Journal of NeuroVirology, 15: 425–433, 2009

ISSN 1355-0284 print / 1538-2443 online
DOI: 10.3109/13550280903475580

informa
healthcare

Chlamydia pneumoniae–specific intrathecal oligoclonal antibody response is predominantly detected in a subset of multiple sclerosis patients with progressive forms

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The purpose of this study was to verify the actual involvement of *Chlamydia pneumoniae* in multiple sclerosis (MS) by the evaluation of its specific intrathecal humoral immune response in MS. We measured by enzyme-linked immunosorbent assay (ELISA) technique cerebrospinal fluid (CSF) and serum levels of anti–*C. pneumoniae* immunoglobulin G (IgG) in 27 relapsing-remitting (RR), 9 secondary progressive (SP), and 5 primary progressive (PP) MS patients, grouped according to clinical and magnetic resonance imaging (MRI) evidence of disease activity. Twenty-one patients with other inflammatory neurological disorders (OIND) and 21 with noninflammatory neurological disorders (NIND) were used as controls. Quantitative intrathecal synthesis of anti–*C. pneumoniae* IgG was determined by antibody-specific index (ASI), whereas the presence of *C. pneumoniae*–specific CSF oligoclonal IgG bands was assessed by antigen-specific immunoblotting. ASI values indicative of *C. pneumoniae*–specific intrathecal IgG synthesis were present in a small proportion of MS (29.3%), OIND (33.3%), and NIND (4.8%) patients and were significantly more frequent ($P < .05$) in total MS and in OIND than in NIND and in SP ($P < .01$) and PP MS ($P < .05$) than in RR MS. *C. pneumoniae*–specific CSF-restricted OCB were detected only in three SP, one PP, and one RR MS patients. These findings suggest that an intrathecal production of anti–*C. pneumoniae* IgG is part of humoral polyreactivity driven by MS chronic brain inflammation. However, an intrathecal release of *C. pneumoniae*–specific oligoclonal IgG can occur in a subset of patients with MS progressive forms in whom a *C. pneumoniae*–persistent brain infection may play a pathogenetic role. *Journal of NeuroVirology* (2009) **15**, 425–433.

Keywords: *Chlamydia pneumoniae*; multiple sclerosis; cerebrospinal fluid; intrathecal synthesis; oligoclonal IgG bands

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The study was supported by Fondazione Cassa di Risparmio di Ferrara, Italy. The authors thank Dr. Elizabeth Jenkins for helpful corrections to the manuscript.

Received 23 April 2009; revised 13 September 2009; accepted 28 September 2009.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS) that is generally hypothesized to be autoimmune in nature (Hauser and Oksenberg, 2006). Although disease etiology is still largely unknown, epidemiological observations suggest the potential implication of an infectious organism as a causative agent of MS (Casetta and Granieri, 2000). Among the various pathogens proposed as putative candidates for MS autoimmunity, there is also *Chlamydia pneumoniae* (*C. pneumoniae*), a gram-negative, obligate intracellular bacterium that is distributed worldwide, is characterized by a high seroprevalence in adults, and is able to induce a persistent brain infection (Fainardi *et al*, 2008). After the first demonstration of a strong association existing between *C. pneumoniae* and MS (Sriram, 1999), the conflicting results obtained in subsequent seroepidemiological, culture, molecular, and neuropathological studies do not support a central role for this microbial agent as a cause of the disease, but indicate that *C. pneumoniae* could act as a cofactor in MS pathogenesis (Contini *et al*, 2004; Dong-Si *et al*, 2004; Sriram *et al*, 2005; Fainardi *et al*, 2008; Contini *et al*, 2008). A similar interpretation was raised after the analysis of the controversial findings concerning the production of anti–*C. pneumoniae* immunoglobulin G (IgG) within the CNS that was absent or observed in a variable proportion of MS patients and controls (Derfuss *et al*, 2001; Krametter *et al*, 2001; Yao *et al*, 2001; Rostasy *et al*, 2003; Fainardi *et al*, 2004; Franciotta *et al*, 2005b). In addition, intratecally released anti–*C. pneumoniae* high-affinity antibodies were found in patients with MS progressive forms (Fainardi *et al*, 2004).

