SHORT REPORT

Increased concentration of C4d complement protein in CSF in amyotrophic lateral sclerosis

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Abstract

Plasma and CSF concentrations of C4d and the circulating immune complex to C1q were measured in 27 patients with amyotrophic lateral sclerosis (ALS) or cervical spondylosis. There was no significant difference among groups in plasma C4d or in plasma or CSF concentrations of the circulating immune complex to C1q. The ALS group, however, had a significantly higher CSF concentration of C4d than the group with cervical spondylosis, as well as a higher C4d index (CSF to plasma C4d ratio × serum to CSF albumin ratio). These results suggest that augmented complement activation in the CNS occurs in ALS. Increased CSF concentration of C4d or raised C4d index may serve as a basis for differentiating ALS from cervical spondylosis.

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The cause of amyotrophic lateral sclerosis (ALS) remains unknown, but several authors have reported immunological abnormalities. These include detection of immune complexes in serum,[1 2] increased concentrations of C4,[2] deposits of IgG and C3 in the motor cortex and spinal cord,[3 4] an IgG subclass deficiency,[5] and lymphocyte infiltration into the spinal cord.[6 7] Furthermore, on the basis of an immune animal model of motor neuron disease, Smith *et al*[8] speculated that the pathogenesis of ALS may be initiated by circulating antibodies directed against specific membrane constituents.

We reported earlier the activation of complement proteins of the classical pathway in affected oligodendroglia (complement activated oligodendroglia[9 10]) in several neurological diseases, including ALS.[6 9] Both these complement activated oligodendroglia and damaged myelinated axons are readily stained by antibodies to C4d and we speculated that one role of complement proteins in these structures is opsonisation.[9 10]

The frequent appearance of C4d positive structures in various brain areas in some neurodegenerative disorders suggests that increased concentrations of this protein might be found in CSF. Recently, this possibility has been confirmed in patients with progressive supranuclear palsy who showed both increased concentrations of C4d in the CSF and a higher C4d index than were found in cases of Parkinson's disease or cervical spondylosis.[11] Here, we found that concentrations of C4d were increased in ALS, whereas those of the circulating immune complex to C1q were not.

Patients and methods
Fifteen patients with ALS and 12 with cervical spondylosis were examined. The table gives the profiles for the ALS and cervical spondylosis groups. A Mann-Whitney U test showed that there was no significant difference in age between the groups. The diagnosis of ALS was based on the presence of both upper and lower motor neuron symptoms and signs with or without bulbar signs and with a progressive course. In the ALS group, the severities of the weakness in the bulbar muscles and in the muscles of the upper and lower extremities, and of spasticity were each rated by semiquantitative indices (0, none; 1, slight; 2, moderate; and 3, severe). The table shows the mean severity scores.

After informed consent was obtained from each patient, 5 ml of CSF was obtained by lumbar puncture. Blood was obtained at the same time. Albumin and IgG in both serum and CSF were measured. Two millilitres of CSF with 10 mM EDTA and the plasma were used for complement analysis.

A circulating immune complex enzyme immunoassay kit (Quidel) (sandwich assay) was used for the measurement of the concentration of the circulating immune complex to C1q. Plasma specimens were diluted (1 to 50) before assay, but CSF samples were not. After incubation of the specimen in the microtitre well for one hour at room temperature, horseradish peroxidase conjugated goat anti-human IgG was added to each well and the mixtures incubated for 30 minutes at room temperature. After reaction with the chromogenic substrate according to the supplier's directions, the absorbances (A405 values) were measured spectrophotometrically (MRP-A4i, TOSOH). A standard curve was generated by plotting the A405 values obtained at various concentrations of the standard. Results were expressed as μg (serum) or ng (CSF) of heat aggregated human γ-globulin equivalents per ml

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Summary of the profile (mean age (SD) and sex distribution) and the means (SDs) of concentrations of complement proteins, C4d and IgG indexes, and Q albumin values in patients with ALS and cervical spondylosis

	Age (y)	Sex M:F	Plasma		CSF		C4d index	IgG index	Q albumin	Duration of illness (months)	Score of severity
			CIC/C1q (μg Eq/ml)	C4d (μg/ml)	CIC/C1q (ng Eq/ml)	C4d (ng/ml)					
ALS (n = 15)	60·5 (10·4)	9:6	2·5 (2·7)	43·0 (11·5)	12·1 (5·7)	1173 (348)**	5·6 (2·0)*	0·4 (0·1)	6·5 (3·0)	11·4 (5·9)	5·2 (2·0)
Cervical spondylosis (n = 12)	54·5 (11·3)	8:4	1·7 (1·6)	41·1 (24·7)	11·7 (6·4)	468 (178)	2·8 (1·7)	0·4 (0·2)	7·0 (3·0)		

*$p < 0.01$; **$p < 0.001$ for differences between the ALS and cervical spondylosis groups. Score of severity was calculated by addition of the ratings (0, none; 1, slight; 2, moderate; 3, severe) assigned to indicate the degree of weakness in the bulbar muscles and those of the upper and lower extremities, as well as the degree of spasticity (maximum possible = 12). CIC: circulating immune complex.

