Intrathecal antibody production against *Chlamydia pneumoniae* in multiple sclerosis is part of a polyspecific immune response

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Summary

Chronic intrathecal immunoglobulin (Ig) production is a hallmark of multiple sclerosis characterized by the presence of oligoclonal IgGs and, in addition, polyspecific recognition of different pathogens such as measles, rubella, and herpes zoster virus. While the antigen specificity of the oligoclonal IgGs in multiple sclerosis is largely unknown, the oligoclonal IgGs arising during CNS infectious diseases are reactive against the specific pathogen. Recently, a link between *Chlamydia pneumoniae* and multiple sclerosis has been claimed. To test the possible role of *C. pneumoniae* in multiple sclerosis, we analysed (i) whether there is intrathecal IgG production against *C. pneumoniae* in multiple sclerosis and (ii) if the oligoclonal IgGs 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; other inflammatory neurological diseases, 35; other neurological diseases, 27) by enzyme-linked immunosorbent assay, we found that 24% of all patients with definite multiple sclerosis, but only 5% of patients with other inflammatory or non-inflammatory diseases, produced IgGs specific for *C. pneumoniae* intrathecally (definite multiple sclerosis versus other inflammatory neurological diseases: \( P = 0.027 \)). The presence of intrathecal IgGs 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 towards purified elementary bodies and reticulate bodies of *C. pneumoniae* on affinity-mediated immunoblot following isoelectric focusing (IEF-western blots). In contrast, the IgGs in the CSF of control patients with neuroborreliosis strongly reacted with their specific pathogen, *Borrelia burgdorferi*, by IEF-western blot analysis. Concomitant analysis of the CSF of 23 patients with a nested polymerase chain reaction for *C. pneumoniae* was negative in all cases. Together, our 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 for *C. pneumoniae* in multiple sclerosis.

Keywords: multiple sclerosis; oligoclonal bands; *Chlamydia pneumoniae*; cerebrospinal fluid; CNS infection

Abbreviations: AI = antibody index; CP = chronic progressive; ECL = enhanced chemiluminescence; ELISA = enzyme-linked immunosorbent assay; IEF = isoelectric focusing; Ig = immunoglobulin; MRZ = measles–rubella–zoster; OD = optical density; OIND = other inflammatory neurological diseases; OND = other neurological diseases; PCR = polymerase chain reaction, RR = relapsing–remitting

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Introduction

Both infectious agents and pathological autoimmune reactions to CNS antigens have been implicated in the aetiology 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 aetiology of multiple sclerosis has been suspected for more than a century, and over the years a large number of different viruses have been linked to it (Meinl, 1999). 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. These polyclonal anti-viral IgGs 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, Chlamydia 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 the C. pneumoniae genome in 97% of multiple sclerosis patients versus 18% of controls with other neurological diseases (OND) (Sriram et al., 1999).

Chlamydia pneumoniae is an obligate intracellular bacterium that infects alveolar macrophages, monocytes and endothelial cells. Serology indicates that about half of the population in developed countries have had contact with C. pneumoniae (Hargreaves et al., 1994). Chlamydia bacteria have been linked to a chronic encephalopathy in cows, which is called sporadic bovine encephalomyelitis (Harshfield, 1970; Sriram et al., 1998). 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 appeared in the meantime, shedding doubt on the Chlamydia hypothesis. The number of patients studied in these scattered reports was small, and data were based mainly on PCR analysis (Boman et al., 2000; Hammerschlag et al., 2000; Layh-Schmitt et al., 2000; Li et al., 2000; Pucci et al., 2000; Treib 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, the amount of CSF drawn, the 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 ~2 weeks, the PCR is usually negative, but the diagnosis can be established retrospectively 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 blot). This serological feature is very stable and reliable (Sauерbrei et al., 2000). Likewise, in neuroborreliosis, the diagnostic sensitivity of the PCR from CSF was estimated to be just 17%, and the diagnosis is based routinely on an intrathecal IgG production to Borrelia 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 blot and PCR. Immunoglobulins are stable outside the body and the experiments are not affected by handling of the CSF, the amount of CSF drawn, the cell 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.

