Detection of Chlamydial Bodies and Antigens in the Central Nervous System of Patients with Multiple Sclerosis

Subramaniam Sriram, Asa Ljunggren-Rose, Song-Yi Yao, and William O. Whetsell, Jr.
Departments of Neurology and Pathology, Vanderbilt University Medical Center, Nashville, Tennessee

(See the correspondence by Hammerschlag et al. and Sriram et al., on pages 1305–7 and 1307–8, respectively.)

To examine a possible relationship between *Chlamydia pneumoniae* infection and multiple sclerosis (MS), we undertook an immunohistochemical (IHC), molecular, and ultrastructural comparison of central nervous system (CNS) tissue and cerebrospinal fluid (CSF) sediment from patients with MS and control individuals with other neurological diseases (ONDs). In 7 of 20 MS cases, IHC staining was seen in association with ependymal surfaces and periventricular regions of formalin-fixed brain tissue, by use of 3 different antichlamydial antibodies. There was no staining with any of the 3 antichlamydial antibodies in formalin-fixed brain tissue from OND controls (n = 17). With available frozen CNS tissue, polymerase chain reaction (PCR) studies for the presence of *C. pneumoniae* genes were performed. The presence of a PCR signal was confirmed in 5 of 8 MS cases and in 3 of 18 OND controls. In an examination of CSF sediment by electron microscopy, we observed electron-dense structures resembling chlamydial organisms in CSF sediments from 11 of 20 MS cases and 2 of 12 OND controls. The presence of immunogold-labeled electron-dense bodies was correlated with the presence of a PCR signal in 10 of 11 MS cases. Results of studies using these different approaches support our suspicion of the presence of chlamydial organisms in the CNS, in a subset of patients with MS.

*Chlamydia pneumoniae* is an obligate intracellular microorganism that is a common cause of respiratory infections in humans [1, 2]. The ability of this organism to persist in infected tissue for extended periods has suggested that it may participate in either the development or the progression of chronic inflammatory disorders—specifically, atherosclerosis and reactive arthritis [3–6]. A number of studies have reported development of acute neurological syndromes after *C. pneumoniae* infection [7–15]. There is considerable debate regarding the role of *C. pneumoniae* persistence and infection in chronic diseases of the central nervous system (CNS).

Nonetheless, a recent study has suggested that *C. pneumoniae* infection may be associated with Alzheimer disease, and we and others have suggested that *C. pneumoniae* infection may play a role in the pathogenesis of multiple sclerosis (MS) [10, 11, 16, 17].

Although the etiology of MS is not known, clinical observations suggest an interaction between an infectious agent and an autoimmune response to neural antigens in the development of the disease [18]. MS shows close similarities to autoimmune experimental allergic encephalitis, and epidemiologic studies have implicated infectious agents as a contributing element in the development of MS [19]. To reconcile the 2 hypotheses pertaining to autoimmunity and infection, it has been suggested that an infectious agent may act as a trigger in initiating an autoimmune process in the CNS [20–23].

In the Nurses Health Study, antibody titers to *C. pneumoniae* were found to be higher in patients with progressive MS than in control individuals, suggesting that *C. pneumoniae* infection may be a factor in disease
failure, 1 who had died of multiorgan failure after renal trans-
cephalitis. Three patients—1 who had died of bone marrow
renoleucodystrophy, cytomegalovirus encephalitis, and HIV en-
of subacute sclerosing panencephalitis, CNS lymphoma, ad-
p
   as follows: Parkinson disease ( ), Alzheimer disease ( )
and the number of cases examined for the control group were

progression [24]. Our studies have shown that the major outer
membrane protein (MOMP) DNA of C. pneumoniae is seen
in the cerebrospinal fluid (CSF) from some patients with MS
(hereafter referred to as “MS cases”) but not in patients with
other neurological diseases (hereafter referred to as “OND controls”). We and others have also noted the presence of anti-
bodies to C. pneumoniae in CSF from some MS cases [16, 25].
Some investigators have confirmed the presence of C. pneumoniae DNA in CSF from MS cases [15, 19, 26–30]; others have
been unable to detect chlamydial DNA in MS cases or in control
individuals [31–33]. Technical differences in the methodologies
of polymerase chain reaction (PCR) assays may account for the
discrepancies.

