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Increased prevalence of and gene transcription by *Chlamydia pneumoniae* in cerebrospinal fluid of patients with relapsing-remitting multiple sclerosis

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Abstract Microbial agents may play a role in the pathogenesis of multiple sclerosis (MS). *C. pneumoniae* has been recently associated with MS; however, study results are at variance. We tested the hypothesis that *Chlamydia pneumoniae*-specific DNA and RNA are more often detected in cerebrospinal fluid (CSF) of patients with multiple sclerosis than patients with other neurological diseases (OND). We investigated CSF samples from 84 patients with definite MS and 89 OND patients (n = 62 with normal CSF; n = 27 with pathological CSF) using a nested polymerase chain reaction (PCR) to detect *ompA* gene sequences of *C. pneumoniae*. In subjects with positive PCR, we probed for chlamydial heat shock protein 60-mRNA and 16S-rRNA by reverse transcriptase (rt)-PCR.

C. pneumoniae-specific DNA was more often detected in MS patients (50 %) than in all OND patients combined (28.1 %, p = 0.003) and in OND patients with normal CSF (24.2 %, p = 0.003) but not than in OND patients with pathological CSF (37 %, p = 0.24). In relapsing-remitting MS (n = 55), the prevalence of *C. pneumoniae* DNA was higher (66.7 %) than in both OND subgroups ($p \leq 0.05$). In MS patients (n = 20), chlamydial heat shock protein 60-mRNA (75 %) and 16S-rRNA (70 %) were more often detected than in OND patients (n = 16; 18.8 %; $p < 0.005$).

Although more often detected in remitting-relapsing MS, *C. pneumoniae* DNA in CSF is not specific for MS owing to its high prevalence in OND controls. However, the higher rate of gene transcription suggests a more active metabolism of *C. pneumoniae* in MS patients.

Key words multiple sclerosis · cerebrospinal fluid · infection · *Chlamydia pneumoniae*

Introduction

The interest in an infectious etiology of multiple sclerosis (MS) has waxed and waned during the last decades. Clinical and epidemiological observations and results from animal experiments suggest that infections may contribute to the pathogenesis of MS [6, 11, 20]. However, so far, no microbial agent has been consistently identified in MS patients. More recently, the gram-negative intracellular pathogen *Chlamydia pneumoniae* was linked to MS but study results have been at variance.

Initially, Sriram and coworkers detected *C. pneumoniae* by polymerase chain reaction (PCR) in cerebrospinal fluid (CSF) of 36 out of 37 MS patients (97 %) but only in 5 out of 27 controls with other neurological diseases (OND; 18 %) [30]. Layh-Schmitt and coworkers detected *C. pneumoniae* in 23 % of their MS patients (n = 30) but in none of the OND controls (n = 56) [22]. Other research groups have also reported the identification of *C. pneumoniae* in MS patients although the

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prevalences of positive results varied widely [13, 17, 29]. Gieffers et al. identified *C. pneumoniae* in 21 % of MS patients (n = 58), in 43 % of OND controls (n = 47) but not in neurologically healthy subjects (n = 67) and suggested that the presence of *C. pneumoniae* in CSF is not specific for MS [10]. In contrast, several research groups have detected *C. pneumoniae* in none or in only a small minority of their MS patients [4, 7, 8, 25, 27, 28]. A multicenter comparative study on split CSF samples confirmed conflicting results on *C. pneumoniae*-DNA detection by different laboratories [18]. Similarly, in serological studies some [8, 21, 30] but not other research groups [4] reported an intrathecal antibody production against *C. pneumoniae* in MS patients, possibly as part of a polyspecific immune response [8].

We compared DNA extraction techniques for detection of *C. pneumoniae* from CSF samples and investigated the so far largest number of MS and OND patients in order to test the hypothesis that *C. pneumoniae* is encountered more often in MS than in other neurological diseases. For the first time in a study on MS, we furthermore used reverse transcriptase PCR (rt-PCR) to investigate whether there is specific gene transcription and therefore active metabolism by *C. pneumoniae*.

