Analysis of intrathecal antibody production against *Chlamydia pneumoniae* in multiple sclerosis patients

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Abbreviations

AI = antibody index, *B. burgdorferi* = *Borrelia burgdorferi*, CSF = cerebrospinal fluid, *C. pneumoniae* = *Chlamydia pneumoniae*, CP = chronic progressive, EAE = Experimental autoimmune encephalomyelitis, ECL = enhanced chemiluminescence, ELISA = Enzyme-linked-immunosorbent assay, IEF = Isoelectric focusing, Ig = immunoglobulin, MBP = Myelin Basic Protein, MOG = Myelin Oligodendrocyte Glycoprotein, MS = Multiple Sclerosis, OD = Optical density, OIND = other inflammatory neurological diseases, OND = other neurological diseases, PCR = Polymerase chain reaction, RR = relapsing remitting,
Summary

Multiple Sclerosis (MS) is one of the most frequent organic diseases of the nervous system, with a prevalence of 30-60 per 100,000 inhabitants. It is characterized by an inflammatory destruction of the myelin sheaths in the white matter of the central nervous system, which may lead to severe disability and death. The underlying mechanism has not been clearly elucidated yet, but involves an attack of the body’s immune system against some of its own neural tissue antigens. One of the immunopathologic hallmarks of MS is the chronic intrathecal production of immunoglobulin (Ig). This contains IgG of very restricted variability, i.e. oligoclonal IgG, and in addition, recognizes a panel of different pathogens such as measles, rubella, and herpes zoster virus. While the antigen-specificity of the largest part of oligoclonal IgG in multiple sclerosis is unknown, the oligoclonal IgG arising during CNS infections are reactive against the specific pathogen. Recently, a link between Chlamydia (C.) pneumoniae and multiple sclerosis has been claimed. To test the possible role of C. pneumoniae in multiple sclerosis, we analyzed a) whether there is intrathecal IgG production against C. pneumoniae in multiple sclerosis and b) whether the oligoclonal IgG in the CSF of multiple sclerosis patients recognize C. pneumoniae.

By studying paired serum/CSF samples from 120 subjects (definite multiple sclerosis: 46; probable multiple sclerosis: 12; OIND: 35; OND: 27) by ELISA, we found that 24% of all patients with definite multiple sclerosis, but only 5% of patients with other inflammatory or non-inflammatory diseases produced IgG specific for C. pneumoniae intrathecally (definite multiple sclerosis versus OIND: p = 0.027). The presence of intrathecal IgG to C. pneumoniae was independent of the duration of disease and relatively stable over time. The major CSF oligoclonal IgG bands from multiple
sclerosis-patients with an intrathecal Ig-production to *C. pneumoniae* did not react to *C. pneumoniae* by IEF-Western as seen by isoelectric focusing and subsequent affinity-mediated immunoblot (IEF-Western) towards purified elementary bodies and reticulate bodies of *C. pneumoniae*. By contrast, the IgG in the CSF of control patients with neuroborreliosis strongly reacted with their specific pathogen, *Borrelia burgdorferi* by IEF-Western. Concomitant analysis of the CSF of 23 patients with a nested PCR for *C. pneumoniae* was negative in all cases. Together, these findings strongly suggest that the immune response to *C. pneumoniae* is part of a polyspecific intrathecal Ig production, as is commonly observed with other pathogens. This argues against a specific role of *C. pneumoniae* in multiple sclerosis.
Zusammenfassung

Die Multiple Sklerose (MS) ist eine der häufigsten organischen Erkrankungen des zentralen Nervensystems, mit einer Prävalenz von 30-60 pro 100.000 Einwohner. Sie zeichnet sich durch eine entzündliche Zerstörung der Myelinscheiden aus. Eine prominente immunpathologische Veränderung bei der MS ist die intrathekale Produktion von Immunglobulinen (Ig). Diese umfassen sogenannte oligoklonale Banden, die mit IEF darzustellen sind und deren Vorhandensein auch für die Diagnosefindung von Bedeutung ist. Darüberhinaus produzieren MS-Patienten intrathekal Ig gegen eine Vielzahl verschiedener Pathogene, wie zum Beispiel Masern-, Röteln- und Varizella-Zoster-Viren. Während die Antigenspezifität der oligoklonalen IgG bei der MS weitestgehend unbekannt ist, reagieren die während einer Infektion des ZNS gebildeten oligoklonalen IgG mit dem spezifischen Pathogen. Kürzlich wurde ein Zusammenhang zwischen Chlamydia (C.) pneumoniae und der Multiplen Sklerose postuliert. Um diesen Erreger auf seine mögliche Rolle bei der Multiplen Sklerose hin zu prüfen, analysierten wir a) ob die Multiple Sklerose mit einer intrathekalen Produktion von IgG gegen C. pneumoniae verbunden ist und b) ob die oligoklonalen IgG im Liquor cerebrospinalis (CSF) von MS-Patienten C. pneumoniae erkennen.

Indem wir gepaarte Serum-/CSF-Proben von 120 Patienten (gesicherte MS: 46, wahrscheinliche MS: 12, andere entzündliche neurologische Erkrankungen: 35, andere nichtentzündliche neurologische Erkrankungen: 27) mit dem Enzyme-linked Immunosorbenent Assay (ELISA) untersuchten, fanden wir, dass 24% aller Patienten mit gesicherter MS aber nur 5% der Patienten mit anderen entzündlichen oder nichtentzündlichen Erkrankungen des ZNS intrathekal IgG spezifisch gegen C. pneumoniae produzierten (gesicherte MS versus andere entzündliche Neurologische...
Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) affecting 30-60 out of 100,000 inhabitants of Central Europe, the etiology of which remains unknown. Both infectious agents and pathological autoimmune reactions to CNS antigens have been implicated in the etiology of multiple sclerosis (Noseworthy et al., 2000). Recent neuropathological data have not only provided evidence for an important role of anti-myelin autoantibodies (Genain et al., 1999), but have also pointed to a considerable immunopathological heterogeneity of multiple sclerosis (Lucchinetti et al., 1998). Specifically, two patterns of multiple sclerosis plaques were identified that would be consistent with infectious or toxin-induced demyelination (Lucchinetti et al., 2000). The pathogenetic heterogeneity of multiple sclerosis has obvious therapeutic consequences.