One of the requirements for demonstrating a causal or in-
fluencing relationship between an infectious agent and a disease
is demonstration of the presence of the organism in involved
tissue. In the study presented here, we have examined CNS
tissue and CSF sediment from MS cases and OND controls for
evidence of the presence of C. pneumoniae.

PATIENTS, MATERIALS, AND METHODS

Patients. With institutional review board approval, CSF was
obtained from MS cases and OND controls. Postmortem brain
tissue samples for immunohistochemical (IHC) staining and
PCR analysis were obtained from archived specimens at the
Vanderbilt Medical Center and from archived material at the Vanderbilt Medical Center and from
the Rocky Mountain Brain Bank. In 11 cases, neither immu-
nosuppressive nor immunomodulatory drugs were adminis-
tered immediately before death. In the remaining 9 cases, pertin-
ent clinical information on the use of immunosuppressive

drugs during the period immediately before death was not
available. In these cases, information on duration, progression,
and severity of disease was also not available. The diagnosis
and the number of cases examined for the control group were
as follows: Parkinson disease (n = 4), Alzheimer disease (n = 3), systemic lupus erythematos (n = 2), and 1 case each of
subacute sclerosing panencephalitis, CNS lymphoma, ad-
renoleucodystrophy, cytomegalovirus encephalitis, and HIV en-
cephalitis. Three patients—1 who had died of bone marrow
failure, 1 who had died of multiorgan failure after renal trans-
plant, and 1 who had died of end-stage renal hypertension—
were also studied. In 5 MS cases and 4 OND controls, frozen
tissue was available for PCR assays. Additional frozen tissue
from 3 MS cases and 14 OND controls was obtained from
archived material at the Vanderbilt Medical Center and from
the Rocky Mountain Brain Bank for PCR assays. CSF from MS
cases and OND controls was obtained as part of the diagnostic
studies performed to evaluate patients seen in the MS clinic.

Reagents. Anti-chlamydial antibodies were purchased from
commercial sources and used at the concentrations shown in table
1. CF-2 antichlamydial lipopolysaccharide (LPS) antibody and
antichlamydial anti-hsp60 antibodies have been recommended as
optimal reagents for tissue staining [34, 35].

IHC staining of formalin-fixed CNS tissue. Five-microm-
eter sections of formalin-fixed brain tissue was deparaffinized,
blocked with peroxidase and casein, and incubated overnight
with 1 of the 3 antichlamydial antibodies or their isotype-
matched controls at dilutions shown in table 1. Control anti-

bodies were used at the same concentrations as the antichla-
mydial antibodies. After overnight incubation, the sections were
washed, and goat anti-mouse antibodies were added for 30 min
at room temperature. The slides were washed, and the AEC
binding to the primary antibody. The efficacy of the different
antibodies was ascertained by staining mouse lungs infected
with C. pneumoniae (mouse tissue blocks were a gift from T.
Nagy, Department of Pathology, Vanderbilt Medical Center).

Immunogold staining of CSF sediment. Twenty milliliters
of CSF from each of 20 MS cases and 12 OND controls were
spun at 65,000 g for 30 min, and the sediment was resuspended
in 100 µL of PBS. CSF sediments were placed on formvar-
coated nickel grids and dried at 4°C. The grids were fixed in
4% paraformaldehyde-PBS for 15 min, washed and incubated
with 50 mmol/L glycine for 10 min, and washed and blocked
with 5% bovine serum albumin. Slides were incubated over-
night with monoclonal antibody (MAb) 807 at a dilution of 1:
50. After washing, a 1:50 dilution of gold-conjugated (10 nm
 gold; EM Sciences) anti-mouse antibody was added for 18 h.
After washing, the grids were postfixed in 2% glutaraldehyde

