibodies against neuron-specific octapeptide predominates in the intrathecal compartment. Further results obtained by comparison of tubulin octapeptide/antibodies to bovine tubulin quotients (anti-TUs/anti-TUb quotients) in CSF and serum corroborate that neuron-specific anti-tubulin antibodies significantly prevail in CSF, especially in the MS group. No relationships between serum and CSF anti-tubulin antibodies were observed. It seems that anti-TU levels in serum and CSF proved to be independent. Therefore, determination of anti-TU antibodies in serum cannot replace CSF analysis.

In conclusion, we confirmed a high correlation between levels of CSF antibodies against the intact form and the neuron-specific part of the same tubulin protein as well as that the neuron-specific anti-tubulin antibodies seemed to be synthesized predominantly intrathecally. The estimation of anti-tubulin antibodies in CSF can contribute to the overall assessment of axonal damage. Serum anti-tubulin antibodies were divergent and not useful for differential purposes in MS.

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Cerebrospinal fluid and serum antibodies against neurofilaments in patients with amyotrophic lateral sclerosis

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amyotrophic lateral sclerosis, anti-neurofilament antibodies, cerebrospinal fluid, ELISA, serum

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Background: The aim of the study was to assess autoimmune involvement in amyotrophic lateral sclerosis (ALS).

Methods: We measured IgG antibodies against light (NFL) and medium (NFM) subunits of neurofilaments using ELISA in paired cerebrospinal fluid (CSF) and serum samples from 38 ALS patients and 20 controls.

Results: Serum levels of anti-NFL were higher in ALS patients than in controls ($P < 0.005$). Serum anti-NFL antibodies and intrathecal anti-NFM antibodies were related to patient disability (serum anti-NFL: $P < 0.05$; intrathecal anti-NFM: $P < 0.05$). Anti-NFL levels were significantly correlated with anti-NFM levels in ALS ($P < 0.001$) and the control group ($P < 0.0001$) in the CSF, but not in serum. Anti-NFL and anti-NFM antibodies significantly correlated between serum and CSF in the ALS group (anti-NFL: $P < 0.0001$; anti-NFM: $P < 0.001$) and in the control group (anti-NFL: $P < 0.05$; anti-NFM: $P < 0.05$).

Conclusions: Autoimmune humoral response to neurocytoskeletal proteins is associated with ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by damage to upper and lower motor neurons. Despite great efforts devoted to understanding the pathogenesis of ALS, the exact mechanisms causing the degeneration of motor neurons remain unclear. Some studies, both past and recent, suggest a possible contribution of cytoskeletal abnormalities and autoimmune mechanisms [1–3].

In an attempt to understand the pathogenesis of ALS and to find suitable biomarkers for monitoring ALS, a variety of analytes in cerebrospinal fluid (CSF) and serum have been explored. Several candidate markers of neuro-axonal damage, such as tau protein or neurofilaments, have been evaluated [4–7]. Autoimmune humoral responses, as a result of the release of certain cytoskeletal proteins, may accompany motor neuron damage and autoantibodies to neurofilaments have been reported in the CSF and/or serum of ALS patients [2,8].

The aim of this study was to evaluate antibodies to light (NFL) and medium (NFM) subunits of neurofilaments in the serum and cerebrospinal fluid of patients with ALS. Moreover, we were interested in the relationship between anti-NF antibodies in the serum and in the CSF as well as between the two anti-NF antibody subtypes.

Patients and methods

Subjects

Paired CSF and serum samples were obtained from 38 amyotrophic lateral sclerosis patients. The diagnoses of ALS (done at the time of the lumbar puncture) were classified using the revised El Escorial criteria [9]. Disability of patients was assessed according to the Amyotrophic Lateral Sclerosis Functional Rating Scale

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(ALSFRS) by an experienced neurologist (PR) [10]. Patients with ALS were divided into a bulbar onset (BO) subgroup and a limb onset (LO) subgroup. The control group of 20 individuals included predominantly patients with headache (spondylogenic or non-specific), vertebrogenic syndromes, cranial facial or oculomotor palsy and neurotic or fatigue syndromes. There was no evidence of any structural, hemorrhagic or inflammatory etiology in control patients. Basic characteristics of ALS patients and controls are shown in Table 1.

All subjects gave a written informed consent regarding study participation. The Postgraduate Medical Education Ethics Committee, Prague, approved the study. Specimens were stored at −80°C until analysis.

Methods

Anti-neurofilament antibodies in serum and cerebrospinal fluid were determined by enzyme-linked immunosorbent assay (ELISA) according to Silber *et al.* [11] with additional modifications [12,13]. Sera were diluted 1:400, whilst CSF samples were analyzed undiluted. The serum concentrations were multiplicated by the dilution factor (400×).