In this setting, the nature of *C. pneumoniae*–specific oligoclonal IgG bands restricted to cerebrospinal fluid (CSF) of MS patients needs to be clarified. In both MS and chronic CNS infections, the presence of CSF oligoclonal antibodies represents a key feature of the disease (Gilden, 2005). Brain infections are characterized by a targeted intrathecal antibody production in which only 20% of oligoclonal IgG produced within the CSF compartment are specific to the causative agent (Conrad *et al*, 1994). Conversely, antigenic specificity of oligoclonal IgG in MS still remains elusive because although this antibody reaction is considered to be driven by a disease-relevant antigen (Smith-Jensen *et al*, 2000; Pachner *et al*, 2007), local production of IgG within the CSF compartment is widely believed to be part of a bystander response directed against many different pathogens unrelated to the cause of the disease and promoted by a polyspecific B-cell activation (Reiber *et al*, 1998).

Therefore, in this study we aimed to investigate *C. pneumoniae*–specific intrathecal humoral immune response in MS patients and controls in order to establish whether the frequency of CSF-restricted anti–*C. pneumoniae* oligoclonal IgG detected in MS patients is consistent with the existence of a CNS persistent infection sustained by this pathogen.

Results

*CSF and serum levels of anti–*C. pneumoniae *IgG in MS patients and controls*

As summarized in Table 1, the percentages of CSF and serum with detectable levels of anti–*C. pneumoniae* did not statistically differ among total MS, OIND,[1] and NIND, and between MS patients grouped according to clinical course and evidence of clinical and MRI activity. Accordingly, no significant differences were found for CSF and serum mean concentrations of anti–*C. pneumoniae* IgG among total MS, OIND, and NIND, and between MS patients categorized according to clinical forms and appearance of clinical and MRI activity (Figure 1A and B). However, the analysis of *C. pneumoniae*–specific CSF antibody profile revealed values approaching a statistical significance. More precisely, CSF measurable titers of anti–*C. pneumoniae* IgG were more frequent in total MS (chi-square; P =.0796) and OIND (chi-square; P =.06) than in NIND (Table 1). Similarly, CSF mean levels of anti–*C. pneumoniae* IgG were higher in total MS (38.7 ± 58.7 AU; Mann-Whitney; P =.0676) and OIND (41.8 ± 68.8 AU; Mann-Whitney; P =.058) than in NIND (13.6 ± 37.3 AU).

Table 1 Percentages of CSF and serum samples with detectable levels of anti–*C. pneumoniae* IgG

	CSF Positive/total (%)	Serum Positive/total (%)
NIND (n = 21)	6/21 (28.6%)	10/21 (47.6%)
OIND (n = 21)	13/21 (61.9%)	12/21 (57.1%)
Total MS (n = 41)	23/41 (56.1%)	28/41 (68.3%)
RR MS (n = 27)	13/27 (48.1%)	17/27 (63%)
SP MS (n = 9)	8/9 (88.9%)	7/9 (77.8%)
PP MS (n = 5)	3/5 (60%)	4/5 (80%)
CA MS	13/27 (48.1%)	16/27 (59.3%)
CS MS	7/9 (77.8%)	8/9 (88.9%)
Gd+ MS	12/20 (60%)	13/20 (65%)
Gd− MS	8/21 (38.1%)	13/21 (61.9%)

Note. Samples were from subjects with noninflammatory neurological disorders (NIND) and other inflammatory neurological disorders (OIND), and from patients with multiple sclerosis (MS) considered as a whole and stratified according to clinical course and evidence of clinical and magnetic resonance imaging (MRI) disease activity.RR = relapsing remitting; SP = secondary progressive; PP = primary progressive; CA = clinically active (presence of relapse at entry); CS = clinically stable (absence of relapse at entry); Gd+ = MRI appearance of gadolium-enhancing lesions; Gd− = no MRI evidence of gadolium-enhancing lesions.

