CSF oligoclonal bands in MS include antibodies against Chlamydombila antigens

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Article abstract—Background: Considerable evidence suggests the role of an infectious agent in MS. The presence of Chlamydombila pneumoniae in CSF from patients with MS was shown earlier; to further examine this association the reactivity of the oligoclonal antibody response in the CSF of patients with MS to C pneumoniae antigens was determined and compared with other antigens. Methods: Seventeen patients with MS and 14 control subjects with other neurologic disease were studied. Affinity-driven immunoblot studies and solid-phase adsorption of CSF oligoclonal bands by elementary body antigens of C pneumoniae, viral antigens (measles and herpes simplex virus-1), bacterial antigen (Escherichia coli and Staphylococcus aureus), and heat shock protein-60 were performed. Results: Affinity-driven immunoblot studies demonstrated reactivity of oligoclonal bands in CSF samples from 16 patients with MS against elementary body antigens of C pneumoniae. None of the control subjects showed a prominent reactivity to elementary body antigens of C pneumoniae. In 14 of 17 patients with MS examined, oligoclonal bands were adsorbed either partially or completely from the CSF by elementary body antigens of C pneumoniae, but not by myelin basic protein, heat shock protein-60, or bacterial or viral antigens. In three patients with subacute sclerosing panencephalitis, adsorption of oligoclonal bands was seen with measles virus antigens but not with elementary body antigens of C pneumoniae. Conclusions: Oligoclonal bands in CSF of patients with MS include antibodies against Chlamydombila antigens.

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Although the etiology of MS is not known, indirect and circumstantial evidence suggests the role of an infectious agent in the disease process.1 We chose to examine a possible link between chronic CNS infection with Chlamydombila pneumoniae and MS because of our initial observation of CNS infection with C pneumoniae in a patient with rapidly progressive MS.2 In extending these observations to a larger number of patients with established relapsing-remitting and progressive (primary and secondary) MS, we noted the presence of C pneumoniae in a majority of patients with MS.3 Chlamydombila belongs to a genus of intracellular pathogens. This family includes at least five species: C pneumoniae, Chlamydombila psittaci, Chlamydombila abortus, Chlamydombila pecorum, and Chlamydombila felis. Of these, C pneumoniae is infectious to humans, and is recognized as causing chronic persistent diseases, including those that affect the central nervous system.4–11 We and others have noted the presence of C pneumoniae in CSF from patients with MS.12–14 Furthermore, we observed antibody responses to C pneumoniae antigens in the CSF of patients with MS, suggesting that chronic infection with C pneumoniae may be occurring in these patients.

To further examine the association between the development of MS and the presence of C pneumoniae infection in the CNS, we analyzed the reactivity of oligoclonal bands from patients with relapsing-remitting and progressive MS against C pneumoniae antigens.15,16 In virtually every chronic CNS infection, increased levels of immunoglobulins that recognize the pathogen are synthesized exclusively within the CNS compartment and are seen as oligoclonal bands by isoelectric focusing (IEF) methods.17–19 In MS, oligoclonal bands are a hallmark of the disease, although the antigenic specificity(s) of these bands remains unknown.20–22 Our present study examined the pattern and reactivity of oligoclonal bands (representing intrathecal antibody synthesis) to C pneumoniae antigens.
measles, human heat shock protein-60 (HSP-60), *Escherichia coli*, *Staphylococcus aureus*, and herpes simplex virus-1 (HSV-1) antigens in patients with MS and control subjects with other neurologic disease (OND).

**Materials and methods.** Patient population. Patients who satisfied the criteria of definite MS were recruited for the study. A total of 17 patients with MS (nine secondary progressive, two primary progressive, and six relapsing remitting) were studied. Ten patients were receiving either interferon beta or methylprednisolone at the time of lumbar puncture. Patient 13 had received no immunosuppressive therapy at any time before the spinal tap. Patients in whom CSF was obtained for diagnostic studies served as OND control subjects. In all patients, CSF and, when possible, serum, was divided into aliquots (0.5-mL freezing vials) and stored at −70 °C before use. CSF samples from patients with subacute sclerosing panencephalitis (SSPE) were a kind gift of Dr. B. Weissbrich (Würzburg, Germany) and J. Rose (Salt Lake City, UT). Dr. S. Jacobson from the NIH kindly provided CSF samples from patients with human T-cell lymphotropic virus-1 (HTLV-1) myelopathy.