Material and methods

Patients

Paired serum and CSF samples from a total of 120 patients were analysed. Of these, 12 patients were diagnosed as possible multiple sclerosis, 35 as relapsing–remitting (RR)
Table 1 IgG response to C. pneumoniae in serum and CSF

<table>
<thead>
<tr>
<th></th>
<th>Probable MS</th>
<th>Definite RR MS</th>
<th>Definite CP MS</th>
<th>OIND</th>
<th>OND</th>
</tr>
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<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. pneumoniae</td>
<td>5/12 (42%)</td>
<td>16/35 (46%)</td>
<td>7/10 (70%)</td>
<td>16/3 (46%)</td>
<td>16/255 (64%)</td>
</tr>
<tr>
<td>Intraethical IgG to C. pneumoniae</td>
<td>1/2 (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 were seropositive for C. pneumoniae and who displayed an intrathecal IgG production to C. pneumoniae with an AI > 2 are shown. MS = multiple sclerosis.

Serology
An enzyme-linked immunosorbent assay (ELISA) specifically detecting IgGs reactive with C. pneumoniae was obtained from Hain Diagnostics (Nehren, Germany, manufactured by Savoy 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 and 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. pneumoniae IgGs in the CSF, the spinal fluid was diluted 1:2. Analogously to evaluation of serum samples, CSF samples were scored positive for C. pneumoniae if the optical density (OD) was at least twice the negative control, which was measured around an OD of 0.2.

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 IgGs. The specific intrathecal IgG production antibody index (AI) was then calculated as AI = OD_{CSF} / OD_{serum}. In the case of an intrathecal IgG production, the corrected AI was calculated as AI × Q_{IgG}/Q_{lim}. Q_{lim} was calculated as described (Reiber and Lange, 1991). An AI > 2 was considered to indicate a significant and reliable intrathecal IgG production against the pathogen studied.

Culture and processing of C. pneumoniae and B. burgdorferi
Buffalo green monkey kidney cells were cultured in OptiMEM medium supplemented with 10% FCS (foetal calf serum) in 50 ml tissue culture flasks. For infection, the monolayers were centrifuged with 5 × 10^7 infection forming units of C. pneumoniae (strain HK) in 6 ml of PBS (phosphate-buffered saline). Cells and C. pneumoniae were centrifuged for 30 min at 2000 g and 37°C. After centrifugation, the supernatant was substituted with OptiMEM supplemented with 10% FCS. After 3–5 days in culture (5% CO_2), C. pneumoniae was harvested. After careful abrasion of the cell layer from the flask bottom, the infected cells were sonicated to release C. pneumoniae. The sonicate was centrifuged for 3 min at 4000 g and 6°C and the supernatant was centrifuged further as an overlay on 8 ml of 35% Urografin (Schering, Berlin, Germany) for 50 min at 8°C and 19 300 r.p.m. (Optima L, rotor SW 28 Ti, Beckmann Coulter, USA). The sediment was collected and overlaid on a gradient consisting of 40, 44 and 52% Urografin. After ultracentrifugation for 60 min at 19 300 r.p.m. and 10°C, the centrifugation tube was tapped with a cannula and the band between the 40 and 44% phase, which contains the reticular bodies, and that between the 44 and 52% phase, which contains the elementary bodies, were aspirated. In a third ultracentrifugation step (17 500 r.p.m., 5°C, 50 min), the remaining Urografin was removed. The resulting sediment was resuspended in PBS and stored at −80°C until further use. The elementary body preparation of C. pneumoniae was tested for its quality by microimmunofluorescence assay with positive and negative control sera. In addition PCR was performed to exclude mycoplasma contamination of cell culture. To break up the reticular and elementary bodies, the antigen preparation was boiled for 20 min in a buffer containing 1% sodium dodecylsulphate, 2.5% 2-ME (2-mercaptopethanol), 0.5 mM EDTA (ethylene diamine tetra-acetic acid) and 31 mM Tris, pH 6.8.