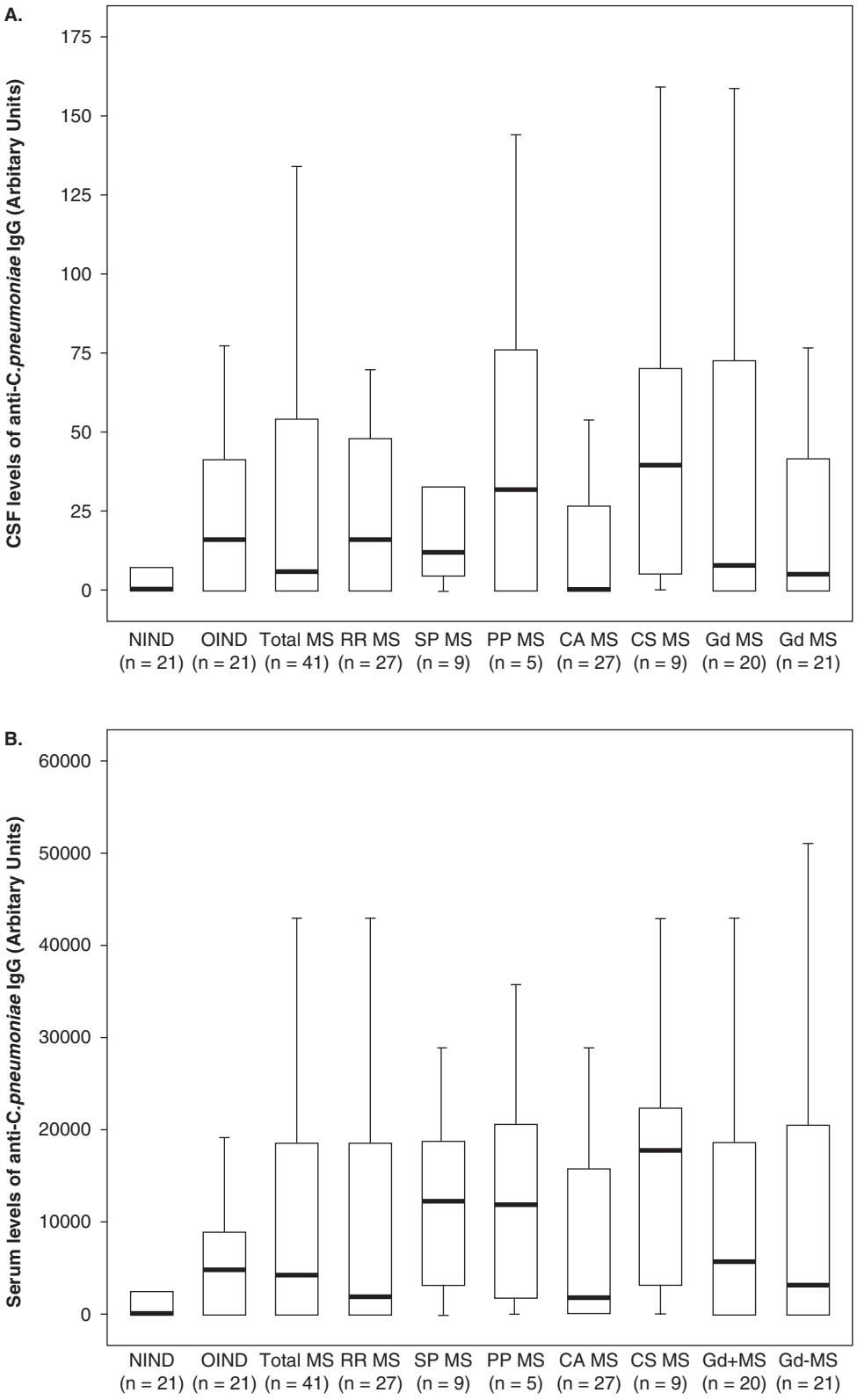

Figure 1 (*Continued*)

C. pneumoniae–*specific intrathecal humoral immune response*

As illustrated in Figure 2A, the presence of ASI abnormal values indicative of a *C. pneumoniae*–specific intrathecal IgG synthesis was significantly more frequent (chi-square; $P < .05$) in total MS and in OIND than in NIND. In addition, an intrathecal synthesis of anti–*C. pneumoniae* IgG, as suggested by ASI values above 1.5, was significantly more represented in SP (chi-square; $P < .01$) and PP (chi-square; $P < .05$) than in RR MS. There were no significant differences for ASI abnormal values between clinically active and clinically stable MS as well as between MS with and without MRI appearance of disease activity. As shown in Figure 2B, *C. pneumoniae*–specific CSF-restricted oligoclonal IgG bands were detected in only five patients with MS. Of these, two were clinically and MRI inactive SP, one was a clinically and MRI active SP, one was a MRI inactive PP, and one was a clinically and MRI active RR MS. Therefore, the presence of *C. pneumoniae*–specific CSF oligoclonal IgG bands was significantly more frequent in SP than in RR MS (chi-square; $P < .05$). In all MS patients with *C. pneumoniae*–specific CSF oligoclonal IgG bands, antigen-specific immunoblotting recognized a local synthesis pattern. All these patients had ASI values greater than the cut-off of 1.5. None of the patients with OIND and NIND showed oligoclonal IgG bands specifically directed against *C. pneumoniae*.