An infectious etiology of multiple sclerosis has been suspected for more than a century, results from different areas of investigation pointing in this direction. Epidemiologic analysis of all cases of MS on the Faroe Islands from 1920 to 1977 indicated a point source epidemic of MS, probably introduced by British troops or their baggage. Thus, MS on the Faroe Islands appeared to be a transmissible, most likely infectious, disease. Furthermore, studies of identical twins of which one has MS have shown that the disease develops in only 30% of second twins, indicating that more than a susceptible genotype determines disease (Spielman et al., 1982). Over the years a large number of different viruses have been linked to multiple sclerosis (Meinl, 1999), including measles virus, herpesviruses such as Epstein-Barr virus and human herpesvirus 6, human retroviruses (especially human T-cell-leukemia virus type 1), JC polyoma virus. To confuse matters further, anti-viral immune responses,
including intrathecal antibody production to such common pathogens as measles, rubella and herpes zoster viruses, are quite common in multiple sclerosis patients: they can be found in 60%, 60% and 50% of MS patients, respectively, and are present individually or in combination in more than 90% of MS patients (Reiber, 1995). These polyspecific anti-viral IgG do not correspond to the major oligoclonal IgG bands of the CSF and are considered as a bystander reaction (Measles-Rubella-Zoster (MRZ-) reaction) (Sindic et al., 1994; Luxton et al., 1995; Reiber et al., 1998). Furthermore, patients with multiple sclerosis have predominantly low affinity antibodies against these pathogens in the CSF, whilst patients with a primary viral infection have predominantly high affinity antibodies against the causative organism (Luxton et al., 1995).

Very recently, C. pneumoniae has been linked to multiple sclerosis. Sriram and colleagues reported on a multiple sclerosis patient who failed to respond to immunosuppressive treatment, but had C. pneumoniae in the CSF and improved dramatically after antibiotic treatment (Sriram et al., 1998). In a larger cross-sectional study, the same group reported that C. pneumoniae could be cultured from 64 % of multiple sclerosis patients versus 11 % of controls and polymerase chain reaction (PCR) allowed the detection of C. pneumoniae genome in 97 % of multiple sclerosis patients versus 18 % of OND controls (Sriram et al., 1999).

C. pneumoniae is an obligate intracellular bacterium that infects alveolar macrophages, monocytes and endothelial cells, including human brain microvasculature endothelial cells (MacIntyre et al., 2002) and infects only humans, generally causing pharyngitis, bronchitis and pneumonia. Over the last decade, substantial evidence of a causative role of C. pneumoniae in atherosclerosis has accumulated. In the field of neurology, C. pneumoniae has been detected in affected
brain tissue from patients with Alzheimer disease, the pathology of which is distinctly different from MS. Several case reports (summarized in Yucesan et al., 2001) of acute CNS manifestations such as Encephalitis, meningitis, Guillain-Barré-Syndrome associated with *C. pneumoniae* infection hint toward a possible neurotropism of the organism. Chlamydia bacteria have been linked to a chronic encephalopathy in cows, which is called sporadic bovine encephalomyelitis (Sriram et al., 1998; Harshfield, 1970). Serology indicates that about half of the population in developed countries have had contact to *C. pneumoniae* (Hargreaves et al., 1994) and that seroconversion to *C. pneumoniae* interestingly usually occurs at 5 to 15 years of age (Thom DH et al., 1991), the same time epidemiologic studies have indicated that MS is acquired, even though disease does not usually develop until after puberty. If *C. pneumoniae* were linked to the pathogenesis of certain subtypes of multiple sclerosis, this would have striking therapeutic consequences. Indeed, based on previous results (Sriram et al., 1999), clinical trials of antibiotic therapy are now underway (Treib et al., 2000). Several contradictory brief reports, mostly in the form of letters, have meanwhile appeared, shedding doubt on the Chlamydia hypothesis. The number of patients studied in these scattered reports was small, and data were mainly based on PCR analysis (Layh-Schmitt et al., 2000; Boman et al., 2000; Treib et al., 2000; Pucci et al., 2000; Hammerschlag et al., 2000; Li et al., 2000). However, the PCR detection of *C. pneumoniae* in the CSF is not standardized and the contradictory results might be explained by different PCR protocols, different strategies to extract DNA, different handling of the CSF, amount of CSF drawn, cell number in the CSF, and other variations.

On the basis of experience with other infectious CNS diseases, it is evident that a negative PCR in the CSF does not exclude an infectious agent as a cause of
disease. For example, in herpes simplex encephalitis the PCR is positive only at the beginning of the disease. After about two weeks the PCR is usually negative, but the diagnosis can retrospectively be established by specific IgG production to herpes simplex virus and by specific reactivity of oligoclonal bands to this virus assessed by isoelectric focusing (IEF) with subsequent affinity mediated immunoblot (IEF-Western). This serological feature is very stable and reliable (Sauerbrei et al., 2000).

Likewise, in neuroborreliosis the diagnostic sensitivity of the PCR from CSF was estimated to be just 17 %, and the diagnosis is routinely based on an intrathecal IgG production to *Borrelia (B.)burgdorferi* (Lebech et al., 2000).

The aim of our present study was to test the *Chlamydia* hypothesis. We studied a total of 120 patients, using a combination of techniques, including conventional serology, IEF Western, and PCR. Immunoglobulins are stable outside the body and the experiments are not affected by the amount of CSF obtained and the speed of further processing. Most importantly, we examined the oligoclonal bands, which are present in 97% of MS patients (Zeman et al., 1995) and despite their not being restricted to MS, have been considered the “holy grail” in the search for the solution of the MS problem. Because, if *C. pneumoniae* infection was pathogenetically relevant, it would be expected that the major CSF oligoclonal bands are directed to this pathogen (Gilden, 1999).