Table 1. Isotype and specificity of antichlamydial antibodies used in immunohistochemical staining assays.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Dilution(s)</th>
<th>Concentration, mg/mL</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal antibody 807</td>
<td>Chlamydia LPS and MOMP</td>
<td>IgG2b/IgG3</td>
<td>1:1000 and 1:1500</td>
<td>1</td>
<td>Chemicon</td>
</tr>
<tr>
<td>CF-2</td>
<td>Chlamydia LPS</td>
<td>IgG2a</td>
<td>1:100</td>
<td>0.1</td>
<td>Washington Research Foundation</td>
</tr>
<tr>
<td>hsp60</td>
<td>Chlamydia hsp60 antigen</td>
<td>IgG1</td>
<td>1:500</td>
<td>1</td>
<td>Affinity Bioreagents</td>
</tr>
<tr>
<td>10-E13</td>
<td>Anti–Escherichia coli LPS</td>
<td>IgG3</td>
<td>1:750</td>
<td>1.4</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>MOPC-141</td>
<td>Control</td>
<td>IgG2b</td>
<td>1:500</td>
<td>0.5</td>
<td>Sigma</td>
</tr>
<tr>
<td>UPC-10</td>
<td>Control</td>
<td>IgG2a</td>
<td>1:100</td>
<td>0.1</td>
<td>Sigma</td>
</tr>
<tr>
<td>MS284</td>
<td>Control</td>
<td>IgG1</td>
<td>1:100</td>
<td>0.2</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

NOTE. LPS, lipopolysaccharide; MOMP, major outer membrane protein.
Inflammation

moderate; 3, marked; 4, severe.

controls for the study included elementary bodies that were
positively stained electron-dense bodies on 5 squares in the
CSF specimens. We arbitrarily chose the presence of at least 4
spheres on the surface were considered to be positive. The total
examined, and electron-dense bodies bearing at least 15 gold
cells in 5 squares was counted.

NOTE

PCR assays were performed with frozen tissue from 8 MS cases.
For cases 1–5, formalin-fixed tissue was available, and IHC staining was performed.
PV (a), PV (b), and PV (c) represent different areas of periventricular
regions obtained for PCR studies; CBL, cerebellum; FH, frontal horn; IVV, fourth
ventricle; LV, lateral ventricle; PV, periventricular region including ependyma;
VW, ventricular wall; WM, white matter.

a +, staining with all 3 antichlamydial antibodies was positive.
b Degree of inflammation seen on hematoxylin-eosin sections: 1, mild; 2, moderate; 3, marked; 4, severe.
c Represents 2 separate regions of frontal horn.

for 10 min, washed, air-dried, and examined by electron micros-
ycopy (EM).

Five randomly selected squares on each Formvar grid were
examined, and electron-dense bodies bearing at least 15 gold
spheres on the surface were considered to be positive. The total
number of immunogold-stained cells in 5 squares was counted.
The evaluator was blinded as to the diagnosis of each of the
CSF specimens. We arbitrarily chose the presence of at least 4
positively stained electron-dense bodies on 5 squares in the
formvar grids as a cutoff number for positive samples. Positive
controls for the study included elementary bodies that were
isolated from human lung cells infected with *C. pneumoniae*.

PCR of *C. pneumoniae* DNA from frozen brain tissue of MS
cases and OND controls. Frozen tissue stored at −20°C was
thawed to −20°C and dissected in a sterile hood. *C. pneumoniae* is a ubiquitous organism, and, therefore, DNA isolation
was performed using aseptic techniques [36]. Where possible,
subependymal (periventricular) lesions were identified and care-
fully dissected for PCR assays. Tissue (2.5 mg) was homogenized
and lysed in 500 µL of PCR lysis buffer; 25 µL of proteinase K
was then added, and the mixture was incubated overnight at
37°C. DNA was extracted from the tissue by use of the chlo-
roform/phenol method [12].