*Correlation between CSF and serum levels and intrathecal synthesis of anti–*C. pneumoniae *IgG and MS clinical features*

No definite associations were observed between disease duration and severity of the disease, as expressed by EDSS, and CSF and serum levels and intrathecal synthesis of anti–*C. pneumoniae* IgG (data not shown).

Discussion

In this study we sought to investigate the nature of intrathecally produced *C. pneumoniae*–specific antibodies, which were reported in a recent meta-analysis to be strongly related to MS (Bagos *et al*, 2006). We here found that a local release of anti–*C. pneumoniae* IgG within the CNS, as revealed by ASI abnormal values, occurred in a minority of MS patients and controls and was preponderant in MS and OIND compared to NIND and in SP and PP with respect to RR MS. More important, we showed for the first time that *C. pneumoniae*–specific CSF oligoclonal IgG bands predominated in SP and PP rather than in RR MS. In addition, although a non-significant slight prevalence was identified for CSF detectable and mean amounts of anti–*C. pneumoniae* IgG in total MS and OIND compared to NIND, we did not find any additional statistical difference for CSF and serum levels, and intrathecal synthesis of anti–*C. pneumoniae* IgG among MS and controls. Likewise, no difference was observed among the various MS subgroups for these laboratory parameters, which were not significantly associated to duration and severity of the MS disease. In this context, the reciprocal distribution observed for ASI abnormal values in MS patients grouped according to clinical activity and MRI activity could be explained by the earlier appearance of Gd enhancement in active lesions with respect to clinical symptoms (Kermode *et al*, 1990) or, alternatively, by a methodological artifact due to the low number of clinically stable MS patients. These results were substantially concordant with those obtained in previous studies (Yao *et al*, 2001; Derfuss *et al*, 2001; Krametter *et al*, 2001; Rostasy *et al*, 2003; Fainardi *et al*, 2004; Franciotta *et al*, 2005b; Fainardi *et al*, 2008), with some divergences likely due to methodological differences in determination techniques and in patient selection.

Overall, this study indicates that an intrathecal production of anti–*C. pneumoniae* IgG is recognized only in a limited number of MS patients and is not restricted to MS, but is shared by several inflammatory neurological conditions. These data argue against a role of *C. pneumoniae* as a unique causative agent of MS and imply that *C. pneumoniae*–specific intrathecally produced antibodies are simple innocent bystanders reflecting a polyspecific humoral reactivity promoted by the overactive chronic immune stimulation associated to MS brain inflammation (Reiber *et al*, 1998). However, a *C. pneumoniae*–targeted intrathecal humoral immune response may exist in MS progressive forms because *C. pneumoniae*–specific CSF-restricted oligoclonal IgG bands were almost exclusively found in patients

Figure 1 Anti–*C. pneumoniae* IgG levels in CSF (A) and serum (B) from subjects with noninflammatory neurological disorders (NIND) and other inflammatory neurological disorders (OIND), and from patients with multiple sclerosis (MS) considered as a whole and classified according to clinical course and evidence of clinical and magnetic resonance imaging (MRI) disease activity. RR = relapsing remitting; SP = secondary progressive; PP = primary progressive; CA = clinically active (presence of relapse at entry); CS = clinically stable (absence of relapse at entry); Gd+ = MRI appearance of gadolium enhancing lesions; Gd− = no MRI evidence of gadolium enhancing lesions. The boundaries of the box represent the 25th to the 75th quartiles. The line within the box indicates the median. The whiskers above and below the box correspond to the highest and lowest values, excluding outliers. The corresponding CSF and serum mean values were 13.6 ± 37.3 and 6473.8 ± 13210.0 AU, respectively, in NIND; 41.8 ± 68.8 and 7046.6 ± 10410.2 AU, respectively, in OIND; 38.7 ± 58.7 and 11791.8 ± 14423.1 AU, respectively, in total MS; 40.2 ± 65.6 and 11484.7 ± 16056.8 AU, respectively, in RR MS; 28.1 ± 34.8 and 11709.8 ± 9821 AU, respectively, in SP MS; 50.3 ± 60.7 and 13597.7 ± 14380.6 AU, respectively, in PP MS; 34.6 ± 62.2 and 9932.9 ± 14759.8 AU, respectively, in CA MS; 44.7 ± 51.7 and 16365.1 ± 13837.8 AU, respectively, in CS MS; 39.0 ± 54.6 and 11905.3 ± 14885.4 AU, respectively, in Gd+ MS; 38.6 ± 63.8 and 11683.7 ± 14336.3 AU, respectively, in Gd− MS.