Borrelia burgdorferi strain PKo, a German skin isolate, was cultivated at 33°C in modified Kelly medium (Preac-Mursic et al., 1991). Washed borreliae (three times in PBS with 0.005 M MgCl_2) were sonicated for 3 min using a Branson Sonifier, Cell disruptor B15, microtip 5 mm (Branson, Danbury, Conn., USA) on ice. The sonicate was controlled for complete destruction of the borrelia cells by dark field microscopy. The protein content of the sonicate was determined by the method of Bradford (Bradford, 1976).

IEF gels
An IEF of Ig in the CSF can be performed in an agarose or a polyacrylamide gel. Agarose gels are more suitable for
to visualize the oligoclonal bands with this method. The gels were run according to the 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 h at 1500 V and 10°C. Subsequently, proteins were silver stained as described (Wurster, 1983).

**PCR to detect C. pneumoniae genome in the CSF**

CSF samples from 23 patients were analysed by nested PCR for the presence of *C. pneumoniae*. DNA of 100 µl of CSF was extracted and purified using the Qiagen DNA extraction kit according to the manufacturer’s instructions. DNA was eluted into 200 µl of buffer AE (Qiagen, Hilden, Germany). A 5 µl aliquot was subjected to a nested PCR, amplifying DNA for 16S rRNA of *C. pneumoniae*. Briefly, in the first PCR, an 828 bp (base pair) fragment was amplified (primers: 5′-CGT GCC GGC GTG GAT G-3′ and 5′-CGA CAC GGA TGG GGT TG-3′; 30 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 60 s and elongation at 72°C for 90 s). In the second PCR, a 570 bp product was reamplified from this fragment (primers 5′-TGG CGG AAG GGT TAG TAG-TGG CGC CGC GTG GAT G-3′ and 5′-CGA CAC GGA TGG GGT TG-3′; 30 cycles of denaturing at 94°C for 30 s, annealing at 68°C for 60 s and elongation at 72°C for 60 s). PCR products were visualized by gel electrophoresis.

**Results**

**Seroprevalence and intrathecal production of IgGs 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: ~50% of all studied patients showed serological evidence of prior infection with *C. pneumoniae* (Table 1). 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* IgGs in the CSF. The ELISA was not sensitive enough to detect transudation of IgGs into the CSF of seropositive patients. Conversely, among the 15 patients identified with an intrathecal IgG production to *C. pneumoniae*, 11 scored positive for anti-*C. pneumoniae* IgGs in the serum.

In contrast, 26% of patients with RR-multiple sclerosis and 20% of those 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 intrathecal IgG response to *C. pneumoniae* in the definite
multiple sclerosis group (11 out of 46) and the OIND group (two out of 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: 11 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* IgGs by repeat CSF analysis in nine patients. The time interval between the two CSF collections ranged from 3 weeks to 8 months. Five of these nine patients showed an intrathecal IgG production to *C. pneumoniae*. In all nine patients, the responder status (i.e. 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

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**Table 2** Longitudinal stability of intrathecal IgG production against different pathogens

<table>
<thead>
<tr>
<th>Corrected AI</th>
<th>Date of lumbar puncture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>November 1999</td>
</tr>
<tr>
<td><em>C. pneumoniae</em></td>
<td>2.8</td>
</tr>
<tr>
<td><em>B. burgdorferi</em></td>
<td>Negative</td>
</tr>
<tr>
<td>Measles virus</td>
<td>9.1</td>
</tr>
<tr>
<td>Rubella virus</td>
<td>22.2</td>
</tr>
<tr>
<td>Varicella zoster virus</td>
<td>17.6</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>1.9</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>2.6</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 AIs that describe the intrathecally produced IgG against the different pathogens are shown.
one OIND patient, the corrected AI to \textit{C. pneumoniae} dropped from 8.1 to 3.4 after 5 months.