The intra-assay variation was 4.6% for anti-NFL and 4.1% for anti-NFM. The inter-assay variation was 8.4% and 7.4% for anti-NFL and anti-NFM, respectively.

Serum and CSF samples both from ALS patients and controls were stored at −80°C within 1–2 h after collection. Both control and ALS samples were treated in a similar way before freezing. Because samples from ALS patients and controls were recruited simultaneously, storage time was similar. We avoided the repeated freeze-thaw cycles, so that the samples were thawed only once.

The concentrations of total IgG and albumin in serum and cerebrospinal fluid were assayed using immunonephelometry. The intrathecal synthesis of anti-neurofilament IgG antibodies was estimated using the following formula for the antibody specificity index (ASI): (CSF anti-NFL IgG or anti-NFM IgG/serum anti-NFL IgG or anti-NFM IgG)/(CSF IgG total/serum IgG total). Intrathecal synthesis was considered to be present when ASI ≥ 1.5 [14].

Determination of differences amongst all groups was performed using the Mann–Whitney U test. Relationships between variables were analyzed using the Spearman's correlation coefficient. A $P < 0.05$ was considered statistically significant.

Results

Serum anti-NFL levels were significantly elevated in ALS patients in general as well as both ALS subgroups compared with controls (ALS LO versus controls, $P < 0.005$; ALS BO versus controls, $P < 0.05$; ALS total versus controls, $P < 0.005$) (Fig. 1a). The serum levels of anti-NFM were significantly elevated only in the bulbar onset subgroup of ALS (ALS BO versus controls $P < 0.05$) (Fig. 1b).

The CSF levels of both anti-NFL and anti-NFM antibodies were higher in the ALS group, but not significantly (Fig. 2a and b).

Intrathecal synthesis of anti-NFL antibodies was observed in eight patients (three patients with bulbar onset and five patients with limb onset). However, only a slight elevation of ASI values (1.51 and 1.55) were noticed in two of these patients. Five patients (one patient with bulbar onset and four patients with limb onset) had intrathecal synthesis of anti-NFM antibodies. However, intrathecal synthesis did not vary within the ALS group as a whole, or between ALS subgroups or in comparison with the control group (Fig. 3a and b).

In the CSF, anti-NFL levels were significantly related to anti-NFM levels in the ALS and control group. A similar relationship was not found for anti-neurofilament antibodies in serum. There was a significant relationship between serum anti-NFL and anti-NFM and CSF anti-NFL and anti-NFM in the ALS group and the control group (Table 2). We found a relationship between the levels of anti-NF antibodies in cerebrospinal fluid and the blood–CSF barrier function

Table 1 Basic characteristics of ALS patients and controls

Diagnostic group	ALS (total)	ALS BO	ALS LO	Controls
Number (*N*)	38	22	16	20
Age (years)*	62 ± 9	65 ± 8	60 ± 9	46 ± 17
Sex (female/male)	18/20	14/8	4/12	11/9
Disease duration (months)**	9.0 (7.0–12.0)	10.0 (7.0–20.0)	8.0 (6.5–11.5)	na
ALSFRS score**	35.0 (33.0–37.0)	35.5 (33.0–38.0)	34.5 (31.5–37.0)	na

ALS, patients with amyotrophic lateral sclerosis; ALS LO, limb onset form of ALS; ALS BO, bulbar onset form of ALS; ALSFRS, Amyotrophic Lateral Sclerosis Functional Rating Scale; na, not applicable; *Data are expressed as mean age ± SD; **Data are expressed as median (25th–75th percentile).