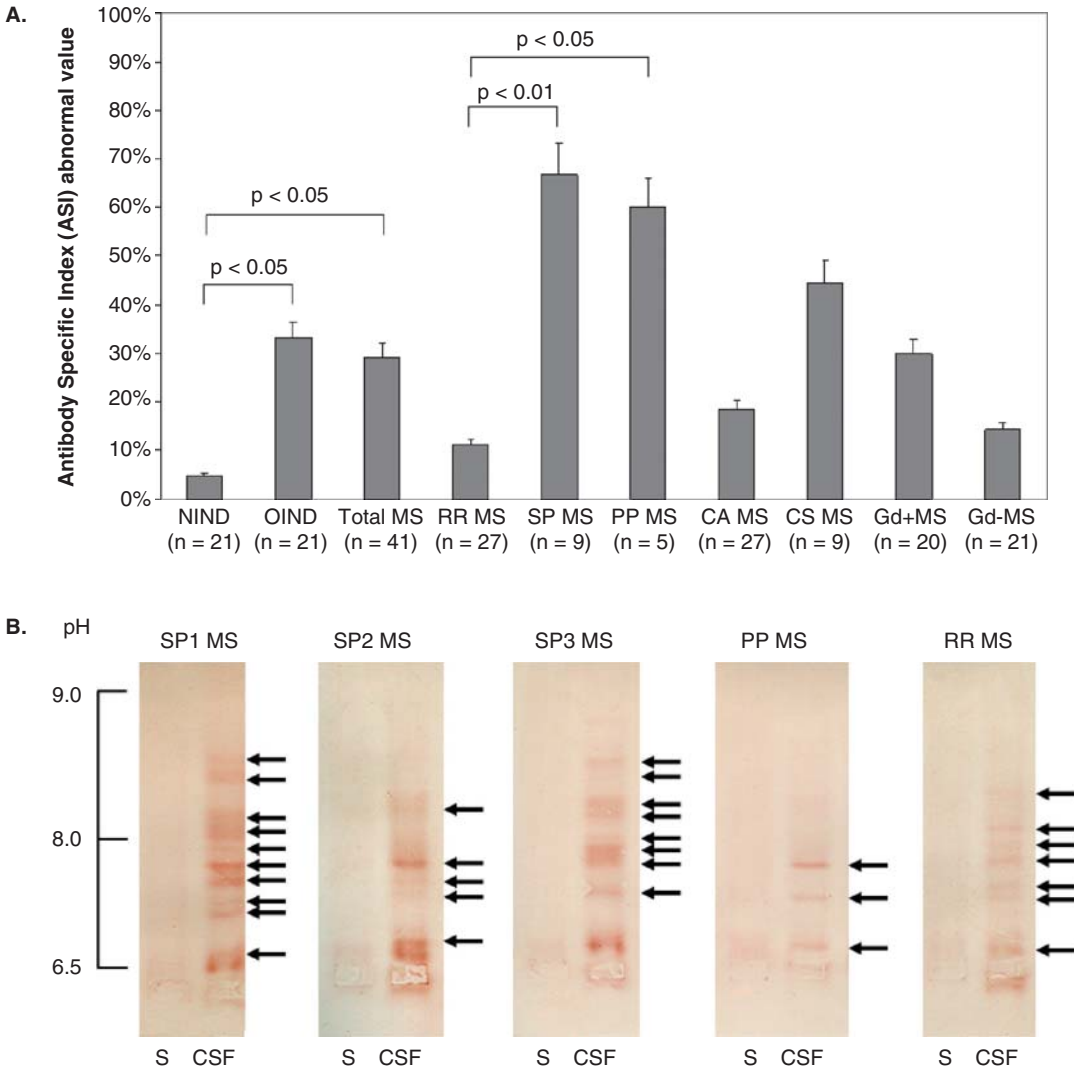

Figure 2 Intrathecal synthesis of anti–*C. pneumoniae* IgG, as espressed by antibody specific index (ASI) abnormal values and the occurrence of CSF-restricted oligoclonal bands, in subjects with noninflammatory neurological disorders (NIND) and other inflammatory neurological disorders (OIND) and in patients with multiple sclerosis (MS) considered as a whole and divided, according to clinical course and evidence of clinical and magnetic resonance imaging (MRI) disease activity, in the following subgroups: RR = relapsing remitting; SP = secondary progressive; PP = primary progressive; CA = clinically active (presence of relapse at entry); CS = clinically stable (absence of relapse at entry); Gd+ = MRI appearance of gadolium enhancing lesions; Gd− = no MRI evidence of gadolium enhancing lesions. (A) The distribution of ASI values above 1.5, which showed significantly higher rates (P <.05) in total MS (12/41; 29.3%) and in OIND (7/21; 33.3%) than in NIND (1/21; 4.8%) as well as in SP (6/9; 66.7%) and PP (3/5; 60%) than in RR (3/27; 11.1%) MS ($P < .01$ and $P < .05$, respectively). (B) The local synthesis pattern detected by antigen-specific immunoblotting in two clinically and MRI inactive SP (SP1 and SP2), one clinically and MRI active SP (SP3), one MRI inactive PP, and one clinically and MRI active RR MS. S = serum; CSF = cerebrospinal fluid. Arrows indicate *C. pneumoniae*–specific CSF oligoclonal IgG bands.

with SP and PP MS who also had ASI values consistent with a more pronounced intrathecal production of anti–*C. pneumoniae* IgG. In fact, it is currently accepted that qualitative analysis is more sensitive than quantitative in the determination of an intrathecal IgG synthesis (Thompson, 2004). This assumption seems to be further supported by the recent demonstration that intrathecally produced anti–*C. pneumoniae* high-affinity IgG were more represented in SP and PP MS than in RR MS patients and controls (Fainardi *et al*, 2004). Thus, as cerebral infections are considered to be marked by CSF high-affinity oligoclonal IgG specifically directed to the causal agent (Chapman *et al*, 2007), our findings suggest that a *C. pneumoniae* persistent brain infection could be present in a subset of MS patients with progressive clinical courses in whom *C. pneumoniae* may act as a cofactor in the maintenance of the disease.