Antigens. Human HSP-60 was purchased from Stress-Gen (Vancouver, BC, Canada). Guinea pig myelin basic protein (MBP) was prepared from spinal cords as previously described. HSV-1 and measles antigen were commercially obtained (Bio-Whittaker, Walkersville, MD). *E. coli* and *S. aureus* were obtained from American Type Culture Collection (Manassas, VA) (numbers 25922 and 25923), and grown in Bacto-agar plates (Becton, Dickenson, Franklin Lakes, NJ) overnight. The colonies were pooled, sonicated at 30 kHz for 30 seconds, and spun down at 9000g for 30 minutes. The proteins from the supernatants were resuspended in phosphate-buffered saline at a concentration of 1 mg/mL and used in the adsorption assays. Protein determination was done using a protein assay kit (Bio-Rad, Hercules, CA).

Preparation of elementary body (EB) antigens of C pneumoniae. Elementary body antigens of *C pneumoniae* were prepared from concentrated elementary bodies by treatment with a reducing agent to break the disulfide bonds and thus dissolve the outer membrane proteins. This was done by incubating 25 mM dithiothreitol and 2% mercaptoethanol at room temperature for 30 minutes followed by boiling for 5 minutes. Elementary body antigens were then sonicated and centrifuged (500g for 30 minutes at room temperature). Elementary body antigens were resuspended (20 μg/mL protein) in phosphate-buffered saline (pH 7.4) and used for all experiments. Concentrated *C pneumoniae* elementary bodies were obtained by growing *C pneumoniae* (VR-1310; American Type Culture Collection) in human lung endothelial (HL) cell line. Elementary bodies were harvested and resolubilized in Iscove’s minimal essential medium and the presence of *C pneumoniae* antigens was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blotting with anti–*C pneumoniae* antibody (Accurate Chemical & Scientific Corp, Westbury, NY). *E. coli* and *S. aureus* antigens were isolated from culture stocks following sonication and centrifugation.

**Affinity-driven immunoblot technique for detection of antigen reactivity of oligoclonal bands.** IEF of CSF immunoglobulins followed by affinity-driven transfer of antibodies onto antigen-coated membranes was undertaken. IEF was performed with 0.25 μg of immunoglobulin from CSF obtained from patients with MS and control subjects with OND using an Isoelectric Focus Units System (Wallac Inc., Akron, OH). Focusing was carried out in agarose gel (pH 3.0 to 10.0) according to the manufacturer’s protocol. Capillary transfer was used to move the IEF gel to nitrocellulose paper (Trans-blot [0.45 μm]; Bio-Rad, Hercules, CA) precoated with antigen. The membrane was precoated with *C pneumoniae* elementary body or viral antigens at a concentration of 5 μg/mL and incubated overnight with gentle rocking at 4 °C. Control antigens for blotting experiments were measles and HSV-1. Unoccupied sites were blocked with 5% fat-free milk. Antibody bound to antigen was probed with peroxidase-conjugated goat antihuman IgG (1:10,000) (Sigma Chemical Corp., St. Louis, MO) using a chemiluminescent detection assay.