We found that an intrathecal IgG production to *C. pneumoniae* is indeed more common in multiple sclerosis patients than controls. However, in contrast to a previous report (Sriram et al., 1999), all our PCR results were negative. More importantly, we report the novel finding that in multiple sclerosis patients, the major CSF oligoclonal bands are not directed to *C. pneumoniae* as assessed by IEF-Western. This is in striking contrast to another chronic bacterial CNS infection,
neuroborreliosis, where IEF-Western analysis revealed a strong reactivity of *B. burgdorferi*-specific oligoclonal bands. Taken together, our results suggest that the intrathecal immune response to *C. pneumoniae* in multiple sclerosis is probably a bystander reaction, very similar to the well-known Measles-Rubella-Zoster (MRZ) reaction (Reiber et al., 1998).
Materials and Methods

Patients

Paired serum and CSF samples from a total of 120 patients were analyzed. Of these, 12 patients were diagnosed as possible multiple sclerosis, 35 as RR-multiple sclerosis, and 10 as CP-multiple sclerosis (Poser et al., 1983). Thirty-five patients with diseases such as viral encephalitis, Guillain-Barré syndrome, meningitis, and chronic inflammatory demyelinating polyneuropathy were classified as other inflammatory neurologic diseases (OIND). Twenty-seven patients with diseases such as disc prolaps, headache, vertigo, and cerebral infarct were classified as other neurological diseases (OND) (Table 1). The serum and CSF samples were analysed in a blinded manner. For analysis of the antigen-specificity of the oligoclonal bands by IEF-Western, four additional patients with definite neuroborreliosis were examined.

Serology

An ELISA specifically detecting IgG reactive with C. pneumonieae was obtained from Hain Diagnostics (Nehren, Germany, manufactured by Savyon Diagnostics, St Ashdod, Israel). Purified elementary bodies of strain TW-183 were used as antigen. In a recent comparative analysis this ELISA performed well in comparison with complement fixation and microimmunofluorescence (Persson, Boman, 2000). Seropositivity was assessed according to the instructions of the manufacturer using a 1:100 dilution of the serum. To determine the presence of anti-C. pneumonieae IgG in the CSF, the spinal fluid was diluted 1:2. In analogy to evaluation of serum samples, CSF samples were scored positive for C. pneumonieae, if the measured optical density
was at least twice the optical density of the negative control, which was measured around an OD of 0.2.

**Determination of intrathecal IgG production**

To assess the presence of an intrathecal IgG production against *C. pneumoniae*, CSF was diluted 1:2 and the corresponding serum was diluted to the same concentration of IgG. The specific intrathecal IgG production antibody index (AI) was then calculated as $\text{AI} = \frac{\text{OD}_{\text{CSF}}}{\text{OD}_{\text{serum}}}$. In the case of an intrathecal IgG production, the corrected AI was calculated as $\text{AI} \times Q_{\text{IgG}/Q_{\text{lim}}}$. $Q_{\text{lim}}$ was calculated as described (Reiber, Lange, 1991). An AI > 2 was considered to indicate a significant and reliable intrathecal IgG production against the pathogen studied.

Immunoglobulins (Ig) in the CSF are either blood-derived or have been produced intrathecally.

The proportion of blood-derived Ig is dependent upon blood-CSF-barrier function, measured as the CSF/serum concentration quotient of Albumin (Alb), $Q_{\text{Alb}}$.

$$Q_{\text{Alb}} = \frac{c(Alb_{\text{CSF}})}{c(Alb_{s})}$$

$$Q_{\text{IgG}} = \frac{c(IgG_{\text{CSF}})}{c(IgG_{s})}$$

In patients that show no intrathecal Ig production, the relation between the CSF/Serum concentration quotients of Ig ($Q_{\text{Ig}}$) and Albumin ($Q_{\text{Alb}}$) has been empirically determined.

Based on these measurements and on the laws of diffusion (of molecules across the blood-CSF barrier), Reiber developed a formula by which one can calculate a maximum value for $Q_{\text{Ig}}$, termed $Q_{\text{lim}(Ig)}$, in the absence of intrathecal Ig production.
A $Q_{Ig}$ exceeding $Q_{lim}(Ig)$ indicates that part of Ig in the CSF originates from antibody forming cells within the CSF space.

$Q_{lim}(Ig)$ = the statistical upper limit of a blood-derived Ig fraction in CSF (comprising +/- 3 standard deviations or 99% of cases)

$$Q_{lim}(Ig) = 0.93\sqrt{(Q_{Alb})^2 + 6 \cdot 10^{-6}} - 1.7 \cdot 10^{-3}$$

The specific intrathecal immune response of a certain antibody species is expressed as Antibody Index (AI).

$AI = \frac{Q_{spec}}{Q_{Ig}}$

If there is intrathecal antibody synthesis, we refer to $Q_{lim}(Ig)$ instead of $Q_{Ig}$:

$$AI' = \frac{Q_{spec}}{Q_{lim}}$$

Example of a patient (GCE) with an intrathecal immune response to Chlamydia pneumoniae:

$c(Alb_{CSF}) = 0.223 \text{ g/l}$
$c(Alb_S) = 45.6 \text{ g/l}$
$\Rightarrow Q_{Alb} = 4.89 \cdot 10^{-3}$

$c(IgG_{CSF}) = 0.124 \text{ g/l}$
$c(IgG_S) = 12.8 \text{ g/l}$
$\Rightarrow Q_{IgG} = 9.69 \cdot 10^{-3}$
Specific antibodies directed against Chlamydia pneumoniae were detected by ELISA:

$Q_{\text{lim}}(\text{IgG}) = 0.93 \sqrt{\left[ (4.89 \times 10^{-3})^2 + 6 \times 10^{-6} \right]} - 1.7 \times 10^{-3} = 3.50 \times 10^{-3}$

$\text{OD}_{450\text{nm}} (\text{CSF}) = 0.691$

$\text{OD}_{450\text{nm}} (\text{Serum}) = 0.460$

$\Rightarrow Q_{\text{spec}} = \frac{0.691}{0.460} = 1.53$

Since CSF and Serum were diluted to equivalent concentrations of IgG for ELISA,

$A_I = Q_{\text{spec}} = 1.53$

$A_I$ must be corrected for the intrathecal IgG synthesis:

$A_{I'} = A_I \cdot \frac{Q_{\text{IgG}}}{Q_{\text{lim}}} = 1.53 \cdot 2.77 = 4.24$

Since $A_{I'}$ is greater than 2, this patients’ CSF contains intrathecally produced IgG against C.p.