Subjects and methods

We investigated 84 patients with definite multiple sclerosis according to the Guidelines from the International Panel on the Diagnosis of Multiple Sclerosis [24]. Patients had relapsing-remitting MS (n = 55), secondary chronic progressive MS (n = 11) or primary chronic progressive MS (n = 18; Table 1). For secondary prevention, patients were treated with beta-interferon (n = 11), mitoxantron (n = 11) and azathioprin (n = 2). Exclusion criteria were inability to give informed consent and other diseases of the central nervous system (CNS) in addition to MS. As control group, we investigated 89 patients with other neurological diseases (OND) that were frequency matched to patients for age and gender. Patients with possible MS or CNS disorders with a possible autoimmunological pathogenesis (e. g. myelitis) were excluded. Twenty-seven of OND patients had pathological CSF examinations with pleocytosis, intrathecal immunoglobulin production or blood-brain barrier disruption. These patients suffered from meningoencephalitis of unknown origin (n = 10), peripheral or cranial neuropathy (n = 7), neuroborreliosis (n = 2), CNS malignancies (n = 2), zoster encephalitis, ischemic stroke, suspected neurosarcoidosis, syphilis, headache of unknown origin and orofacial dyskinesia (n = 1, respectively). Patients with normal CSF examination had neurodegenerative diseases (e. g. motoneuron disease, early onset Parkinson's syndrome or cerebellar atrophy; n = 16), headaches and other pain disorders (n = 13), peripheral or cranial neuropathy (n = 11), psychosomatic and neuropsychiatric disorders (n = 6), epileptic seizures (n = 5), cerebral or spinal ischemia (n = 5*), Borellia burgdorferi* infection (n = 3) or brain tumours (n = 3). The study protocol was approved by the local people's subject committee and patients gave informed consent.

Extraction of DNA and polymerase chain reaction

DNA was extracted from 0.5 ml of frozen (–86°C) CSF either using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions or by phenol/chloroform extraction that was performed as follows. CSF was mixed with trypsin/EDTA (0.1 % and 0.4 mM, final concentrations), incubated at 37°C for 30 minutes and centrifuged for 45 minutes (18,000 g, 4 °C). The pellet was resuspended in 200 µl of lysis buffer (0.5 % sodium dodecyl sulfate, 0.5 % Nonidet P-40, 0.2 M NaCl, 40 mM dithiothreitol, 10 mM EDTA, 20 mM Tris-HCl at pH 7.5) and left over night at 37 °C after addition of 20 µl of proteinase K (10 µg/ml; Roche Diagnostics, Mannheim, Germany). From this specimen, DNA was purified using phenol/chloroform/isoamyl alcohol (25:24:1) followed by two extraction steps with chloroform (Sigma, Munich, Germany). To the aqueous DNA fraction, 3M sodium acetate (22.5 µl) was added and precipitated in 555 µl cold absolute ethanol. Finally, the DNA was washed with 75 % ethanol, centrifuged for 10 minutes at 18,000 g and resuspended in 25 µl water. The *ompA* gene of *C. pneumoniae* was used as target for a nested PCR applying external primers as described by Tong and Sillis (sense: 5' TTACAAGCCTTGCCTGTAGG 3'; antisense: 5' GCGATCCCAAATGTTTAAGGC 3'; touchdown PCR with 32 cycles) [31]. We used the following internal primers (sense: 5' GAACCCTTCTGATCCAAGCT 3'; antisense: 5' AATGAAGCCTGCATT AGTGA 3'; 40 cycles, annealing temperature 60 °C, denaturation temperature 94 °C, extension temperature 72 °C, holding time 1 min). The reaction mixture consisted of 5 µl DNA, 5µl 10xPCR buffer, 1 µl of each primer (0.4 µM final concentration), 0.5 µL Taq polymerase (2.5 U). 1µL dNTPs (100 µM), $MgCl_2$ (1.5 mM in the first and 3 mM in the second PCR) and water to a final volume of 50 µl. Amplification products had a size of 292 bp and were visualized by agarose (3 %) gel electrophoresis and ethidium bromide staining [22]. As compared with the primers initially described by Tong and Sillis, this protocol resulted in better visible PCR products without differences in sensitivity. A BLAST search showed the amplified sequence to be specific for *C. pneumoniae*. Sequencing of 10 PCR-products confirmed the specificity of the PCR (310 ABI-Prism genetic analyser; Perkin Elmer, Weiterstadt, Germany). *C. pneumoniae* TW-183 was used as positive control. Water without DNA as negative control was included in each PCR. Most PCRs were repeated twice yielding identical results and the pyruvate dehydrogenase gene was amplified to detect an inhibition of the PCR [16].