In one multiple sclerosis patient, we compared the time course of \textit{C. pneumoniae}-specific IgGs with the reactivity against several other infectious agents (measles, rubella, varicella zoster, cytomegalovirus and herpes simplex virus). This patient, who had had clinically definite RR-multiple sclerosis for 12 years, had elevated AIs to all these agents. This type of response has been described as the 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 \textit{C. pneumoniae}, the patient was treated with doxycycline (200 mg per day orally) for 2 weeks.

\textbf{Antigen specificity of oligoclonal bands}

Next, we tested the antigen specificity of CSF oligoclonal bands by IEF-western blotting, using \textit{C. pneumoniae}, \textit{B. burgdorferi} and milk powder as antigens. Low-fat milk powder is used commonly as a blocking reagent and served here to control the specificity and evaluate unspecific transfer.

The analysis was done in all 15 samples with an intrathecal IgG production to \textit{C. pneumoniae} (Table 1), in three samples with a high \textit{C. pneumoniae}-specific OD in the ELISA in both serum and CSF and additionally as a control in eight samples without anti-\textit{C. pneumoniae} IgGs 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 blot. A representative example is shown in Fig. 1A. In one of these 11 patients, weak reactivity to \textit{C. pneumoniae} was evident by IEF-western blot (Fig. 1B).

Two of the four control patients with an intrathecal IgG production to \textit{C. pneumoniae} displayed reactivity to \textit{C. pneumoniae} in the IEF-western blot. One representative example (patient Hu.M.) of these two is shown (Fig. 2B). This patient presented with hypoglycaemic coma, having a 32-year history of type 1 diabetes. PCR to detect \textit{C. pneumoniae} in the CSF was not done in this patient. The other patient (N.M.) 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 blot 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*.

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 blot. Two of them (PoVe with probable multiple sclerosis, LuAl with definite CP-multiple sclerosis) showed reactivity in both serum and CSF. The third (ZiSa with RR-multiple sclerosis) did not react by IEF-western blot. It is of note that IEF-western blot 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 Fig. 2A. The patient PoVe, who had probable multiple sclerosis, showed the strongest response to *C. pneumoniae* by IEF-western blot. However, even in this patient, the very sensitive detection system was required (Fig. 3). Importantly, since neither 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 blot 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.

For additional specificity control of the IEF-western blot analysis, eight patients (four 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 eight patients were analysed by IEF-western blot 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 validate our IEF-western blot system further, we analysed serum–CSF pairs from patients with definite neuroborreliosis by IEF-western blot. The four control patients with neuroborreliosis showed a strong intrathecal IgG production to *B. burgdorferi* with a mean AI of 43.4.

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

As reported in the previous section, most of the patients who had an intrathecal IgG production to *C. pneumoniae*
detectable by ELISA showed only weak or no reactivity of their oligoclonal bands to *C. pneumoniae* by IEF-western blot analysis, even with the sensitive technique. To exclude 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 IgGs of patients PoVe and HuM. reacted by IEF-western blot to *C. pneumoniae*, but not to *B. burgdorferi* (Fig. 2 and data not shown). CSF of patient PoVe was run in parallel as a positive control in all IEF-western blot experiments searching for *C. pneumoniae* reactivity. Secondly, to evaluate further the efficiency of binding of *C. pneumoniae*, membranes were coated with *C. pneumoniae* and blocked with 10% milk powder as for the IEF-western blot, 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 blot 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 *C. pneumoniae* antigens bind efficiently to the nitrocellulose membrane and can be recognized readily by patients’ antibodies.