**Culture and processing of C. pneumoniae and B. burgdorferi**

The *C. Pneumoniae* and *B. Burgdorferi* antigens being used in the IEF-Western blots have been prepared by M. Hartmann, Institute for Medical Microbiology, FSU Jena, and B. Wilske, Max-v-Pettenkofer-Institute, Munich, respectively, as described (Derfuss, Gürkov *et al.*, 2001)
An isoelectric focusing (IEF) of Ig in the CSF can be performed in an agarose gel or a polyacrylamide gel. Agarose gels are more suitable for affinity mediated transfer, but polyacrylamide gels allow a better resolution of individual bands. Since the purpose of this part of the study was to perform a sensitive IEF Western analysis, agarose gels were chosen for these experiments. In addition, oligoclonal banding was revealed at high resolution in a subset of patients using polyacrylamide gels.

To perform the IEF in agarose gels, unconcentrated CSF and serum were subjected to an IEF gel pH 3 – 10 according to the manufacturer’s guidelines (Titan Gel Electrophoresis Kit, Helena BioSciences, UK). All agarose IEF gels were run under the same conditions. Briefly, the IgG in CSF were diluted to 50 µg/ml in 0.9 % NaCl if they had a concentration higher than 50 µg/ml, and were used undiluted if they had a concentration lower than 50 µg/ml. The IgG in the serum was diluted to the respective CSF concentration. Five µl of the dilutions of CSF and serum were used for the IEF gel. After focusing, the gels were blotted on nitrocellulose paper for 30 min with a weight of 1 kg. The blotted IgGs were detected with anti-human-IgG-HRP and ECL as a substrate.

To determine the antigen-specificity of oligoclonal bands in the CSF, IEF and a subsequent affinity mediated immunoblot were performed. To this end the nitrocellulose paper was coated with 10 µg/ml antigen (5 ml / 12 cm²) (C. pneumoniae or B. burgdorferi) in 0.1 M carbonate buffer pH 9.5 overnight at 4°C before blotting. To block non-specific binding the nitrocellulose paper was subsequently incubated in 10% low fat milk powder for 1 h at room temperature. As a control, a part of the membrane was coated only with 10% milk powder for 1 h at room temperature. The IEF-Western protocol was adjusted such that no IgG blotted to
the membrane coated with milk powder. After blotting the IgG were detected with anti-human-IgG-peroxidase (supplied with the kit) or goat anti-human-IgG-biotin (Sigma) and streptavidin-peroxidase (Jackson). Anti-human-IgG-peroxidase was used to detect *B. burgdorferi* specific oligoclonal bands. For detection of *C. pneumoniae* specific oligoclonal bands the sensitivity had to be increased and the biotin-streptavidin system was used. Using this biotin-streptavidin system the sensitivity could be increased by a factor of around 10. The blots were developed with the enhanced chemiluminescence (ECL) system. The signals were detected with X-ray films or the LAS 1000 (Fuji, Straubenhardt, Germany). For pI determination a calibration kit from Amersham Pharmacia was used. The marker was stained in the agarose gel with Coomassie.

To validate the quality of the antigen and the coating procedure, a dot blot assay was performed. To this end, 2.5 µg of the antigen preparation were dotted on the membrane. After the spot had dried the membrane was blocked with 10 % milk powder for 1 hour at room temperature. Then the membrane was incubated with different dilutions of sera for 30 min at room temperature. Bound IgG were detected with goat-anti-human-peroxidase and the blot was developed with ECL.

IEF was also performed in polyacrylamide gels (Amersham Pharmacia Biotech, Uppsala, Sweden) in a subset of patients to visualize the oligoclonal bands with this method. The gels were run according to manufacturer’s instructions. Briefly, 20 µl of serum and CSF diluted to an IgG concentration of 0.02 mg / ml were added on sample application pieces located 4 cm from the anode. The gel was run for 1.5 hours at 1500 V and 10°C. Subsequently, proteins were silver stained as described (Wurster, 1983).
Results

Seroprevalence and intrathecal production of IgG specific for C. pneumoniae

First, we screened all patients and controls for the presence or absence of a serum and CSF IgG response to *C. pneumoniae*. The proportion of seropositive patients was similar in the multiple sclerosis and control groups: About 50 % of all studied patients showed serological evidence of prior infection with *C. pneumoniae* (Table 1).

Table 1. IgG response to *C. pneumoniae* in serum and CSF

<table>
<thead>
<tr>
<th>Group</th>
<th>Probable MS</th>
<th>Definite RR-MS</th>
<th>Definite CP-MS</th>
<th>OIND</th>
<th>OND</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>35</td>
<td>11</td>
<td>35</td>
<td>27</td>
</tr>
<tr>
<td>Seropositive to C.p.</td>
<td>5/12 (42%)</td>
<td>16/35 (46%)</td>
<td>7/10 (70%)</td>
<td>16/35 (46%)</td>
<td>16/25 (64%)</td>
</tr>
<tr>
<td>Intrathecal IgG to C.p.</td>
<td>1/12 (8%)</td>
<td>9/35 (26%)</td>
<td>2/11 (18%)</td>
<td>2/35 (6%)</td>
<td>1/26 (4%)</td>
</tr>
</tbody>
</table>

This table shows the number of patients studied and their classification. The number and percentage of patients who are seropositive for Cp and who displayed an intrathecal IgG production to Cp with an AI > 2 are shown. C.p. = *Chlamydia pneumoniae*. MS = multiple sclerosis

In this regard, there was no difference between multiple sclerosis patients and controls. Sixty patients were seropositive for *C. pneumoniae*. Out of these, 37 % had anti-*C. pneumoniae* IgG in the CSF. This ELISA is not sensitive enough to detect
transudation of IgG into the CSF of marginally seropositive patients. Conversely, among the identified 15 patients with an intrathecal IgG production to *C. pneumoniae*, 11 scored positive for anti-*C. pneumoniae* IgG in the serum.

In contrast, 26 % of patients with RR-multiple sclerosis and 20 % of patients with CP-multiple sclerosis (mean = 24 % for all patients with definite multiple sclerosis), but only 6 % of the OIND and 4 % of the OND patients showed an intrathecal IgG production to *C. pneumoniae* (Table 1). The specific AI in all patients with an intrathecal IgG response to *C. pneumoniae* was between 2.1 and 8 with a mean AI of 3.86. The difference between the proportion of patients with an intrathecal IgG response to *C. pneumoniae* in the definite multiple sclerosis group (11/46) and the OIND group (2/35) was statistically significant (Chi$^2$ test: p = 0.027). The presence or absence of an intrathecal IgG production to *C. pneumoniae* did not correlate with disease duration: Eleven multiple sclerosis patients with intrathecal IgG production to *C. pneumoniae* had a mean disease duration of 10.4 years, whereas 29 multiple sclerosis patients without intrathecal IgG production to *C. pneumoniae* had a mean disease duration of 10.0 years. Taken together, the results demonstrate that there is a difference between multiple sclerosis and controls in the intrathecal, but not in the serum IgG response to *C. pneumoniae*.