Table 1 Demographic data in patients with multiple sclerosis and patients with other neurological diseases and their respective subgroups

Disease	No.	Gender (female/male)	Age (years) (mean ± SD)	Duration (years) (mean ± SD)	EDSS (median, range)	CSF Cells/µL (mean ± SD)	Oligoclonal bands (positive/all tested)
Definite MS	84	54/30	39.2±12.5	4.7±7.0	3.0 [0–8.5]	6.6±9.5	70/78 (89.8 %)
Relapsing remitting MS	55	37/18	34.1±9.8	2.8±3.8	2.0 [0–7]	8.6±11.0	47/52 (90.4 %)
Secondary chronic progressive MS	11	8/3	47.0±10.1	9.7±10.1	6.0 [3.0–8.0]	3.3±3.2	6/8 (75.0 %)
Primary chronic progressive MS	18	9/9	49.9±12.2	7.3±9.8	4.0 [2.5–8.5]	2.3±2.3	17/18 (94.4 %)
OND patients	89	46/43	40.8±11.7	–	–	42±315	10/62 (16.1 %)
Pathological CSF	27	14/13	43.3±12.8	–	–	140±576	10/19 (52.6 %)
Normal CSF	62	32/30	39.8±11.1	–	–	0.8±0.7	0/43

RNA Extraction and Reverse transcriptase PCR (RT-PCR)

RNA was extracted with TRIzol LS® Reagent (Invitrogen Life Technologies, Karlsruhe, Germany) as recommended by the manufacturer. After CSF (1 mL) was spun down (5 min at 18,000 g), 800 µl TRIzol LS® Reagent and 160 µl chloroform were added to the pellet and centrifuged together (15 min, 10,000 rpm, 2 °C). After addition of 10 µg glycogen (AGS Hybaid, Ulm, Germany) to the aqueous phase, RNA was precipitated with isopropanol for 10 min at 30 °C. The RNA pellet was washed once in 75 % cold ethanol and dissolved in 20 µl DEPC water. *C. pneumoniae* TW-183 served as positive control and water as negative control. We applied a one-step rt-PCR system using Superscript reverse transcriptase (Life technologies, Karlsruhe, Germany) to detect *C. pneumoniae*-specific 16S-rRNA as described recently [1] and heat shock protein 60 (HSP60)-mRNA. The primers (5'-AGACAGAGTAGATGATGCACAACA-3'; 5'-TCCTGCGTCAATCATATCTGTATAAG-3') were specific for HSP60 from *C. pneumoniae*.

After DNA digestion (RNase-Free DNase Set, Qiagen) for 30 min at room temperature, cDNA (48 °C for 30 min) was synthesized and PCR was performed as follows: denaturation temperature 94 °C for 2 min, annealing temperature 55 °C for 16S-rRNA and 65 °C for HSP60 (30s, respectively), elongation temperature 72 °C for 6min, 40 cycles. Each rt-PCR was also performed without the Superscript reverse transcriptase and using only *Taq*-polymerase to control for complete digestion of genomic DNA. Amplification products had a size of 463 bp (16S-rRNA) and 304 bp (HSP60) and were visualized on 2 % agarose gels with ethidium bromide staining. Specificity of amplifications was confirmed by sequencing of PCR products (HSP60, n = 8; 16 s-rRNA, n = 6). RT-PCR amplification of the human β-actin gene was used to confirm the presence of undegraded and amplifiable RNA and only samples with detectability of human β-actin mRNA were used for investigation of chlamydial RNA.

Separate rooms were used for preparing specimens, setting up PCRs, and analysing products and all measures required were applied to avoid carryover contamination of specimens by amplified products. Investigators were blinded to the patients' status and to other study results. We compared continuous variables by Student's t-test or Mann Whitney *U* test and categorical variables by Fisher's exact test or Chi^2 test, as appropriate.

Results

In a pilot study, we examined CSF samples from 25 patients, 15 patients with MS and 10 OND patients, to compare two standard methods for DNA extraction, the QIAamp DNA Blood Mini Kit and the phenol/chloroform extraction. Both tests were performed at random order. *C. pneumoniae* was not detected by nested PCR from any sample using the QIAamp kit. In contrast, 10 of the 25 samples were tested positive after DNA extraction with the phenol/chloroform method. These results prompted us to use only the phenol/chloroform extraction method in subsequent experiments.