(μg Eq/ml or ng Eq/ml). The detection limit of the assay, taken as 3 SD above the mean absorbance of the zero standard, was 2 ng Eq/ml.

To measure the C4d concentration, we developed a sandwich assay. Microassay plates were coated with human C4d antibody (mouse monoclonal, 1:100, Quidel). The specificity of the antibody has been reported earlier.[11] The specimen was diluted 1 to 50 for CSF and 1 to 400 for plasma. In the second stage, we used a horseradish peroxidase conjugated human C4 antibody (goat polyclonal, 1:2000, binding site). The third stage was reaction with the same chromogenic substrate used in the circulating immune complex to C1q assay, followed by reading the absorbance at A450. Results, obtained by comparison with a standard curve generated with various known concentrations of C4d (Quidel), were expressed as μg/ml of C4d for plasma and ng/ml of C4d for CSF. The detection limit of the assay was 10 ng/ml.

All assays were done in duplicate. The Q albumin—CSF albumin concentration × 10³/serum albumin concentration—was calculated as an indicator of blood-brain barrier function. The CSF IgG index ((CSF IgG concentration × serum albumin concentration)/(serum IgG concentration × CSF albumin concentration)) and the CSF C4d index ((CSF C4d concentration × serum albumin concentration)/(serum C4d concentration × CSF albumin concentration)) were taken as measures of the intrathecal synthesis of IgG or C4d respectively.

One of the patients died of respiratory failure and was confirmed pathologically as having ALS. Small blocks from the precentral cortex and high cervical spinal cord were dissected and were fixed in 4% paraformaldehyde for two days in the cold. We also used spinal cord tissue from non-neurological cases aged 65 and 70. The cryoprotected blocks were cut at 20 μm on a freezing microtome and stained by human C4d antibody (mouse monoclonal, Quidel, 1:500) as previously described.[10]

Results

The table shows the group means and SDs for the concentrations of the circulating immune complex to C1q and C4d in plasma and CSF, the C4d and IgG indexes, and the value of Q albumin.

Mann-Whitney U tests showed no significant difference between the groups in the concentrations of plasma C4d, plasma circulating immune complex to C1q, or CSF circulating immune complex to C1q, or in the IgG index. The value of the IgG index in all the ALS cases was below 0·7, which may indicate normal IgG synthesis.[2] Of the variables measured, only the CSF concentrations of C4d ($p < 0.001$) and the CSF C4d index ($p < 0.01$) showed significant differences.

The values of Q albumin ranged from 2·8 to 13·9 in the ALS and from 3·2 to 11·8 in the group of cervical spondylosis, with the mean values not being significantly different. If a value of < 7·0 is taken as normal,[12] six (40%) of the cases and eight (67%) of the cervical spondylosis cases had slight to moderate damage to the blood brain barrier.

In the ALS group, the CSF C4d correlated significantly with severity scores ($r = 0.76$, $p < 0.01$), with CSF IgG ($r = 0.62$, $p < 0.05$), and with Q albumin ($r = 0.64$, $p < 0.01$). The Q albumin also correlated significantly with CSF IgG ($r = 0.85$, $p < 0.01$) and with the duration of illness ($r = 0.72$, $p < 0.01$). In the group with cervical spondylosis, no correlations were found among the variables.