**Solid phase adsorption of cationic antibodies in CSF to *C pneumoniae* antigens.** Elementary bodies of *C pneumoniae* were heated to 100 °C for 5 minutes, sonicated for 30 seconds, resuspended in carbonate buffer (pH 9.6), and coated (20 μg/well) overnight onto microtiter 96-well plates. The wells were washed in phosphate-buffered saline and unoccupied sites blocked using 1% bovine serum albumin for 2 hours. CSF samples containing 0.8 μg immunoglobulin in 200 μL saline (pH 7.4) were added to the 96-well plates. Control antigens (20 μg/well) to which CSF immunoglobulin samples also were added included cell lysates of uninfected HL cells, MBP, HSP-60, *E. coli*, *S. aureus*, measles, and HSV-1. After overnight incubation at 4 °C, CSF containing unbound immunoglobulins was carefully removed, frozen, and lyophilized. In view of the small volumes of CSF, care was taken to ensure that fluid remained frozen following placement in the lyophilizer. Immunoglobulins were redisolved in 30 μL water immediately prior to running an IEF gel. Samples containing 0.25 μg immunoglobulin (10 μL) were loaded into an IEF gel, and IEF was performed. The gel was transferred onto nitrocellulose membranes, and the presence of immunoglobulin bound to antigen on the membranes was probed with peroxidase-conjugated goat antihuman IgG (Sigma Chemical Corp.) using a chemiluminescence detection assay (Amersham, Arlington Heights, IL).

**Results.** Affinity-driven immunoblots to determine antigen reactivity of oligoclonal bands in patients with MS and control subjects. In 16 of 17 patients with MS, cationic antibodies (seen as oligoclonal bands) showed binding to *C pneumoniae* antigens following affinity-driven immunoblot transfer (table 1). Representative patterns of the immunoblots after transfer onto antigen-coated membranes are shown in figure 1 (four patients with MS) and figure 2 (four control subjects with OND). In all patients with MS, the binding of *C pneumoniae* antigens to the cathodal antibodies closely reflected the CSF immunoglobulin pattern seen on IEF gels transferred onto membranes not coated with antigen. The prominence of the signal of individual oligoclonal bands following transfer onto elementary body–coated membranes differed in intensity, although not in their overall pattern, suggesting differences in the affinity of the individual oligoclonal bands to the antigen. When reactivity to other antigens was examined, weak binding to
measles antigen was seen in nine of 15 patients (see figure 1, A and B; table 1). In three patients, binding to HSV-1 was seen (see figure 1C; table 1); in one of these patients, affinity-driven immunoblots showed prominent bands reactive to HSV-1.

Representative patterns of four affinity-driven immunoblots for control subjects are shown in figure 2. In two patients with SSPE, as expected, oligoclonal bands seen on IEF gels were bound to measles antigen following transfer. In SSPE Patient 2, reactivity of some of the cathodal antibodies to C pneumoniae antigens was seen. Weak binding to C pneumoniae antigens was seen for Patient 9, who presented with clinical features of HSV-2 myelitis. In Patient 4 (table 2), who had CNS syphilis, weak bands to measles and C pneumoniae were seen. No binding to either viral antigen was seen in the patient with CNS vasculitis.

Solid-phase adsorption of oligoclonal bands with elementary body antigens of C pneumoniae. If oligoclonal bands represent the dominant CNS humoral response to C pneumoniae infection, we predicted adsorption of these bands by antigens of C pneumoniae. Adsorption was carried out in solid phase with a 25-fold excess of antigen over antibody (0.8 μg CSF IgG plated onto microtiter wells incubated overnight with 20 μg/well of antigen). In parallel experiments, CSF samples containing 0.8 μg of IgG were added to wells coated with 25-fold excess of MBP, HSP-60, E coli, S aureus, measles, or HSV-1 antigens, which served as antigen specificity controls. In addition, we examined the adsorption of CSF immunoglobulins to cell lysates prepared from uninfected HL cells. In 14 of 17 patients with MS, partial to complete adsorption of antibodies in the cationic region of the gel was seen following adsorption

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<th>Immuno-therapy</th>
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<th>OC bands</th>
<th>Immunoblot, measles</th>
<th>Immunoblot, HSV-1</th>
<th>Immunoblot, C pneumoniae</th>
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* Patients 1 through 11 had progressive MS; Patients 12 through 17 had relapsing-remitting MS.

EDSS = Expanded Disability Status Scale score; IgG = immunoglobulin G; OC = oligoclonal; HSP = heat shock protein; ND = not done; MTX = methotrexate; IFNβ = interferon beta; NA = not available; HSV-1 = herpes simplex virus-1.