Demographic data of the patient cohorts of the final study are depicted in Table 1. The rate of *C. pneumoniae* detection by PCR was higher in patients with definite MS (50 %) than in all OND controls together (28.1 %; p = 0.003; Fig. 1). This difference was significant as compared with OND patients with normal CSF (24.2 %; p = 0.002) but not with those with pathological CSF (37 %; p = 0.24). In MS-patients with relapsing-remitting disease, the rate of *C. pneumoniae*-positivity was particularly high (66.7 %) and significantly increased over the rate in OND patients with normal (p < 0.001) and pathological CSF (p = 0.050) (Table 2). Among patients with relapsing-remitting MS, those with positive and negative PCR were not different regarding age (34.0 ± 10.1 vs. 34.2 ± 9.4 years; p = 0.78), disease duration (2.4 ± 3.2 vs. 3.4 ± 4.6 years, p = 0.98) or disease severity (EDSS 2.0, 0–7 [median, range] versus 2.0, 0–4.5; p = 0.35). *C. pneumoniae* was not more often detected in patients with primary (33.3 %) or secondary chronic progressive MS (27.3 %) than in OND controls. OND patients tested positive for *C. pneumoniae* suffered from neurodegenerative diseases (n = 6), headaches and other pain disorders (n = 4), peripheral or cranial neuropathy (n = 4), meningoencephalitis of unknown origin (n = 4), CNS malignancies (n = 3), neu-

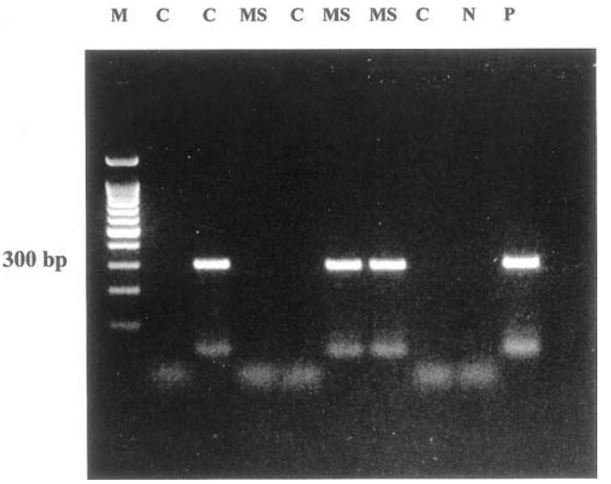

Fig. 1 Ethidium bromide-stained agarose gel of nested polymerase chain reaction products after amplification of the ompA gene of *Chlamydia pneumoniae* out of cerebrospinal fluid samples (*M* marker, *C* control patient, *MS* multiple sclerosis patient, *N* negative control, *P* positive control)

Table 2 Detection of *Chlamydia pneumoniae* genomic DNA by nested PCR

Disease	Positive nested PCR
Definite MS	42/84 (50.0 %)[a, b]
Relapsing-remitting MS	33/55 (66.7 %)[a, b, c]
Secondary chronic progressive MS	3/11 (27.3 %)
Primary chronic progressive MS	6/18 (33.3 %)
OND patients	25/89 (28.1 %)
OND patients with pathological CSF	10/27 (37.0 %)
OND patients with normal CSF	15/62 (24.2 %)

PCR polymerase chain reaction; *MS* multiple sclerosis; *OND* other neurological diseases

[a] significantly different compared to all OND patients combined

[b] significantly different compared to OND patients with normal CSF

[c] significantly different compared to OND patients with pathological CSF

roborreliosis (n = 2), neuropsychiatric disorders (n = 1) and epileptic seizures (n = 1).

We performed rt-PCR for detection of *C. pneumoniae*-specific mRNA in 20 MS patients (relapsing-remitting course n = 15; primary chronic progressive course n = 3, secondary chronic progressive course n = 2) and 16 OND patients with positive nested PCR, detection of human β-actin mRNA and a sufficient sample of CSF (Fig. 2). In 14 of the 20 MS patients (relapsing-remitting course n = 10, primary and secondary chronic progressive course n = 2, respectively), both HSP60-mRNA and 16S-rRNA could be detected and in one patient with relapsing-remitting MS, only HSP60 rt-PCR turned positive. In the 4 other MS patients both rt-PCRs were negative. Among OND-patients, 3 tested positive and 13 negative for both HSP60-mRNA and 16 s-rRNA. Therefore, detection of HSP60-mRNA (75 % vs. 18.8 %; p = 0.002) and 16S-rRNA (70 % vs. 18.8 %; p = 0.003) was more common in MS patients than OND controls. In addition, we performed rt-PCR in 7 MS patients and 10 OND controls with negative nested PCR. In one MS patient and one OND control both chlamydial 16S-rRNA and HSP60-mRNA were detected.