Immunohistochemistry with the antibody to C4d showed abundant complement activated oligodendroglia in the precentral cortex, especially in the grey matter. Fewer complement activated oligodendroglia were seen in the spinal cord, but numerous deposits of C4d on fibrous structures were found in the anterior horn (figure (A)). In the
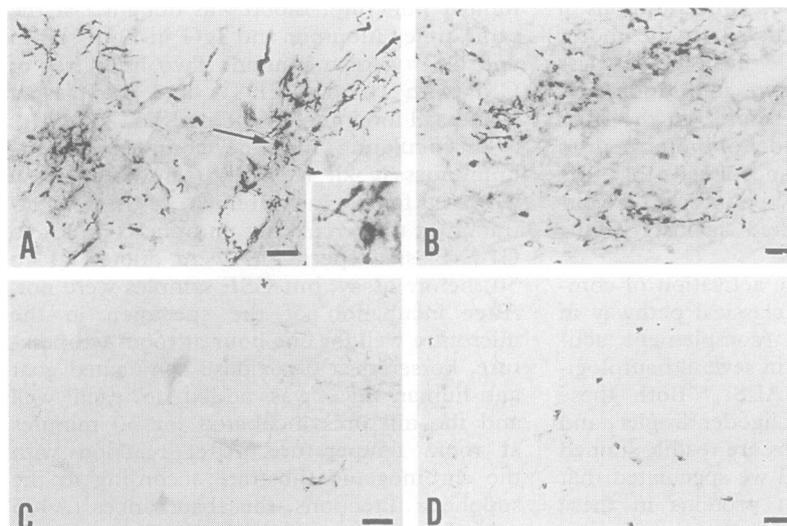
Immunostaining by the antibody to C4d of spinal cord tissue from an ALS case (A,B) and a neurologically normal control (C,D). (A) The staining in the anterior horn shows abundant C4d positive fibrous structures. Arrow indicates an oligodendroglial cell. High magnification in this area is shown in the right corner. (B) Numerous C4d positive structures in the lateral funiculus may be related to myelinated long tracts (round dots). (C) Only a few C4d positive fibres are present in the anterior horn of a control. Negatively stained, neurons containing lipofuscin are present in the background. (D) Dot like structures in the lateral funiculus reflect C4d attached to myelinated axons. Bar = 50 μm.

lateral funiculus, dotted deposits of C4d seemed to be attached to the surface of corticospinal fibres running longitudinally (figure (B)), the same as in our previous report.[6] By contrast, only a few C4d-positive structures were seen in neurologically normal control tissue (figure (C,D)).

Discussion

A major result of this study is the indication that the C4d concentration in the CSF and the CSF C4d index might be used to differentiate ALS from cervical spondylosis. Occasionally, the differential diagnosis between ALS and cervical spondylosis is difficult in the early stages, especially if the cervical spondylosis presents predominant motor or pure motor signs. Moreover, ALS sometimes occurs in association with cervical spondylosis.

An increased serum C4 concentration has been reported in ALS cases.[2] In our study, the plasma C4d concentration was not significantly higher in ALS than in cervical spondylosis, but both the C4d concentrations in the CSF and the C4d index were. The exact mechanism for the increased concentrations of C4d in the CSF is unknown. These high concentrations are not surprising, however, in view of the many complement activated oligodendroglia and deposits of C4d seen immunohistochemically in ALS.[6] We have not yet done immunohistochemical studies on cervical spondylosis tissue, but the low values of C4d in the CSF suggest that few complement activated oligodendroglia would be found, and this is consistent with its restricted pathology. We believe that C3d and C4d attachment to oligodendrocytes may be an early event in demyelination.[9 10] If this is true, there may be continuous complement activation in progressive diseases with widespread pathology such as ALS or progressive supranuclear palsy. It is still not clear how the complement cascade is activated, but it may be either by IgG deposition[4] or damaged myelin.[13]

The correlation analyses on the ALS data suggest that the blood brain barrier breakdown progresses as the disease develops, and that increases in the CSF C4d concentration reflect progressive pathology in the CNS. The evidence that CSF IgG and CSF C4d both increase as the blood brain barrier breakdown becomes more severe (as evidenced by their significant correlations with Q albumin) might suggest their passage from the systemic circulation into the brain. Therefore, the increased C4d in CSF might be determined by both increased intrathecal C4d synthesis and by an influx from the systemic circulation in ALS. On the contrary, cervical spondylosis had low C4d synthesis and no clear evidence of C4d influx from the systemic circulation. These results show that CSF C4d assay may be a major diagnostic adjunct in ALS.

In our study, the circulating immune complex of C1q in plasma and the IgG index were not increased. These findings agree with one previous report[14] but conflict with some others.[1 2] A possible role for autoimmunity in the pathogenesis of ALS has been suggested by several groups.[8 15 16] Alterations of cellular (lymphocyte infiltration in the spinal cord) and humoral immunity in ALS have recently been extensively studied by Appel's group.[4 7 8 16 17] According to their hypothesis, there is a possibility that complement proteins play a part in the degeneration of motor neurons. The other possibility, however, that complement activation merely reflects a secondary response to motor neuron degeneration caused by some other process[14] still remains. Our data do not prove a pathogenetic role for complement proteins in ALS, but suggest they are an important phenomenon of the disease.

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