Figure 1. Affinity-driven immunoblot studies of four patients with MS. Lanes 1 to 4 represent the banding pattern of oligoclonal antibodies following affinity-driven transfer onto untreated (lane 1), Chlamyphilla pneumoniae antigen–coated (lane 2), measles antigen–coated (lane 3), and herpes simplex virus-1 antigen–coated (lane 4) nitrocellulose membranes and probed with antihuman immunoglobulin antibody. Note the similarities in the banding pattern (but differences in banding intensity) between CSF immunoglobulins on isoelectric focused gel and following transfer to C pneumoniae–coated membranes in all four patients.
Table 2 Clinical profile and antibody characteristics of patients in the OND control group

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<th>Patient</th>
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<th>Immunoblot, C pneumoniae</th>
<th>Adsorption, C pneumoniae</th>
<th>Adsorption, measles</th>
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OND = other neurologic diseases; OC = oligoclonal; SSPE = subacute sclerosing panencephalitis; ND = not done; HSV = herpes simplex virus; HTLV-1 = human T-cell lymphotropic virus-1.

with C pneumoniae antigens (figures 3 and 4). Adsorption of oligoclonal bands was not seen to any of the control antigens. No adsorption of CSF immunoglobulins was seen with cell lysates of uninfected HL cells in the 10 patients examined (data not shown).

In three patients with MS, adsorption of oligoclonal bands was not seen when a 25-fold excess of antigen over CSF IgG was used (figures 5, A through C, and 6). In Patient 4 (see table 1), weak adsorption of at least one oligoclonal band was noted (see figure 6). To determine if higher concentrations of antigen plated to microtiter wells would improve adsorption, C pneumoniae antigens were plated at concentrations of 20 μg, 40 μg, and 60 μg in each well and 0.8 μg CSF IgG was added. Measles antigen at concentration of 60 μg/well was added as a control. As seen in figure 6, increasing the amount of antigen coated onto the well improved the efficiency of adsorption and at least three bands in the cathodic region either disappeared or were decreased in intensity. In Patient 1, no significant adsorption was seen when C pneumoniae antigen was added at concentrations of 50 μg/well (data not shown). This would suggest that in some patients, differences in the affinity of the antibodies or the availability of the appropriate epitopes may require higher antigen–antibody ratios for optimal adsorption. Alternatively, antibodies that recognize Chlamydophila antigens and that focus in the cathodal region of the IEF gel may not be present in all patients with MS.

Nine patients in the OND group were studied; representative patterns from six are shown in figure 7. No changes in the chemiluminescence signal of the oligoclonal bands were seen following adsorption with C pneumoniae antigens. Oligoclonal bands were adsorbed with excess measles antigen in all three patients with SSPE, but not with HSV-1 or EB antigens of C pneumoniae, suggesting that the antimeasles antibody response in the CSF constituted the major antibody response in patients with SSPE. In SSPE Patient 2 (see table 2), cathodal antibodies reactive to EB antigens of C pneumoniae were seen on affinity-driven immunoblots (see figure 2). Incubation of CSF from SSPE Patient 2 with EB antigens of C pneumoniae did not alter the IEF gel, suggesting that the anti-C pneumoniae antibodies did not comprise the major antibody response in the CSF. In the remaining five patients with inflammatory

![Figure 2](image-url)
disease of the CNS, no difference in the banding pattern of cathodal antibodies was seen following adsorption with EB antigens of C pneumoniae. These results suggest that oligoclonal bands in CSF of patients with MS represent in part antibodies to Chlamydophila antigens. Nonspecific adsorption of antibodies to C pneumoniae antigens in patients with MS was an unlikely explanation, because antibodies present in the anodal region did not bind to C pneumoniae antigens. Also, no decrease in the intensity of the oligoclonal bands was seen among nine OND control subjects following incubation with C pneumoniae antigens.