**Presence of *C. pneumoniae* in the CSF**

The presence of *C. pneumoniae* genome in the CSF was assessed by a sensitive nested PCR. Ten CSF samples from patients with definite RR multiple sclerosis, three from patients with definite CP-multiple sclerosis, five from patients with OIND and another five from OND patients were investigated. Using this method, *C. pneumoniae* was not detected in any of the 23 analysed CSF samples. One CSF sample from an OND patient initially gave a positive PCR signal in a first test but, since this could not be confirmed in two subsequent PCR assays of the same CSF sample, this material was scored negative.

**Discussion**

Our analysis of 120 paired CSF and serum samples from multiple sclerosis patients and controls revealed the following: (i) a positive serum IgG response to *C. pneumoniae* was observed in ~50% of multiple sclerosis patients and controls; (ii) in 20–25% of multiple sclerosis patients, but only 4–6% of controls, there is clear evidence for intrathecal synthesis of antibodies directed against *C. pneumoniae*; (iii) IEF-western blotting demonstrated that in multiple sclerosis the major CSF-specific oligoclonal bands are not directed against *C. pneumoniae*; and (iv) our CSF PCR results were consistently negative for *C. pneumoniae*.

**Seroprevalence and intrathecal IgG production**

The overall seroprevalence of *C. pneumoniae* in multiple sclerosis patients and control OIND and OND patients was similar. About 50% of the patients and controls were seropositive for *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 pre-existing antibodies to *C. pneumoniae* (formerly called TWAR strain) (Hargreaves et al., 1994). This finding alone, that only about half of the multiple sclerosis patients show serological evidence of previous infection with *C. pneumoniae*, would argue against a role for *C. pneumoniae* in 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 *C. pneumoniae*. In agreement with our results, another recent publication described a similar seropositivity in multiple sclerosis patients versus controls and an intrathecal IgG production to *C. pneumoniae* in a subset of multiple sclerosis patients. The authors did not detect any association between disease activity and the immune response to *C. pneumoniae* (Krametter et al., 2001). In their study, the intrathecal IgG production to *C. pneumoniae* was associated with elevated synthesis of total polyclonal IgG and correlated with the intrathecal synthesis of virus-specific IgG (Krametter et al., 2001). This finding raises the question as to whether in this subgroup of multiple sclerosis patients, *C. pneumoniae* is directly linked to the pathogenesis or whether the intrathecal IgG production to *C. pneumoniae* is part of a bystander
immune response known as the ‘MRZ reaction’ (Luxton et al., 1995; Reiber et al., 1998).

**Specificity of oligoclonal IgGs in the CSF of multiple sclerosis patients**

Intrathecal IgG production and oligoclonal bands in the CSF represent typical laboratory features of multiple sclerosis and other inflammatory and infectious diseases of the CNS. The antigen specificity of the oligoclonal IgGs in multiple sclerosis 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 and ter Meulen, 1984), *B. burgdorferi* and neuroborreliosis (Martin et al., 1988), and other infections of the CNS (reviewed by Gilden, 1999). Furthermore, the oligoclonal IgGs in the CSF are temporarily stable in multiple sclerosis patients, suggesting that they are caused by a specific and chronic activation of B cells (Walsh and Tourtellotte, 1986), presumably by an antigen-driven response (Smith-Jensen et al., 2000). Therefore, we tested whether the oligoclonal IgGs present in multiple sclerosis CSF were specific for *C. pneumoniae*. For comparison, we analysed paired CSF- and serum samples from patients with neuroborreliosis and determined the reactivity of their oligoclonal bands to the specific pathogen of this disease, *B. burgdorferi*.

The IEF-western blot experiments revealed that the major oligoclonal IgG bands in the CSF of multiple sclerosis patients did not react with *C. pneumoniae*. In contrast, all control patients with neuroborreliosis analysed showed a strong intrathecal IgG production against *B. burgdorferi* and strong reactivity of oligoclonal bands to this bacterium detectable by IEF-western blot.