Discussion

Results from studies on *C. pneumoniae* in MS patients had yielded conflicting results. A main problem appears to be the lack of a reliable and standardized PCR method for detection of *C. pneumoniae* [9, 19]. In a recent comparative trial, one research group identified *C. pneumoniae* in the majority of MS patients but unfrequently in controls whereas three other investigators did not detect *C. pneumoniae* in any sample [18]. This underscores that technical details may play a dominant role in the detectability of *C. pneumoniae* DNA. In our hands, isolation of DNA by phenol-chloroform extraction yielded more sensitive PCR results than isolation with commercial DNA extraction kits used by other research groups [4, 10, 18]. Given the present discrepany in MS, it appears desirable that different DNA extraction and amplification protocols are compared with each other with strict adherence to technical details. Similar to the present controversy in MS, results from detection of *C. pneumoniae* in atherosclerotic plaques have varied widely [12] including the results of a comparative multicenter trial [2]. Despite such conflicting results, detectability of *C. pneumoniae* in atherosclerotic plaques can hardly be disputed today [12].

The high rate of positive PCR results in OND controls and mainly in those with pathological CSF indicates that the presence of *C. pneumoniae* in the central nervous system is not specific for MS although detection rates were higher in MS patients than in all OND controls combined. *C. pneumoniae* has been identified in circulating monocytes of healthy subjects [15] and by passing the blood-brain barrier, these cells could be the source of *C. pneumoniae* detected intrathecally, a hypothesis that may also explain the trend to a higher prevalence in subjects with pleocytosis or other CSF pathologies. *C. pneumoniae*-positivity was increased only in relapsing-remitting MS but not in chronic progressive subtypes, a finding that is in contrast to recent results [30]. It is possible that *C. pneumoniae* contributes to different courses of MS in a varying way. Alternatively, detectability of *C. pneumoniae* may diminish in parallel to extended disease duration although we found no close correlation between duration of relapsing-remitting MS and *C. pneumoniae*-positivity. It is a limitation of our study that only one sample in each patient was available and longitudinal studies are desirable for the future.

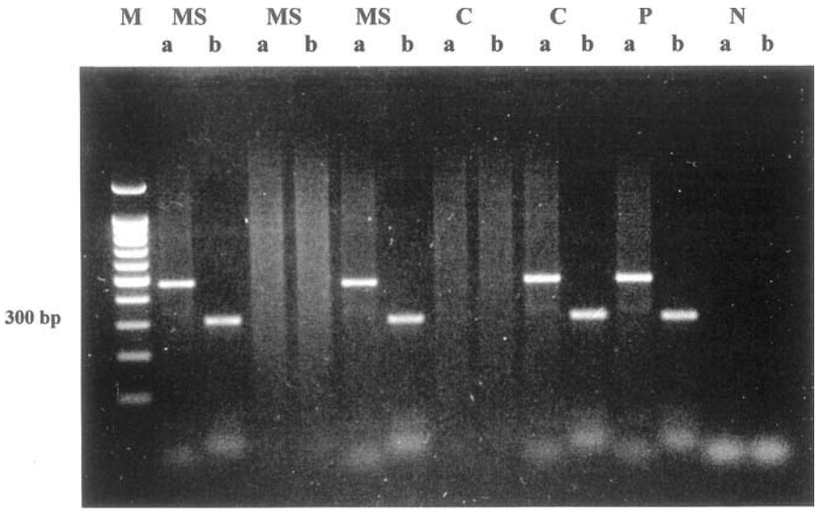

Fig. 2 Ethidium bromide-stained agarose gel of reverse transcriptase polymerase chain reaction products after reverse transcription and amplification of 16S-rRNA (lanes a) and heat shock protein 60-mRNA (lanes b) of *Chlamydia pneumoniae* from cerebrospinal fluid samples (*M* marker, *C* control patient, *MS* multiple sclerosis patient, *N* negative control, *P* positive control)