**Stability of the antigen-specific intrathecal IgG production over time**

We assessed the temporal stability of anti-*C. pneumoniae* IgG by repeat CSF analysis in 9 patients. The time interval between the two CSF collections ranged from three weeks to 8 months. Five of these 9 patients showed an intrathecal IgG production to *C. pneumoniae*. In all 9 patients, the responder status (that is, presence or absence of an intrathecal IgG response) remained unchanged over the observation period. In
eight of the nine cases, the quantitative specific AI to *C. pneumoniae* was very stable over time. In one OIND patient, the corrected AI to *C. pneumoniae* dropped from 8.1 to 3.4 after 5 months.

In one multiple sclerosis-patient, we compared the time course of *C. pneumoniae*-specific IgG to the reactivity against several other infectious agents (Measles, Rubella, VZV, CMV and HSV). This patient, who had had clinically definite remitting-relapsing multiple sclerosis for twelve years, had elevated AIs to all these agents. This type of response has been described as the “Measles-Rubella-Zoster (MRZ)” reaction (Reiber *et al.*, 1998). CSF was obtained at two time points, 6 months apart. After the first CSF sample revealed an intrathecal IgG response to *C. pneumoniae*, the patient was treated with doxycyclin (200 mg per day p.o.) for two weeks. As shown in Table 2, all AIs remained essentially unchanged. Together, the results show that the intrathecal immune response to *C. pneumoniae*, as well as to other infectious agents, is quite stable over time.
Table 2: Longitudinal stability of intrathecal IgG production against different pathogens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Date of lumbar puncture</th>
<th>Corrected AI November 1999</th>
<th>Corrected AI July 2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. pneumoniae</td>
<td></td>
<td>2.8</td>
<td>2.1</td>
</tr>
<tr>
<td>B. burgdorferi</td>
<td></td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>Measles virus</td>
<td></td>
<td>9.1</td>
<td>9.9</td>
</tr>
<tr>
<td>Rubella virus</td>
<td></td>
<td>22.2</td>
<td>18.6</td>
</tr>
<tr>
<td>Varizella zoster virus</td>
<td></td>
<td>17.6</td>
<td>21.3</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td></td>
<td>1.9</td>
<td>2.5</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td></td>
<td>2.6</td>
<td>1.9</td>
</tr>
</tbody>
</table>

CSF and serum of a patient with definite RR-multiple sclerosis was analysed for IgG against different viral antigens and C. pneumoniae at two different time points by ELISA. The corrected AI that describe the intrathecally produced IgG against the different pathogens are shown.

**Antigen-specificity of oligoclonal bands**

Next, we tested the antigen-specificity of CSF oligoclonal bands by IEF-Western blotting, using C. pneumoniae, B. burgdorferi, and milk powder as antigens. Low fat milk powder is commonly used as a blocking reagent and served here to control the specificity and evaluate unspecific transfer. This analysis was done in all 15 samples with an intrathecal IgG production to C. pneumoniae (Table 1), in three samples with a high C. pneumoniae-specific OD in the ELISA in both serum and CSF and
additionally as a control in 8 samples without anti-\textit{C. pneumoniae} IgG as assessed by ELISA.

Eleven of the 15 patients with an intrathecal IgG production to \textit{C. pneumoniae} had definite multiple sclerosis. In none of these 11 multiple sclerosis-patients were the oligoclonal bands in the CSF specific for \textit{C. pneumoniae}. Ten of them did not show any specific response to \textit{C. pneumoniae} in the IEF Western. A representative example is shown in Figure 1A. In one of these 11 patients, weak reactivity to \textit{C. pneumoniae} was evident by IEF-Western (Figure 1B).

**Figure 1: Lack of reactivity of multiple sclerosis oligoclonal bands to \textit{C. pneumoniae}**

**Figure 1.** In A and left panel of B, CSF and serum of two multiple sclerosis-patients were analysed by IEF-Western. Both patients had an intrathecal IgG production to \textit{C. pneumoniae} by ELISA. Patient GCe (A) (AI = 4.6) did not show any reactivity to \textit{C. pneumoniae} in the IEF Western, while patient KoYu (B) (AI = 2.4) displayed a weak reactivity that seemed to be distinct from the major oligoclonal
bands. The membranes were coated with *C. pneumoniae* or milk powder as indicated. Immunoglobulin, which was affinity-mediated to the coated membranes, was detected with anti-human IgG-biotin, streptavidin-peroxidase, and ECL. The immunoglobulins blotted to the uncoated membranes were detected with the less sensitive system using anti-human IgG-peroxidase and ECL. IEF markers, which are indicated left in panel B, were stained in the gel with Coomassie. The right panel in B shows IEF of serum and CSF of patient KoYu in a silver stained polyacrylamide gel. The region between pH 9.3 (indicated by the presence of cystatin C in the CSF) and pH 6.5 is shown.

Two of the 4 control patients with an intrathecal IgG production to *C. pneumoniae* displayed reactivity to *C. pneumoniae* in the IEF Western. One representative example (patient HuM) of these two is shown (Figure 2B). This patient presented with hypoglycaemic coma, having a 32-years-history of type1-diabetes. PCR to detect *C. pneumoniae* in the CSF was not done in this patient. The other patient (NM) was diagnosed with limbic encephalitis of unknown origin and focal epileptic seizures. The CSF of this patient did not contain *C. pneumoniae* as assessed by PCR. It should be noted that the IEF-Western reactivity in these patient samples was only detected by the very sensitive method of applying streptavidin/biotin. This indicates that only a small minority of the IgG in the CSF was directed against *C. pneumoniae*. 
Figure 2: Weak reactivity to *C. pneumoniae* in special cases

**Figure 2.** In A (left panel) the IEF Western of a patient PoVe (probable multiple sclerosis) with a high IgG response to *C. pneumoniae* in both serum and CSF, but without an intrathecal Ig production to *C. pneumoniae* (AI = 1.3) is shown. Both serum and CSF react to *C. pneumoniae* by IEF Western. In B
the IEF Western of patient HuM is shown. This patient, who suffered from a hypoglycemic coma and a long-standing diabetes mellitus, had an intrathecal IgG production against *C. pneumoniae*. In the IEF Western analysis IgG reactive to *C. pneumoniae* both in CSF and serum can be seen. The vertical line in A and B indicates that CSF and serum were run on different gels under the same focusing conditions. In A (left panel) and B membranes were coated with *C. pneumoniae*, milk powder as control, or were left uncoated. IgG was detected with anti-human IgG-biotin, streptavidin-peroxidase, and ECL on coated membranes and with the less sensitive anti-human IgG-peroxidase on uncoated membranes. The right panel in A shows IEF of serum and CSF of patient PoVe in a silver stained polyacrylamide gel. The region between pH 9.3 (indicated by the presence of cystatin C in the CSF) and pH 6.5 is shown.