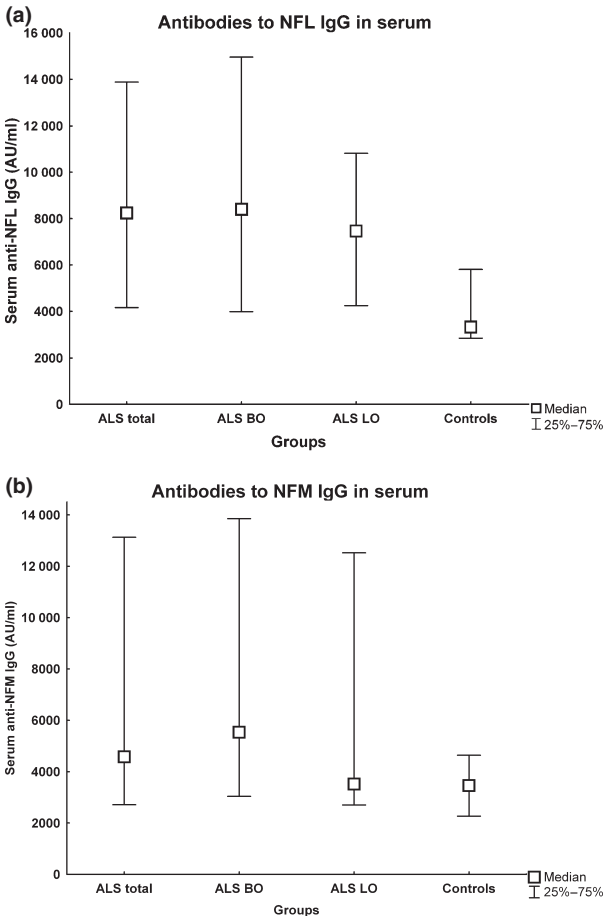

Figure 1 (a, b) Serum levels of anti-NFL IgG and anti-NFM IgG antibodies in the amyotrophic lateral sclerosis (ALS) patients and the controls. (a) There were significant differences in serum anti-NFL antibodies between ALS (total patients) and controls ($P < 0.005$), between ALS BO and controls ($P < 0.05$) and between ALS LO and controls ($P < 0.005$). (b) There was a significant difference between ALS BO and controls ($P < 0.05$). Abbreviations: ALS LO, limb onset form of ALS; ALS BO, bulbar onset form of ALS; anti-NFL, antibodies against light subunits of neurofilaments; anti-NFM, antibodies against medium subunits of neurofilaments; AU, arbitrary units.

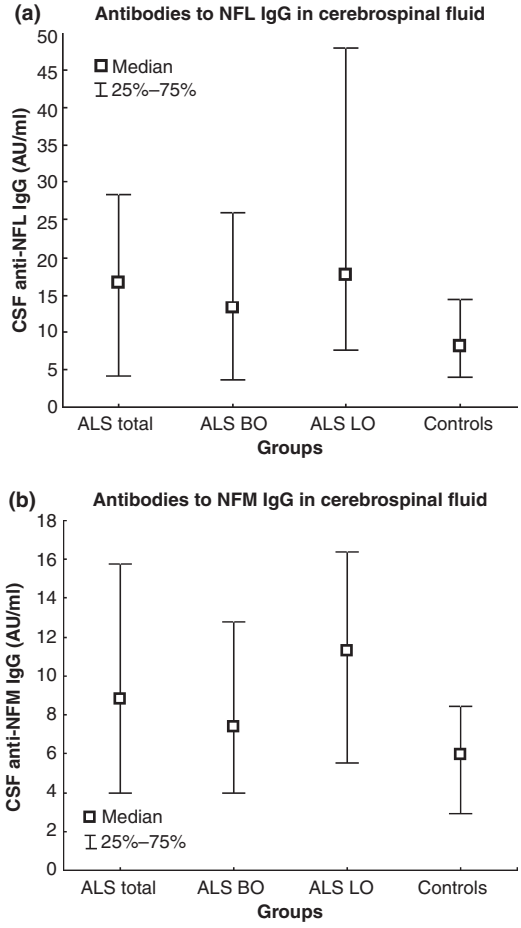

Figure 2 (a, b) CSF levels of anti-NFL IgG and anti-NFM IgG antibodies in the amyotrophic lateral sclerosis (ALS) patients and controls. There was no significant difference between groups in anti-NFL or anti-NFM antibodies. Abbreviations: CSF, cerebrospinal fluid; Other abbreviations are explained in the Fig. 1.

[expressed as Q_{alb} (albumin concentration in CSF related to that in serum)] in ALS patients (anti-NFL: $r = 0.5$, $P < 0.005$; anti-NFM: $r = 0.4$; $P < 0.05$), but not in the control group.

No correlation was found between total IgG and antibodies to NFL and NFM in serum, with the exception of a significant relationship between total IgG and anti-NFL in the control group ($r = 0.5$; $P < 0.05$). A significant correlation between total IgG and anti-NFL in the CSF was observed in the ALS group ($r = 0.5$; $P < 0.005$).

We observed a significant difference in age between ALS patients and controls ($P < 0.01$). However, we found no relationship between age and anti-NFL and anti-NFM levels in serum and CSF in ALS patients or in the control group. Intrathecal synthesis of anti-neurofilament antibodies also failed to correlate with age in any group.

ALSFRS and serum anti-NFL levels were related in the ALS group ($r = -0.3$; $P < 0.05$). Additionally, a relationship between ALSFRS and intrathecal synthesis of anti-NFM was found in the ALS group ($r = -0.4$, $P < 0.05$). No correlation between disease duration and anti-NF in serum or CSF was observed.