Nested PCR studies were performed using primers specific
for the *C. pneumoniae* MOMP and 16s RNA genes. The primers
for the MOMP gene were as follows: external, sense (5′-AAC
TAT ACT ACT GCC GTA GA-3′) and antisense (5′-GTA GTA
GAC AAT GCT GTG G-3′); and nested, sense (5′-ACA CCT
CTT TCT GCT GGA GGC T-3′) and antisense (5′-TAT ATG
GTC GCA GAC TTT GTT C-3′). The primers for the 16s RNA
gene were as follows: external, sense (5′-GCT AAT ACC GAA
TGT AGT GTA A-3′) and antisense (5′-ATC TAT CCT CTA
AGA TAG TT-3′); and nested, sense (5′-GTA AAA GCC
CAC CAA GCC GAT GA-3′) and antisense (5′-CTA CAC GCC
CTT TAC GCC CAA-3′).

Table 2. Comparison of immunohistochemical (IHC) staining
and polymerase chain reaction (PCR) analysis for the presence
of *Chlamydia pneumoniae* DNA in the central nervous system of
patients with multiple sclerosis (MS cases).

<table>
<thead>
<tr>
<th>Sample type, MS case</th>
<th>Tissue location</th>
<th>MOMP PCR</th>
<th>16s RNA PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>WM/PV</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>WM/PV</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1</td>
<td>WM/PV</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>WM/PV</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>CBL</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>PV</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>FH</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>PV (a)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>PV (b)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>PV (c)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>PV</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>FH</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>PV</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>WM</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>WM</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>WM</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>WM</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fixed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>PV</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>CBL</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Pons</td>
<td>−</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>FH</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>IVV</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>LV</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>VV</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>LV</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| NOTE. PCR assays were performed with frozen tissue from 8 MS cases.

For cases 1–5, formalin-fixed tissue was available, and IHC staining was performed.
PV (a), PV (b), and PV (c) represent different areas of periventricular
regions obtained for PCR studies; CBL, cerebellum; FH, frontal horn; IVV, fourth
ventricle; LV, lateral ventricle; PV, periventricular region including ependyma;
VW, ventricular wall; WM, white matter.

a +, staining with all 3 antichlamydial antibodies was positive.
b Degree of inflammation seen on hematoxylin-eosin sections: 1, mild; 2, moderate; 3, marked; 4, severe.
c Represents 2 separate regions of frontal horn.

Table 3. Comparison of immunohistochemical (IHC) staining
and polymerase chain reaction (PCR) analysis for presence of
*Chlamydia pneumoniae* DNA in patients with other neurological
diseases (OND controls).

<table>
<thead>
<tr>
<th>OND control</th>
<th>Diagnosis</th>
<th>Location</th>
<th>MOMP PCR</th>
<th>16s RNA PCR</th>
<th>Formalin-fixed tissue IHC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PD</td>
<td>PV</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>PD</td>
<td>PV</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>Anemia</td>
<td>PV-WM</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>AD</td>
<td>F-PV</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>BMF</td>
<td>CBL</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>MSA</td>
<td>WM</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>ALS</td>
<td>SC</td>
<td>+</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>Acute MI</td>
<td>PVR</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>CA lung</td>
<td>PV</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>10</td>
<td>DD</td>
<td>WM</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>11</td>
<td>VE</td>
<td>WM</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>12</td>
<td>PD</td>
<td>TL</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>13</td>
<td>AD</td>
<td>TL</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>14</td>
<td>AD, PD</td>
<td>TL</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>15</td>
<td>PD</td>
<td>TL</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>16</td>
<td>MSA</td>
<td>PV</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>17</td>
<td>PD</td>
<td>PV</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>18</td>
<td>AD</td>
<td>PV</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>
| NOTE. PCR assays and IHC studies on the same tissue were possible for
OND controls 1–4; additional frozen tissue was obtained from archived speci-
mens at Vanderbilt Medical Center (OND controls 5–18). AD, Alzheimer disease;
ALS, amyotrophic lateral sclerosis; BMF, bone marrow failure; CA, carcinoma;
CBL, cerebellum; DD, Devic disease; F-PV, frontal-PV; FR, frontal region; MI,
myocardial infarction; MSA, multisystem atrophy; ND, not done; PD, Parkinson
disease; PV, periventricular region including ependyma; PVR, PV region; SC, spinal
cord; TL, temporal lobe; VE, viral encephalitis; WM, white matter.

a −, no staining for chlamydial antigen seen with monoclonal antibody 807
and CF-2 and anti-hap60 antibodies.
Figure 1  A, Immunohistochemical (IHC) staining of the brain periventricular region from 2 patients with multiple sclerosis (left [20×] and middle [100×]) and from a control individual with other neurological disease (right [20×]), by use of monoclonal antibody (MAb) 807. Note the granular staining of cytoplasm of ependyma. B, IHC staining of a mouse lung infected with Chlamydia pneumoniae, by use of MAb 807 (left) and isotype control antibody (right). Note the granular staining of C. pneumoniae–infected cells.