Three patients with high reactivity in serum and CSF to *C. pneumoniae* by ELISA, but without an intrathecal IgG production to *C. pneumoniae* (AI = 0.9, AI = 1.0, and AI = 1.3) were also analysed by IEF Western. Two of them (PoVe with probable multiple sclerosis, LuAl with definite CP-multiple sclerosis) showed reactivity in both serum and CSF in the *C. pneumoniae*-directed IEF-Western. The third (ZiSa with RR-multiple sclerosis) did not react by IEF-Western. Of note, IEF-Western analysis also detected bands in the serum of patients PoVe and LuAl. Again, specificity of this reactivity was confirmed by using milk proteins and *B. burgdorferi* as control antigens (data not shown). One representative example (PoVe) is shown in Figure 2A. The patient PoVe, who had probable multiple sclerosis, showed the strongest response to *C. pneumoniae* by IEF-Western. However, even in this patient, the very sensitive detection system was required (Figure 3). Importantly, since none of these two patients showed a quantitative intrathecal IgG response to *C. pneumoniae*, this bacterium is unlikely to be involved in their disease. The AI did not correlate with IEF-Western reactivity. This might be explained by the fact that the AI
reflects the ratio between CSF and serum reactivity and does not necessarily mirror the total amount of IgG in the CSF.

Figure 3: Comparison of the IEF Western response to C. pneumoniae and B. burgdorferi using detection systems with different sensitivities

Figure 3. CSF and serum of patient NB 3 (neuroborreliosis) and CSF of patient PoVe (probable multiple sclerosis) were tested with different detection systems for their reactivity to B. burgdorferi and C. pneumoniae, respectively. Detection was performed either with the more sensitive anti-human IgG-biotin and streptavidin-peroxidase (indicated as B+SA-PO) or with the less sensitive anti-human IgG-peroxidase (indicated as PO). C. pneumoniae reactive oligoclonal bands in the CSF of patient PoVe can be observed only with the more sensitive detection system, whereas a strong reactivity to B. burgdorferi in the CSF of patient NB 3 can already be seen with the less sensitive detection system. As
indicated, membranes were coated with *C. pneumoniae*, *B. burgdorferi*, or milk powder as control. The applied IgG concentrations of samples from NB3 and PoVe were identical.

For additional specificity control of the IEF-Western analysis, 8 patients (4 with RR-multiple sclerosis, two with probable multiple sclerosis, one with CP-multiple sclerosis, and one OIND patient) were studied who did not have reactivity to *C. pneumoniae* in the CSF measured by ELISA. When these 8 patients were analysed by IEF-Western for reactivity to *C. pneumoniae*, none of them showed any reactivity even when evaluated with the highly sensitive detection system.

*Further evaluation of the IEF Western Blot system and comparison with the immune response to B. burgdorferi*

To further validate our IEF-Western system, we analysed serum/CSF pairs from four patients with definite neuroborreliosis by IEF Western. These four control patients with neuroborreliosis showed a strong intrathecal IgG production to *B. burgdorferi* with a mean AI of 43.4.
Figure 4. CSF of patient NB 1 with neuroborreliosis (panel A) was taken at the onset of disease and showed an intrathecal production of IgG against *B. burgdorferi* with an AI of 4. CSF of patient NB 2 (panel B) was taken one year after onset of neuroborreliosis, the specific AI was 22. CSF and serum were separated by IEF and subsequently blotted to a nitrocellulose membrane coated with *B. burgdorferi* or milk powder. The IgG affinity mediated to the coated membranes and the IgG blotted to the uncoated membranes was detected with anti-human IgG-peroxide.

All four patients showed – as expected – *B. burgdorferi*-specific oligoclonal IgG in the CSF (Figures 3 and 4). In addition, we analysed the reactivity of serum from the four patients to *B. burgdorferi* by IEF-Western. In three patients *B. burgdorferi*-specific reactivity was observed by IEF-Western in both serum and CSF. One representative patient is shown in Figure 4. In one patient the IEF-Western reactivity to *B. burgdorferi* was detected in CSF only (Figure 4B). In all 4
neuroborreliosis patients *B. burgdorferi*-specific oligoclonal IgG was readily detected with the less sensitive detection protocol using anti-human Ig-peroxidase, whereas detection of reactivity to *C. pneumoniae* by IEF-Western required the sensitive detection system (Figure 3). One of these neuroborreliosis patients was also cross-examined with *C. pneumoniae* and did not react to this agent by IEF-Western (data not shown). The *B. burgdorferi* specific IgG response in CSF was strong and distributed over a broad range of pI (Figures 3 and 4). That makes a direct comparison of the oligoclonal IgG detected in the uncoated membrane with the *B. burgdorferi*-specific oligoclonal IgG difficult. This observation is in accordance with an earlier study about *B. burgdorferi*-specific oligoclonal IgG in the CSF (Martin et al., 1988).

As reported in the previous section, most of the patients who had a detectable intrathecal IgG production to *C. pneumoniae* in ELISA showed only weak or no reactivity of their oligoclonal bands to *C. pneumoniae* in IEF-Western, even with the sensitive technique. To exclude the possibilities of insufficient coating of the nitrocellulose membrane or alteration of the Chlamydia antigen during or after coating, we performed a series of additional experiments. First, CSF-IgG of patients PoVe and HuM reacted by IEF Western to *C. pneumoniae*, but not to *B. burgdorferi* (Figure 2 and data not shown). CSF of patient PoVe was run in parallel as a positive control in all IEF Western experiments searching for *C. pneumoniae* reactivity. Second, to further evaluate the efficiency of binding of *C. pneumoniae*, membranes were coated with *C. pneumoniae* and blocked with 10 % milk powder as for the IEF-Western, and diluted serum samples were applied directly to the coated membranes without prior IEF-separation. Subsequently, the membranes were developed with the same detection system as used for the IEF-Western method. With this method, reactivity of the serum samples that were positive by ELISA could be detected at
dilutions down to 1:30,000. The results of these experiments show that the \textit{C. pneumoniae} antigens bind efficiently to the nitrocellulose membrane and can be readily recognized by patients´ antibodies.