The lack of specificity argues against a pathogenetic role of *C. pneumoniae* in MS. However, it is possible that differences in the microbial burden, in the host response to *C. pneumoniae* or in the metabolic activity of the microbial agent are important variables that codetermine whether chronic inflammatory CNS disease develops under infection. We analysed specific gene transcripts to investigate whether *C. pneumoniae* is alive and metabolically active. This is an appropriate method as the isolation and propagation of *C. pneumoniae* from clinical specimens by cell cultures is rather insensitive and requires technical expertise [9, 22]. *C. pneumoniae* is an unusual pathogen with a biphasic life cycle consisting of an active, obligate intracellular form, the reticular body, and a metabolically inactive, but infectious form, the elementary body, that can persist in humans for long periods [12]. The more frequent detection of *C. pneumoniae*-specific RNA points towards a more active metabolism in MS patients. We investigated HSP60 gene-transcription as increased HSP expression has been demonstrated in MS lesions and an immune response to HSPs has been suggested as contributing to the pathogenesis of MS, e. g. by antigenic mimicry between closely related human and microbial HSPs [3, 5]. HSP60 was shown to be an important mediator in immune responses during *C. pneumoniae* infection [26]. Our study is limited to the qualitative detection of chlamydial HSP60-mRNA and further studies need to quantify specific mRNA and respective proteins and assess the immunologic response against chlamydial HSP60 in MS.

Neither the mere presence nor the greater prevalence of active gene transcription by *C. pneumoniae* prove that the agent triggers MS. It is possible that detection of *C. pneumoniae* just represents an opportunistic secondary event. But even in this circumstance, the organism could exacerbate pathogenic processes initiated by other means. Future attempts to clarify the role of *C. pneumoniae* in MS pathogenesis may include further animal experiments [23], may focus on the detection of *C. pneumoniae* in CNS specimens that has been unsuccessful so far [14] and address the unresolved question whether oligoclonal bands include antibodies against *C. pneumoniae* [8, 32].

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References

1. Airenne S, Surcel HM, Alakärppä H, et al. (1999) *Chlamydia pneumoniae* infection in human monocytes. Infect Immun 67:1445–1449
2. Apfalter P, Blasi F, Boman J, et al. (2001) Multicenter comparison trial of DNA extraction methods and PCR assays for detection of *Chlamydia pneumoniae* in endarterectomy specimens. J Clin Microbiol 39:519–524
3. Bachmaier K, Neu N, de la Maza LM, Pal S, Hessel A, Penninger JM (1999) Chlamydia infections and heart disease linked through antigenic mimicry. Science 283:1335–1339
4. Boman J, Roblin PM, Sundström P, et al. (2000) Failure to detect *C. pneumoniae* in the central nervous system of patients with MS. Neurology 54:265
5. Brosnan CF, Battistini L, Gao YL, Raine CS, Aquino DA (1996) Heat shock proteins and multiple sclerosis. A review. J Neuropathol Exp Neurol 55:389–402
6. Casetta I, Granieri E (2000) Clinical infections and multiple sclerosis: contribution from analytical epidemiology. J Neurovirol 6(Suppl. 2):S147–S151
7. Chatzipanagiotou S, Tsakanigas C, Anagnostouli M, et al. (2003) Detection of *Chlamydia pneumoniae* in the cerebrospinal fluid of patients with multiple sclerosis by combination of cell culture and PCR: no evidence for possible association. Mol Diagn 7:41–43
8. Derfuss T, Gürkov R, Then Bergh F, et al. (2001) Intrathecal antibody production against *Chlamydia pneumoniae* in multiple sclerosis is part of a polyspecific immune response. Brain 124:1325–1335
9. Dowell SF, Peeling RW, Boman J, et al. (2001) Standardizing *Chlamydia pneumoniae* assays: recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada). Clin Infect Dis 33:492–503
10. Gieffers J, Pohl D, Treib J, et al. (2001) Presence of *Chlamydia pneumoniae* DNA in the cerebral spinal fluid is a common phenomenon in a variety of neurological diseases and not restricted to multiple sclerosis. Ann Neurol 49:585–589
11. Goverman J, Woods A, Larson L, et al. (1993) Transgenic mice that express a Melin Basic Protein-specific T cell receptor develop spontaneous autoimmunity. Cell 72:551–560
12. Grayston JT (2000) Background and current knowledge of *Chlamydia pneumoniae* and atherosclerosis. J Infect Dis 181(Suppl. 3):S402–410
13. Grimaldi LM, Pincherle A, Martinelli-Boneschi F, et al. (2003) An MRI study of Chlamydia pneumoniae infection in Italian multiple sclerosis patients. Mult Scler 9:467–471
14. Hammerschlag MR, Ke Z, Lu F, et al. (2000) Is *Chlamydia pneumoniae* present in brain lesions of patients with multiple sclerosis? J Clin Microbiol 38:4274–4276
15. Haranaga S, Yamaguchi H, Leparc GF, et al. (2001) Detection of *Chlamydia pneumoniae* antigen in PBMNCs of healthy blood donors. Transfusion 41:1114–1119
16. Ho L, Javed AA, Pepin RA, et al. (1988) Identification of a cDNA clone for the β-subunit of the pyruvate dehydrogenase component of human pyruvate dehydrogenase complex. Biochem Biophys Res Commun 150:904–908
17. Ikejima H, Haranaga S, Takemura H, et al. (2001) PCR-based method for isolation and detection of *Chlamydia pneumoniae* DNA in cerebrospinal fluids. Clin Diagn Lab Immunol 8:499–502
18. Kaufman M, Gaydos CA, Sriram S, et al. (2002) Is *Chlamydia pneumoniae* found in spinal fluid samples from multiple sclerosis patients? Conflicting results. Mult Scler 8:289–294
19. Kuoppa Y, Bomann J, Scott L, et al. (2002) Quantitative detection of respiratory *Chlamydia pneumoniae* infection by real-time PCR. J Clin Microbiol 40:2273–2274