The extracted DNA (5 μL) in extraction buffer was added to 10× PCR buffer, 1.5 mmol/L MgCl₂, 200 μmol/L dNTP, 25 pmol of each primer, and 1 U of AmpliTaq polymerase (Roche), in a volume of 25 μL. DNA amplification was performed using a touchdown/nested PCR technique. After a preheating cycle at 94°C for 5 min, amplification of the outside product was performed first by use of a touchdown technique in which the annealing temperature was reduced 0.5°C every cycle, from 58°C to 48°C, and further amplification was performed for 30 cycles at 48°C, followed by an extension of the product at 72°C for 5 min. For amplification of 16s ribosomal DNA, the annealing temperature during the touchdown phase was decreased from 60°C to 50°C. The nested amplification consisted of a denaturation step at 94°C for 45 s, an annealing stage for 45 s (58°C for MOMP and 60°C for 16s ribosomal DNA, respectively), and an extension phase at 72°C for 1 min. The PCR product was run on a 1.5% agarose gel and stained with ethidium bromide. CSF or water spiked with 0.4 inclusion-forming units (IFUs) of C. pneumoniae elementary bodies was used as a positive internal control.

RESULTS

IHC staining of chlamydial antigens in the CNS. We examined 5-μm sections of 30 paraffin blocks from 20 MS cases and 25 blocks from 17 OND controls, using IHC techniques for identifying the presence of C. pneumoniae antigens (a representative selection of cases for which both PCR and IHC techniques were performed is shown in tables 2 and 3). Staining with all 3 antichlamydial antibodies and the respective control antibodies was performed. The criterion used to conclude that a particular section was positive was confirmation of antibody staining with all 3 antichlamydial antibodies (figures 1A and 2). The specificity of staining was established by performing IHC studies on mouse lungs infected with C. pneumoniae (figure 1B). Among the 20 MS cases, 7 showed staining with all 3 antichlamydial antibodies. Staining was repeated at least 3 times, and similar results were obtained. In 1 MS case, prominent staining for MAb 807 was seen in a tuft of ependyma, but staining with CF-2 and anti-hsp60 antibodies was not performed. In the sections showing positive staining, the antibody staining was seen in some but not all ependymal cells of the ventricular wall. In the positively staining cells, the staining showed a granular pattern (figures 1 and 2). In some sections, the staining appeared to be intranuclear (figure 2), but, since chlamydial antigens are not known to be
Figure 2. Six panels showing immunohistochemical (IHC) staining of ependyma from patient 1 with multiple sclerosis (table 2), stained with CF-2 antibody (upper left), anti-hsp60 antibody (upper middle), and monoclonal antibody (MAb) 807 (upper right). Their respective isotype-matched antibody controls are shown in the lower panels. The staining in these sections shows the signal as arising in either the nuclear or perinuclear region of the cell. In the panel showing staining with MAb 807, weak, diffuse staining is seen in some of the subependymal cells (20×).

Figure 3. Staining of perivascular cells in the subependymal layer with CF-2 antibodies (left) and isotype-matched control antibody (right) (20×).

present in the nucleus, we interpret these findings as indicating that the perinuclear location of chlamydial antigens gives a false appearance of intranuclear localization. Along with staining of ependymal cells, staining was also seen in glial cells and perivascular cells in the subependymal layer (figures 3 and 4). Although many of the parenchymal regions of the white matter showed presence of an active inflammatory response, with rare exception these regions lacked staining with any of the antichlamydial antibodies (figure 5). In the OND controls, we did not detect staining of CNS tissue with any of the antichlamydial antibodies.