\textbf{Discussion}

Our analysis of 120 paired CSF and serum samples from multiple sclerosis patients and controls revealed the following: a) a positive serum IgG response to \textit{C. pneumoniae} was observed in about 50 percent of multiple sclerosis patients and controls; b) in 20-25 \% of multiple sclerosis-patients, but only 4-6 \% of controls, there is clear evidence for intrathecal synthesis of antibodies directed against \textit{C. pneumoniae} and c) IEF Western blotting demonstrated that in multiple sclerosis the major CSF-specific oligoclonal bands are not directed against \textit{C. pneumoniae}.

\textit{Seroprevalence and intrathecal IgG production}

The overall seroprevalence of \textit{C. pneumoniae} in multiple sclerosis patients and control OIND and OND patients was similar. About 50 \% of the patients and controls were seropositive for \textit{C. pneumoniae}. This is within the range of expected seropositivity in the normal population. For example 43 \% of basic trainees of the US airforce had preexisting Abs to \textit{C. pneumoniae} (formerly called TWAR strain) (Hargreaves \textit{et al.}, 1994). This finding alone - that only about half of the multiple sclerosis patients show serological evidence of previous infection with \textit{C. pneumoniae} - would argue against a role for \textit{C. pneumoniae} in \textit{all} multiple sclerosis-patients.
In contrast to the proportion of overall seropositivity, which was essentially identical between multiple sclerosis patients and controls, there was a clear difference in the proportion of patients with intrathecal antibody production to \textit{C. pneumoniae}. This finding raises the question as to whether in this subgroup of multiple sclerosis-patients, \textit{C. pneumoniae} is directly linked to the pathogenesis or whether the intrathecal IgG production to \textit{C. pneumoniae} is part of a bystander immune response known as the “MRZ reaction” (Luxton \textit{et al.}, 1995; Reiber \textit{et al.}, 1998).

\textit{Specificity of oligoclonal IgG in the CSF of multiple sclerosis patients}

Intrathecal IgG production and oligoclonal bands in the CSF represent typical laboratory features of MS and other inflammatory and infectious diseases of the CNS. The antigen-specificity of the oligoclonal IgG in MS is largely unknown. In the case of infectious CNS disease, at least part of the intrathecally produced oligoclonal IgG is directed to the specific pathogen. This has been reported for different virus infections of the CNS (Dörries, ter Meulen, 1984), \textit{B. burgdorferi} and neuroborreliosis (Martin \textit{et al.}, 1988), and other infections of the CNS (reviewed by Gilden, 1999). Furthermore, the oligoclonal IgG in the CSF are stable over time in multiple sclerosis patients, suggesting that they are caused by a specific and chronic activation of B cells (Walsh, Tourtellotte, 1986), presumably by an antigen-driven response (Smith-Jensen \textit{et al.}, 2000). Therefore we tested whether the oligoclonal IgG present in multiple sclerosis CSF were specific for \textit{C. pneumoniae}. For comparison, we analysed paired CSF/serum samples from patients with neuroborreliosis and determined the reactivity of their oligoclonal bands to the specific pathogen of this disease, \textit{B. burgdorferi}.
The IEF-Western experiments revealed that the major oligoclonal IgG bands in the CSF of multiple sclerosis-patients did not react with *C. pneumoniae*. In contrast, all four analysed control patients with neuroborreliosis showed a strong intrathecal IgG-production against *B. burgdorferi* and strong reactivity of oligoclonal bands to this bacterium detectable by IEF-Western.

What might be the reason for the strong reactivity of CSF oligoclonal IgG of neuroborreliosis patients to *B. burgdorferi* on the one hand and lack of reactivity of the major oligoclonal IgG of multiple sclerosis-patients to *C. pneumoniae* on the other? The IgG response to a specific pathogen involves a high percentage of the CSF Ig. About 20% of the CSF IgG has been estimated to recognize measles virus in the case of subacute sclerosing panencephalitis (Conrad *et al.*, 1994). In contrast, the concentration of those IgG that belong to the polyspecific immune response such as IgG directed to measles, rubella, and zoster virus represent together only about 2% of the IgG in the CSF (Reiber *et al.*, 1998). In addition, the intrathecal polyspecific IgG in multiple sclerosis is usually of low affinity in contrast to the IgG directed against a specific pathogen (Luxton *et al.*, 1995). Both the low affinity and lower concentration of the anti-*C. pneumoniae* IgG in multiple sclerosis as compared to the anti-Borrelia IgG in neuroborreliosis might account for the weak or absent reactivity to *C. pneumoniae* in the affinity mediated IEF Western.

Our finding that the major oligoclonal IgG in the CSF of multiple sclerosis-patients are not directed against *C. pneumoniae* argues that the intrathecal IgG production against *C. pneumoniae* in a subgroup of multiple sclerosis is part of a polyspecific activation of B cells in the CSF. Longitudinal analysis revealed that the intrathecal IgG production to measles, rubella, and herpes zoster viruses, and at a lower level also to cytomegalovirus, herpes simplex virus, and *C. pneumoniae*, is
quite stable over time. Furthermore, the polyspecific IgG response to measles, rubella and zoster occurs rather independently of disease duration and is usually present at the onset of disease (Reiber et al., 1998).

Two patients (one with probable multiple sclerosis (PoVe) and one with definite CP-multiple sclerosis (LuAl)) showed a few oligoclonal bands specific to *C. pneumoniae* in both serum and CSF. However, a highly sensitive detection system was required to demonstrate this reaction. The ELISA results indicate that these two patients had a rather high concentration of IgG against *C. pneumoniae* in both serum and CSF. Importantly, they did not show a specific intrathecal IgG production to *C. pneumoniae* (AI<2). The finding that these two patients showed *C. pneumoniae*-specific oligoclonal bands in the CSF and serum by IEF-Western validates the IEF-Western analysis. However, since these two patients did not display an intrathecal IgG production to *C. pneumoniae*, there is no evidence that this agent is causally related to their disease.