Discussion

We found that anti-NFL levels in ALS patients and anti-NFM in ALS BO patients were increased in serum, but not in the CSF or intrathecally. We assume that, as a consequence of motor neuron degeneration, the

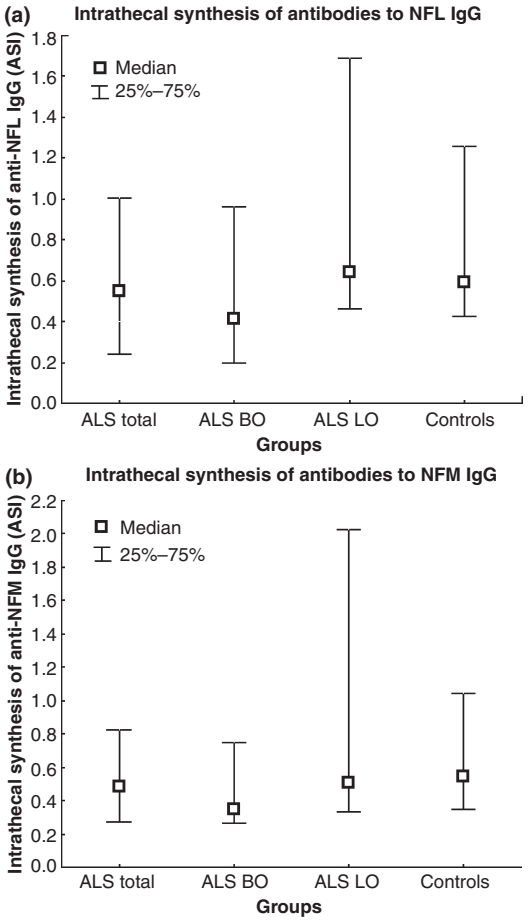

Figure 3 (a, b) Intrathecal synthesis of anti-NFL IgG and anti-NFM IgG antibodies in the amyotrophic lateral sclerosis (ALS) patients and the controls. Intrathecal synthesis of anti-NF antibodies was similar in all groups. Abbreviations: ASI, antibody specificity index; Other abbreviations are explained in the Fig. 1.

released neurofilaments or their fragments leak into the blood and that, therefore, the immune response to neurofilaments is expressed more in the periphery than in the CSF compartment.

Table 2 Correlations among levels of IgG antibodies to NFL and NFM in CSF and serum

	ALS total		Controls	
Correlation	*r*	*P*	*r*	*P*
S-anti-NFL × S-anti-NFM	0.3	0.058	0.3	n.s.
CSF-anti-NFL × CSF-anti-NFM	0.6	< 0.001	0.8	< 0.0001
S-anti-NFL × CSF-anti-NFL	0.6	< 0.0001	0.5	< 0.05
S-anti-NFM × CSF-anti-NFM	0.5	< 0.001	0.5	< 0.05

ALS, amyotrophic lateral sclerosis; Anti-NFL, antibodies against light subunits of neurofilaments; anti-NFM, antibodies against medium subunits of neurofilaments; CSF, cerebrospinal fluid; *r*, Spearman correlation coefficient; S, serum.

The release of neurofilaments into the interstitial fluid and their diffusion into the CSF may induce the synthesis of autoantibodies [15]. This process has been studied in some neurologic diseases associated with axonal damage. Some previous studies have reported elevated anti-NF antibodies in serum and/or CSF or increased intrathecal synthesis in various neurologic diseases including ALS [2,8,11]. In our studies with patients having multiple sclerosis, we demonstrated elevated levels of intrathecal anti-NFM [13]. The immune response is not only directed against cytoskeletal structures, but also other neuron components such as gangliosides [3,16,17].

The absence of a relationship between anti-NFL and anti-NFM levels in serum may be caused by different antigen stimulation depending on the individual neurofilament subunits in the serum and CSF. This view may be supported by the finding of a non-significant correlation between total IgG and anti-NFL and anti-NFM in the serum of ALS patients in contrast to a significant correlation between total IgG and anti-NFL in the CSF. This particular relationship seems to be a common feature. We have found similar differences in other patients with a variety of neurologic diseases as well as in control groups [18].

The correlation of ALSFRS with serum anti-NFL and intrathecal anti-NFM levels indicates that disability is reflected by antibody status. The significance was only weak, and the group of patients was not large enough to draw definite conclusions, but it suggests that anti-NF may play a role in the autoimmune mechanisms of ALS.

It is not clear whether autoantibodies are involved in the pathogenesis of ALS or if their presence is only an epiphenomenon of the immune response. Some experiments suggest a role in the neurodegeneration seen in ALS patients [19]. After injection (into mice) of IgG samples, isolated from sera of ALS patients, IgGs were observed in the axon terminals of lower motor neurons [20]. It was reported that passively transferred IgG from the sera of ALS patients to mice can have cytotoxic effects or induce signs of degeneration [19].

In summary, anti-NF was higher in the serum ALS patients, and serum anti-NFL was inversely correlated with patient disability. These findings contribute to the growing body of knowledge associated with the immune humoral response in ALS patients.

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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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