In contrast, the potential link between a *C. pneumoniae* CNS infection and MS progression is not corroborated by the lack of a significant correlation between intrathecally produced anti–*C. pneumoniae* IgG and the disability rate. In addition, we were not able to provide the direct culture

evidence of a *C. pneumoniae* persistent brain infection in our progressive MS patients because the isolation of the pathogen was not performed. As a consequence, this study did not definitely clarify whether the antigen-driven intrathecal synthesis of IgG anti–*C. pneumoniae* detected in SP and PP MS actually corresponds to a persistent brain infection. On the one hand, the presumed *C. pneumoniae* persistent CNS infection may occur without a significant release of specific CSF and serum antibodies because it could appear in latent form that, unlike chronic form, is characterized by an intermittent instead of a continual antigen stimulation (Lipton *et al*, 2007). On the other hand, *C. pneumoniae*–specific antibody response may be generated by mechanisms other than microbial persistence into the brain, such as molecular mimicry or epitope spreading (Archelos and Hartung, 2000). The use of a respiratory strain as antigen for the detection of *C. pneumoniae*–specific oligoclonal IgG bands could represent a further drawback of our study given that other *C. pneumoniae* strains may reside within the CNS. However, the degree of genetic homology among the different *C. pneumoniae* strains is very high (Sommer *et al*, 2009). Moreover, brain isolates of *C. pneumoniae* seem genetically more similar to respiratory than atherosclerotic strains (Dreses-Werringloer *et al*, 2009). In any case, the most important limitation of this study was the small sample size that could strongly weaken the consistency of our data. Therefore, more extensive research in a larger number of patients is warranted for a better understanding of the role of *C. pneumoniae*–specific humoral immune response in MS.