**Weak reactivity in IEF Western in special cases**

One patient without evidence of an inflammatory CNS disease showed an intrathecal IgG production to *C. pneumoniae* and *C. pneumoniae*-specific oligoclonal bands in the IEF-Western analysis detected in both serum and spinal fluid. This might seem surprising, but it has been reported that an intrathecal IgG synthesis develops in 5-10 % of patients with non-inflammatory neurological diseases (Tourtellotte, Tumani, 1997). An intrathecal IgG synthesis in patients presenting with non-inflammatory neurological diseases is frequently regarded as an immune scar, since an intrathecal IgG synthesis persists for many years after overcoming of an encephalitis or meningitis.
Another patient, who had limbic encephalitis of unidentified origin, showed an intrathecal IgG production to *C. pneumoniae* and *C. pneumoniae*-specific oligoclonal bands exclusively in the CSF, but not in the serum. The CSF of this patient did not contain *C. pneumoniae* detectable by PCR. It should be noted that the reactivity to *C. pneumoniae* by IEF-Western was much weaker than the reactivity of the neuroborreliosis patients to *B. burgdorferi* and required a highly sensitive detection method. It remains to be established whether in this single case of encephalitis of unknown origin *C. pneumoniae* plays a pathogenic role. This patient recovered without a specific therapy.

**Animal models and the Chlamydia hypothesis**

The reports on the presence of *C. pneumoniae* in MS patients that incited us to examine the matter have also led to increased efforts to show a role for *C. pneumoniae* in CNS autoimmunity in experimental allergic encephalomyelitis (EAE), the animal model for multiple sclerosis. One study (Du et al., 2002) could show that EAE in mice induced by immunization with myelin antigens was worsened by concomitant infection of the animals with *C. pneumoniae*. The bacteria were also shown to be present in the CNS by Immunohistochemistry and PCR, but only in the tissue of animals suffering from EAE. Proliferation and IFN-γ production of autoreactive T cells was enhanced in diseased animals, but there was no evidence of cross-reactivity or molecular mimicry between the autoantigens and *C. pneumoniae*. Another group (Lenz et al., 2001) used a *C. pneumoniae*-derived peptide, which shows homology to myelin basic protein, to induce EAE in rats, whereas immunization with a sonicated *C. pneumoniae* preparation produced mild EAE in one out of five rats only. An intriguing study (MacIntyre et al., 2002) examined the effects
of *C. pneumoniae* infection of human brain microvascular endothelial cells on expression of proteins involved with blood brain barrier permeability and found that a downregulation of occludin by 60% at 36-48h post infection could allow for transient fluctuations in the permeability of the blood brain barrier.

**PCR results and comparison of the data with other reports**

Different approaches including the culture of *C. pneumoniae* and PCR analysis of the CSF and autoptic brain have been undertaken to analyse a potential role for *C. pneumoniae* in the pathogenesis of multiple sclerosis. The detection of *C. pneumoniae* by culture is difficult and not very sensitive, because the viability of the organism decreases rapidly outside the host cell (Maass, Dalhoff, 1995). Since *C. pneumoniae* could not be detected by PCR in 23/23 of our CSF samples (Ten patients with definite RR-multiple sclerosis, 3 patients with definite CP-multiple sclerosis, 5 patients with OIND and another 5 with OND) and since the PCR is more sensitive than culture, we did not attempt to culture *C. pneumoniae* (Derfuss, Gürkov et al., 2001).

Others looked at the presence of *C. pneumoniae* in multiple sclerosis patients’ brains. *C. pneumoniae* was not detected by PCR in any of the analysed patient and control specimens (Morre et al., 2000; Hammerschlag et al., 2000). These findings are consistent with the conclusion drawn from our different experimental approach.

Different groups have looked for *C. pneumoniae* in CSF by PCR with highly ambiguous results. While the first report described positivity in 97% of the CSF of multiple sclerosis patients (Sriram et al., 1999), two other studies were completely negative (Boman et al., 2000; Pucci et al., 2000). Another study detected *C. pneumoniae* by PCR in the CSF in 5/10 patients and then in a second series in 2/20
patients by PCR (Layh-Schmitt et al., 2000). Yet another study detected *C. pneumoniae* by PCR in 2/8 multiple sclerosis patients and found intrathecal IgG production in 8/22 multiple sclerosis patients (36%) (no data about control patients were reported) and initiated a placebo-controlled multicenter study to evaluate the efficiency of an antibiotic treatment with Roxithromycin (Treib et al., 2000). In contrast, however, another group detected *C. pneumoniae* in a high percentage of the CSF of both multiple sclerosis-patients and controls (Li et al., 2000). We did not find reproducible evidence for the presence of *C. pneumoniae* genome in any of the studied 23 CSF samples. The diverging results of previous PCR studies may depend on cell number, CSF amount, handling of the probe, and the specific PCR protocol. Still, one must consider that a negative PCR does by no means exclude an involvement of *C. pneumoniae*, since it has been established that in another chronic CNS disease, neuroborreliosis, in only about 17% of the patients can *B. burgdorferi* be detected by PCR (Lebech et al., 2000).

For these reasons we focused our study not on PCR or culture, but rather on the IgG response in the CSF and in serum. IgG is stable outside the body and the results are not affected by the amount of CSF obtained or the speed of further processing. Most importantly, it has been well established that oligoclonal bands specific for the respective pathogen arise in all kinds of infectious CNS diseases (Gilden, 1999). More recently, another group has studied CSF samples from 25 adolescents with MS by ELISA and found intrathecal IgG production against *C. pneumoniae* in seven patients (28%). Furthermore, the authors calculated the proportion of anti-*C. pneumoniae* antibodies within the intrathecally produced total IgG to range from 0.01 to 0.8% only and thereby underpin our observations (Rostasy et al., 2003).
Taken together, our study shows that a subgroup of about 25% of multiple sclerosis-patients produces intrathecal IgG against *C. pneumoniae*. Importantly, even in this fraction of multiple sclerosis patients the major oligoclonal IgG in the CSF do not recognize *C. pneumoniae*. This strongly argues against a pathogenic role of this agent in multiple sclerosis.
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