What might be the reason for the strong reactivity of CSF oligoclonal IgGs of neuroborreliosis patients to *B. burgdorferi* on the one hand and lack of reactivity of the major oligoclonal IgGs 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 IgGs have been estimated to recognize measles virus in the case of subacute sclerosing panencephalitis (Conrad et al., 1994). In contrast, the concentration of those IgGs that belong to the polyspecific immune response, such as IgGs directed to measles, rubella and zoster virus, represent together only ~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* IgGs in multiple sclerosis as compared with the anti-*Borrelia* IgGs in neuroborreliosis might account for the weak reactivity to *C. pneumoniae* in the affinity-mediated IEF-western blot.

Our finding that the major oligoclonal IgGs 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 IgGs 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 blot validates this 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.

In a recent paper, which was accompanied by several critical editorials, Yao et al. (2001) reported that CSF oligoclonal bands include antibodies to *C. pneumoniae*. Reactivity to *C. pneumoniae* was detected in 16 out of 16 multiple sclerosis patients by IEF-western. Oligoclonal bands were adsorbed either partially or completely with *C. pneumoniae* in 14 out of 17 patients (Yao et al., 2001). Presently the discrepancy between their and our results remains unresolved. We note, however, the following points. (i) Yao et al. did not report any ELISA results to quantify intrathecal IgG production of subacute sclerosing panencephalitis (Conrad et al., 1994). In contrast, the concentration of those IgGs that belong to the polyspecific immune response, such as IgGs directed to measles, rubella and zoster virus, represent together only ~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* IgGs in multiple sclerosis as compared with the anti-*Borrelia* IgGs in neuroborreliosis might account for the weak reactivity to *C. pneumoniae* in the affinity-mediated IEF-western blot.

Our finding that the major oligoclonal IgGs 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
Weak reactivity in IEF-western blot 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 blot analysis detected in both serum and CSF. 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 and Tumani, 1997). An intrathecal IgG synthesis in patients presenting with non-inflammatory neurological diseases frequently is regarded as an immune scar, since an intrathecal IgG synthesis persists for many years after overcoming 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 blot 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 any specific therapy.

PCR results and comparison of our data with previous reports

Different approaches including the culture of C. pneumoniae and PCR analysis of the CSF and autopic 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 and Dalhoff, 1995). Since C. pneumoniae could not be detected by PCR in our samples and since the PCR is more sensitive than culture, we did not attempt to culture C. pneumoniae.

Others looked at the presence of C. pneumoniae in multiple sclerosis brains. Chlamydia pneumoniae was not detected by PCR in any of the patient and control specimens analysed (Hammerschlag et al., 2000; Morre 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 five out of 10 patients and then in a second series in two out of 20 patients (Layh-Schmitt et al., 2000). Yet another study detected C. pneumoniae by PCR in two out of eight multiple sclerosis patients and found intrathecal IgG production in eight out of 22 multiple sclerosis patients (36%) (no data about control patients were reported) and initiated a placebo-controlled multicentre 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). Yet another study detected C. pneumoniae in the CSF by PCR in 21% of multiple sclerosis patients and in 43% of controls (Gieffers et al., 2001). We did not find reproducible evidence for the presence of the C. pneumoniae genome in any of the 23 CSF samples studied. 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 by no means excludes an involvement of C. pneumoniae, since it has been established that in another chronic CNS disease, neuroborreliosis, B. burgdorferi can be detected by PCR in only ~17% of the patients (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).

Taken together, our study shows that a subgroup of ~25% of multiple sclerosis patients produces intrathecal IgGs against C. pneumoniae. Importantly, even in this fraction of multiple sclerosis patients, the major oligoclonal IgGs in the CSF do not recognize C. pneumoniae. This argues strongly against a pathogenic role for this agent in multiple sclerosis.

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