Identification of C. pneumoniae DNA by PCR in frozen CNS tissue of MS cases and OND controls. Frozen tissue from 8 MS cases and 18 OND controls were available for analysis. Of these, 5 MS cases and 4 OND controls were also examined by IHC staining (tables 2 and 3). Nested PCR was performed using 2 different primers. Amplification of MOMP and 16s RNA DNA was possible with the frozen tissue from 5 of 8 MS cases. Three of these cases were also positive for chlamydial antigens by IHC staining (P<.05, vs. OND controls). MS case 2 (table 2) was positive by IHC staining, but a PCR signal was detected only with MOMP primers. For MS case 8 (table 2), tissue was positive by PCR, but formalin-fixed tissue was not available for IHC staining. We also studied 18 OND controls in a similar manner. These included OND controls 1–4 (table 3), for whom IHC staining with antichlamydial antibodies were performed.

Of the 18 OND controls studied, 3 were positive by PCR with both primers (table 3). These included 1 with Alzheimer disease, another with multisystem atrophy, and a third with Devic disease. Three controls—1 with amyotrophic lateral sclerosis, another with a diagnosis of Parkinson disease and Alzheimer disease, and a third with bone marrow failure—were positive by PCR after amplification of the MOMP gene alone. Interestingly, 1 of the controls with Alzheimer disease was positive by PCR in the cerebellum.
Immunohistochemical staining of periventricular and subependymal regions with anti-hsp60 antibody. Note the prominent staining of ependymal cells and subependymal cells (glial cells; indicated with arrows) with the antibody (40×).

**Immunogold staining of CSF sediment with antichlamydial antibodies and the detection of a PCR signal for C. pneumoniae DNA in CSF.** Our observation of chlamydial antigens in close proximity to the ventricular system and the presence of *C. pneumoniae* DNA in CSF from MS cases suggested that a direct examination of chlamydial elementary bodies in CSF may be productive. Eleven of 20 MS cases had ≥4 cells in 5 squares stained with MAb 807. In contrast, CSF sediment from only 2 of 12 OND controls was positive. One of these 2 had a diagnosis of acute disseminated encephalomyelitis (ADEM), and the other had an as-yet-undiagnosed CNS disease, with abnormal lesions found on magnetic resonance imaging in the CNS white matter (*P* < .025, vs. OND controls) (figure 6). Many of the electron-dense bodies showed a morphology that was either oval or pear shaped, similar to that seen in the positive controls (figure 7).

We next explored whether the immunogold staining for *C. pneumoniae* in CSF sediment was correlated with a PCR assay for the presence of *C. pneumoniae* MOMP DNA obtained from the same aliquot of CSF (table 4). Of the 20 MS cases, 12 (60%) were positive by PCR for the *C. pneumoniae* MOMP gene. One (MS case 12; table 4) did not show IHC staining of CSF sediment but was PCR positive for MOMP DNA. Of the OND controls, 1 with ADEM (OND control 5; table 4) was found by PCR to be positive for the *C. pneumoniae* MOMP gene, and the remaining 11 were negative (*P* < .02, MS cases vs. OND controls). These studies provide evidence that immunogold-stained electron-dense bodies resembling elementary bodies of *C. pneumoniae* can be detected in CSF from MS cases with greater frequency than in CSF from OND controls, and these observations were correlated with the results of accompanying PCR studies performed with CSF obtained at the same time.

**Direct EM study of CSF sediment from MS cases.** CSF sediment from another set of 10 MS cases and 5 OND controls was examined ultrastructurally. Structures resembling chlamydial bodies were found to be present in samples of CSF sediment from 4 MS cases but not in those from any of the OND controls (figure 8). The diameter of these bodies ranged from 0.25 to 0.70 µm, and the outer membrane showed a trilaminar organization. Furthermore, the surfaces of the outer membrane had knoblike projections that were similar to those described in scanning EM studies of other chlamydial species (figure 8) [37–39]. These bodies were not found in any of the CSF sediment from OND controls.