20. Kurtzke JF (1993) Epidemiologic evidence for multiple sclerosis as an infection. Clin Microbiol Rev 6:382–427
21. Krametter D, Niederwieser G, Berghold A, et al. (2001) *Chlamydia pneumoniae* in multiple sclerosis: humoral immune responses in serum and cerebrospinal fluid and correlation with disease activity marker. Mult Scler 7:13–18
22. Layh-Schmitt G, Bendl C, Hildt U, et al. (2000) Evidence for infection with *Chlamydia pneumoniae* in a subgroup of patients with multiple sclerosis. Ann Neurol 47:652–655
23. Lenz DC, Lu L, Conant SB, et al. (2001) A *Chlamydia pneumoniae*-specific peptide induces experimental autoimmune encephalomyelitis in rats. J Immunol 167:1803–1808
24. McDonald WI, Compston A, Edan G, et al. (2001) Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol 50:121–127
25. Numazaki K, Chibar S (2001) Failure to detect *C. pneumoniae* in the central nervous system of patients with MS. Neurology 57:746 (letter)
26. Prazeres da Costa C, Kirschning CJ, Busch D, et al. (2002) Role of chlamydial heat shock protein 60 in the stimulation of innate immune cells by *Chlamydia pneumoniae*. Eur J Immunol 32:2460–2470
27. Pucci E, Taus C, Cartechini E, et al. (2000) Lack of *C. pneumoniae* infection of the central nervous system in multiple sclerosis. Ann Neurol 48: 399–400
28. Saiz A, Marcos MA, Graus F, et al. (2001) No evidence of CNS infection with *C. pneumoniae* in patients with multiple sclerosis. J Neurol 248: 617–618
29. Sotgiu S, Piana A, Pugliatti M, et al. (2001) *Chlamydia pneumoniae* in the cerebrospinal fluid of patients with multiple sclerosis and neurological controls. Mult Scler 7:371–374
30. Sriram S, Stratton CW, Yao SY, et al. (1999) *Chlamydia pneumoniae* infection of the central nervous system in multiple sclerosis. Ann Neurol 46:6–14
31. Tong CYW, Sillis M (1993) Detection of *Chlamydia pneumoniae* and *Chlamydia psittaci* in sputum samples by PCR. J Clin Pathol 46:313–317
32. Yao SY, Stratton CW, Mitchell WM, Sriram S (2001) CSF oligoclonal bands in MS include antibodies against Chlamydophila antigens. Neurology 56:1168–1176