Materials and methods

Patient selection

Forty-one consecutive patients (26 women and 15 men; mean age = 37.8 ± 11.5 years) satisfying the criteria for definite MS according to the classification of McDonald *et al* (2001) and admitted to the MS Center of the Department of Neurology, University of Ferrara, during the period from November 2005 to January 2008 were prospectively selected for the study. In agreement with the criteria of Lublin and Reingold (1996), this group of patients was divided into three different clinical courses: 27 showed relapsing-remitting (RR) form, 9 secondary progressive (SP) form, and 5 primary progressive (PP) form. Evidence of a relapse at admission, as defined by Poser's criteria (Poser *et al*, 1983), was considered as clinical disease activity. Accordingly, at entry, 27 patients were clinically active and 9 were clinically stable. PP MS patients were not included in these two subgroups because, by definition, they were not experiencing relapses (Lublin and Reingold, 1996). Disease severity was scored in all MS patients at the time of sample collection using Kurtzke's Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) (mean at entry = 2.3 ± 1.3; range from 0 to 6.5). The duration of the disease was expressed in months (mean at entry = 35.4 ± 51.3; range from 0.5 to 246). At entry, none of the patients had fever or other symptoms or signs of acute infections. Moreover, at the time of inclusion in the study, none of the patients had received any potential disease-modifying therapies (e.g., azathioprine or methylprednisolone, interferon-beta, or glatiramer acetate) during the 6 months before the study. Twenty-one patients with other inflammatory neurological disorders (OIND) and 21 subjects with other noninflammatory neurological disorders (NIND) served as neurological controls. The OIND group (12 women and 9 men, mean age = 50.3 ± 16.1 years) included 5 with herpes simplex virus-1 encephalitis, 2 with varicella-zoster virus encephalitis and 3 with acute disseminated encephalomyelitis, 7 with bacterial meningitis, 2 with intracerebral abscess, 1 with neuroSjögren, and 1 with neurolupus. The NIND group (11 women and 10 men, mean age = 52.8 ± 14.0 years) consisted of 6 patients with headache, 4 with migraine, 3 with epilepsy, 2 with cervical spondylosis, 2 with amyotrophic lateral sclerosis, 2 with paresthesias, 1 with hereditary ataxia, and 1 with compression neuropathy. To avoid confounding factors, patients who suffered from human immunodeficiency virus (HIV) encephalopathy, Alzheimer, and cerebrovascular diseases were not included in controls because the potential implication of *C. pneumoniae* in these neurological conditions has recently been suggested (Grayston *et al*, 1995; Danesh *et al*, 1997; Balin *et al*, 1998; Contini *et al*, 2003; Gérard *et al*, 2006). OIND and NIND patients were free of immunosuppressant drugs, including steroids, at the time of sample collection.

CSF and serum sampling

CSF and serum samples were collected under sterile conditions and stored in aliquots at −70°C until assay. Paired CSF and serum samples from MS, OIND, and NIND patients were stored and measured under exactly the same conditions. All CSF and serum analysis were performed within 2 weeks of the onset of clinical symptoms in relapsing MS patients, and at least 2 months after the end of clinical exacerbation in clinically stable MS patients. Informed consent was given by all patients before inclusion and the study design was approved by the Local Committee for Medical Ethics in Research. CSF and serum albumin and IgG levels were measured by immunochemical nephelometry with the Beckman Array Protein System (Beckman Instruments, Fullerton, CA, USA) according to the procedure of Salden *et al* (1988). Blood-CSF barrier (B-CSF-B) dysfunction was determined by CSF/serum albumin quotient (QAlb) (Tibbling *et al*, 1977).

Magnetic resonance imaging analysis
Brain magnetic resonance imaging (MRI) scans were performed at entry using a standard head coil in all patients with a 1-Tesla MRI unit (GE Signa Horizon; General Electric Medical Systems, Milwaukee, WI, USA) within 48 h after CSF sampling. Routinely used T1-weighted axial spin echo images were obtained approximately 10 min after intravenous injection of 0.1 mmol/kg of gadolinium (Gd)-DTPA in each patient. Lesions showing Gd-enhancement on T1-weighted scans were defined as indicative of MRI activity. Accordingly, 20 MS patients (14 RR and 6 SP; 17 with clinically active and 3 with clinically stable disease) were classified as MRI active and 21 (13 RR, 3 SP, and 5 PP; 10 with clinically active and 6 with clinically stable disease) were considered as MRI inactive. All brain MRI scans were evaluated by one investigator (E.F.) blinded to clinical and sample data.

*CSF and serum levels of anti–*C. pneumoniae *IgG determination*
CSF and serum concentrations of anti–*C. pneumoniae* IgG were measured by enzyme-linked immunosorbent assay (ELISA) as previously described (Fainardi *et al*, 2004) by using a commercially available ELISA kit (Cp Quant IgG; Eurospital Diagnostics, Trieste, Italy; catalog no. 9267). CSF and corresponding serum were diluted at 1:2 and at 1:400, respectively. As described by Conrad and associates (Conrad *et al*, 1994), concentrations of anti–C. *pneumoniae* IgG were expressed as arbitrary units (AU). Both intra- and interassay variations, expressed as CV%, were less than 8%.