Discussion. Our results demonstrate that the development of an intrathecal immune response to C pneumoniae antigens is a common occurrence in the population of patients with MS that we have examined. Epidemiologic studies have shown the presence of antibody titers in serum to C pneumoniae in over 50% of individuals over 40 years of age.28 However, the elevated levels in the CSF of patients with MS compared with control subjects with OND strongly suggests a compartmentalization of the elevated antibody response within the CNS. This therefore argues for a persistent infection with C pneumoniae in the CNS of patients with MS.29 The central feature of the study was our ability to adsorb, either partially or completely, the oligoclonal bands with purified elementary body antigens of C pneumoniae in 14 of 17 patients with MS. The inability of human HSP-60, bacterial or viral antigens, or cell lysates from uninfected HL cells to decrease the signal on IEF gels following solid-phase adsorption suggests that the adsorption is seen mainly for Chlamydophila an-

Figure 3. Adsorption studies on CSF immunoglobulins to elementary body antigens of Chlamydia pneumoniae, measles, herpes simplex virus-1 (HSV-1), heat shock protein (HSP)-60, Escherichia coli, and Staphylococcus aureus in patients with MS. In each individual patient, the left two lanes represent isoelectric focused (IEF) gel patterns for 0.25 µg immunoglobulin of unmanipulated serum and CSF. Right lanes represent the IEF gel patterns following incubation with antigens as labeled. In (H) and (I) the adsorption experiments on the viral antigens (lanes 3 to 6) and bacterial antigens, including HSP-60 (lanes 7 to 10), were done at different times. Hence, the differences in intensity of the oligoclonal bands and the location of the isoelectric point markers between lanes 3 to 6 and 7 to 10. All adsorption studies were carried out following coating of 20 µg antigen onto plates followed by the addition of 0.8 µg CSF IgG (ratio 25:1).
tigens. We have not examined the reactivity of antibodies to species of Chlamydophila other than C pneumoniae and hence the presence of cross-reactivity with other bacterial antigens including those in the Chlamydophila genus cannot be excluded. Because serologic cross-reactivity is seen between members of the chlamydia family, reactivity of oligoclonal bands with antigens derived from the different members of the Chlamydophila family is quite likely.\textsuperscript{30,31}

Oligoclonal bands representing intrathecal synthesis of antibody occur in more than 95% of patients with MS. Oligoclonal bands are not specific for MS, as they are also seen in 10% of patients with other inflammatory disease of the CNS.\textsuperscript{20-22,29} In many patients with MS, oligoclonal bands do not have a counterpart in the serum, suggesting solely an intrathecal synthesis of antibody. Although termed oligoclonal, the specific bands on the IEF gel do not necessarily represent a monoclonal B cell response.\textsuperscript{32} In fact, two-dimensional electrophoresis patterns for IEF gels have shown single bands that focus at fractions of 0.1 to 0.3 pH units can be resolved into many different antibodies. These observations suggest that in most patients with MS, the number of B cell clones constituting oligoclonal bands is in excess of the total number of oligoclonal bands seen.\textsuperscript{33}

The significance of oligoclonal bands in MS has remained an enigma. In most CNS disorders (other than MS) in which oligoclonal bands are seen, these
bands are thought to indicate intrathecal synthesis of immunoglobulins and represent an immune response to an infectious agent. Chronic bacterial and fungal CNS infections such as those seen with tuberculosis, syphilis, neuroborreliosis, coccidiodomycosis, and cryptococcosis are all characterized by the presence of oligoclonal bands in the CSF. Similarly, in chronic viral infections such as SSPE, rubella panencephalitis, and HTLV-1 myelitis, oligoclonal bands constitute the major immune response to the respective viral antigens. Oligoclonal bands are also seen following acute monophasic infections of the CNS such as HSV-1 encephalitis and following enteric and picorna viral infections of the CNS. The reactivity of the antibody response to the inciting pathogen, represented as oligoclonal bands, has been shown using affinity-driven immunoblotting techniques and by direct adsorption of antibodies with relevant infectious antigens. Immunoblotting of CSF antibodies onto antigen-coated membranes has shown that the pattern of oligoclonal bands reflect differing affinities of the antibodies to antigen. Thus, for patients with tuberculous meningitis and SSPE, antimycobacterial and antiviral antibodies in CSF represent the major portion of the oligoclonal bands. Adsorption studies of oligoclonal antibodies with antigens from infectious pathogens have been difficult to perform, due to technical difficulties in purifying a sufficient amount of antigen (50- to 100-fold excess of antigen over antibody) to ensure adequate binding to antibody. These limitations notwithstanding, the specificity of the oligoclonal bands to infectious agents has been proven with adsorption studies in CNS for syphilis, SSPE, HTLV-1 myelopathy, and neuroborreliosis.