Calculation of C. pneumoniae*–specific antibody index*
In accordance with Reiber's formula (Reiber and Lange, 1991), production of anti–*C. pneumoniae* IgG was determined by antibody-specific index (ASI). *C. pneumoniae*–specific intrathecal IgG synthesis was assumed for values of ASI ≥ 1.5.

C. pneumoniae *antigen preparation*
C. pneumoniae strain AR-39 (ATCC no. 53592) was cultured and purified as described by Krull *et al* (2004) with some opportune modifications. Briefly, Hep-2 cell line (ATCC CCL-23) cycloheximide-treated was seeded in four flasks (75-cm^2) at the density of 5×10^5 cells/ml, in 20 ml Dulbecco's modified Eagle medium (D-MEM) (Gibco, Invitrogen) containing 2 mM L-glutamine, 1 g/L D-glucose, 10% foetal bovine serum, and antibiotics (streptomycin/penicillin) for 24 h at 37°C and successively the monolayer was inoculated with elementary bodies (EBs) from a frozen stock of *C. pneumoniae* (1.0×10^9 Inclusion-Forming Units (IFU)/ml) (Mukhopadhyay et al, 2004) in 3 ml of D-MEM without serum. Each flask was centrifuged at 37°C for 2 h and incubated for 3 h at 37°C after addition of 6 ml of medium. Finally, all medium was removed from each flask and replaced with 20 ml of fresh medium with serum. The flasks were then incubated in CO_2 at 37°C. Infected monolayers were harvested after 72 h from culture flasks, sonicated 2 times for 30 s, and centrifuged at $32000 \times g$ for 45 min at 4°C. Each pellet was diluted in sucrose-phosphate-glutamate (SPG) buffer slowly up to 10 ml and sonicated as above. After adding other SPG 10 ml, the cellular debris were removed by centrifugation at $500 \times g$ for 5 min at 4°C. Finally, to each supernatant were added 15 ml of SPG, centrifuged at $32000 \times g$ for 45 min at 4°C, and each pellet obtained was diluted with an equal volume of SPG buffer and stored at −80°C until use.

Antigen-specific immunoblotting
C. pneumoniae–specific IgG oligoclonal bands were detected by antigen-specific immunoblotting according to the protocol reported elsewhere (Fainardi *et al*, 2000) using a commercially available isoelectric focusing (IEF) kit (IgG IEF; Helena Laboratories, Gateshead, Tyne and Wear, UK). Equal amounts of undiluted CSF and 1:300 diluted serum samples (5 µl) were applied to the agarose gel. After IEF run, the gel was blotted onto a nitrocellulose (NC) sheet previously coated with *C. pneumoniae* antigen solution at the concentration of 100 µg/ml per 10 cm^3 of NC area. The antigen-specific blotting was then performed according to the manufacturer's instructions. The immunoblotting specificity was evaluated following the protocol of Dörries *et al* (1989). In accordance with the currently accepted guidelines (Franciotta *et al*, 2005a), we identified four different antigen-specific immunoblotting IgG banding patterns ("normal"; "local synthesis"; "mixed"; and "mirror). The results were examined by two independent investigators. Only the detection of antibody-specific immunoblotting "local synthesis" and "mixed" profiles was considered suggestive of intrathecal synthesis of anti–*C. pneumoniae* oligoclonal IgG.

Statistics
The normality of each variable was checked by using the Kolmogorov-Smirnov test. As normality of data distribution was rejected in several variables, statistical analysis was performed by a nonparametric approach. Kruskal-Wallis test was used to compare CSF and serum mean levels of anti–*C. pneumoniae* IgG among the various groups. If significant differences were found, Mann-Whitney *U* test was then used to compare CSF and serum mean concentrations of anti–*C. pneumoniae* IgG between two different groups. Percentages of measurable CSF and serum mean levels of anti–*C. pneumoniae* IgG and intrathecal synthesis of anti–*C. pneumoniae* IgG in the different groups were compared by means of chi-square test. In cases of multiple comparisons, a Bonferroni *post hoc* correction was applied. The

Spearman rank correlation coefficient test was used to identify possible relationships among the different variables. A value of $P < .05$ was considered as statistically significant.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 06 January 2010.