Prior studies attempted to define the nature of the putative infectious agent in MS by determining the antigenic specificity of the intrathecal immune response. Using IEF and affinity-driven immunoblot techniques, investigators noted that CSF

Figure 6. Adsorption of oligoclonal bands with increasing concentrations of elementary body (EB) antigens but not with measles antigen in Patient 4 (see table 1). Left panel represents isoelectric-focused gel of 0.25 μg IgG of unmanipulated CSF. Middle panel shows the lack of adsorption of cationic antibodies following plating of 20 μg/well of antigen. The right panel shows the disappearance of some of the cationic antibody (arrowheads) following incubation of CSF with increasing concentrations of EB antigen. In all lanes shown in the right panel, the concentration of CSF IgG (0.8 μg/well) was kept constant and the amount of EB antigen of Chlamydia pneumoniae and measles increased as the ratios indicate. (The isoelectric focused gels in the middle and left panel were run at different times and hence the difference in the location of the isoelectric point markers.)

Figure 7. Isoelectric focused gel patterns following adsorption studies on CSF immunoglobulins on six control subjects with neurologic diseases other than MS: (A through C) subacute sclerosing panencephalitis, (D) CNS syphilis, (E) CNS vasculitis, (F) chronic meningitis. Note the lack of adsorption by Chlamydia pneumoniae antigens in all six patients. The concentrations of CSF IgG and antigens added for adsorption was the same as those in the other experiments.
antibodies react to a number of infectious agents and self-antigens (HSP) that focus at the cathodal region of the gel.\textsuperscript{46,47} However, these bands did not constitute the major oligoclonal bands, because incubation of CSF immunoglobulins with the antigen did not remove the oligoclonal bands. A number of patients with MS had antimeasles and anti-HSV-1 antibodies that focused in the cathodal region of the gel.\textsuperscript{46,47} The frequency of cathodal antibodies reactive to measles and HSV-1 antigen seen in the patients with MS studied (56% and 30%) was similar to those reported previously.\textsuperscript{48} Like other authors, we noted that the antimeasles antibodies did not represent the major antigenic specificity(s) of the oligoclonal bands in patients with MS, because they were not adsorbed by purified measles antigen and hence represented antibodies of low affinity.\textsuperscript{46}

We have established that a humoral response to \textit{C. pneumoniae} antigens is seen in a majority of patients examined in our laboratory. In three of the 17 patients examined, the oligoclonal antibody was not adsorbed by \textit{C. pneumoniae} antigens, suggesting that the immune response to \textit{Chlamydia phila} antigens is not dominant in all patients with MS. In another patient, adsorption was seen only when the antigen-antibody ratio was increased from 25:1 to 75:1 in the solid-phase adsorption assay. Other investigators have also noted that the presence of PCR signal to \textit{C. pneumoniae} was not present in all patients.\textsuperscript{12-14}

These observations are therefore a caution as to the universality of the association between the presence of an immune response to \textit{C. pneumoniae} and the development of MS. At present, the biochemical nature and structure of the antigens reacting with the antibodies are not known. It is conceivable that among the universe of pathogens, cross-reactivity between \textit{C. pneumoniae} and other as-yet-undetermined antigens is possible, including other members of the \textit{Chlamydia} family. In virtually all infections of the CNS in which oligoclonal bands represent intrathecal antibody synthesis, the antigenic specificity of the immune response is directed against the infectious pathogen. If this paradigm were to be extended to MS, it would indicate that \textit{C. pneumoniae}, or an organism that is a close member of the \textit{C. pneumoniae} family, may play a role in the immunopathology of CNS lesions in some patients with MS.

\textbf{Disclosure statement}

Vanderbilt University, with C.W.S. and W.M.M., hold equity positions in Merlin Technology, Inc., which has in the past supported studies on the role of \textit{Chlamydia phila pneumoniae} in MS.

\textbf{References}


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