**DISCUSSION**

The studies reported here show evidence of the presence of chlamydial antigens in postmortem brain tissue samples from MS cases. Structures that resemble elementary bodies of chlamydial organisms were observed in CSF from MS cases, and IHC studies demonstrated localization of the chlamydial antigens in the ventricular wall and subependymal regions in MS cases but not in OND controls. Parallel studies performed using PCR techniques with CNS tissue showed that the *C. pneumoniae* DNA was found in the same regions. CSF analysis showed the presence of *C. pneumoniae* MOMP DNA in CSF from MS cases more commonly than in CSF from OND controls. It would have been ideal to demonstrate the presence of chlamydial organisms in the CNS by all available methods. However, attempts to demonstrate the presence of chlamydial DNA by use of in situ hybridization and PCR assays on formalin-fixed brain tissue were technically unsuccessful.

IHC staining of paraffin-fixed postmortem brain tissue from MS cases demonstrated specificity as indicated by the similarity in quality and localization of staining with all 3 antibodies (MAb 807, CF-2, and anti-hsp60). The morphological characteristics of the staining were also similar to those published elsewhere showing the presence of *C. pneumoniae* in vascular tissue [3, 40–42]. These antibodies recognize chlamydial antigens that are genus specific but not species specific and are therefore not specific for *C. pneumoniae*; however, since the accompanying PCR studies were performed with primers specific for *C. pneumoniae*, it is likely that the antigens found in the brains are those of *C. pneumoniae*. Although monoclonal antibodies RR402 and TT401, which are specific for *C. pneumoniae*, are available, IHC staining with these antibodies was
inconsistent and required high concentrations (dilutions of 1:2 or 1:5). Although other studies have shown the presence of *C. pneumoniae* antigens in the brains of patients with Alzheimer disease, we did not see staining with our panel of antibodies in brain tissue from the patient with Alzheimer disease included in the present study [10]. However, we performed IHC staining in the periventricular regions but not in the gray matter, and that might account for the discrepancy.

Since we were not able to extract nondegraded DNA from paraffin-embedded tissue, DNA for PCR analysis was obtained from frozen brain tissue in periventricular regions that had been demonstrated to be positive for chlamydial antigens by IHC staining. Although *C. pneumoniae* was not exclusively present in MS brain tissue, it was identified in more MS cases than OND controls. The morphological characteristics of the bodies identified by ultrastructural examination of CSF in this study resemble structures of *Chlamydia* species. These bodies have a bilaminar membrane and show arrays of surface projections that are virtually identical in appearance to those that have been observed in *C. trachomatis* and *C. psittacci* [37, 39].

It is important to point out that we did not identify any IHC staining in regions of active inflammation. This is an intriguing and puzzling observation, since we had anticipated that antigenic evidence of the pathogen’s presence would be seen in close proximity to inflammation. We assumed that migration of monocytes infected with *C. pneumoniae* would represent a conduit for its entry into the CNS and, hence, that its presence would be seen in perivascular regions. In some diseases of the CNS that are well recognized as being infectious, the route of entry of the pathogen is not always clear [43, 44].

![Figure 5](image1.png) **Figure 5.** Lack of staining with CF-2 antibodies in areas of inflammation. *Upper left,* Periventricular region stained with Luxol fast blue, showing areas of inflammation and the demyelination in the periventricular regions (2×). *Upper right,* Same region stained with anti–CF-2 antibody and counterstained with hemotoxylin (2×). *Lower left,* Staining of ependymal cells with anti–CF-2 antibody (10×). *Lower right,* Lack of staining of perivascular lymphocytes with anti–CF-2 antibody (20×).

![Figure 6](image2.png) **Figure 6.** Scatter graph showing the number of immunogold-staining bodies in the cerebrospinal fluid sediment from 20 patients with multiple sclerosis (MS) and 12 control individuals with other neurological disease (OND).
IHC staining for chlamydial antigens in the ependyma and in the subependymal regions may suggest an alternate route of entry. We suggest that the circumventricular organs may provide a route of entry for infected mononuclear cells into the CNS. These regions contain loosely structured vascular tissue and compose the area postrema: the caudal region of the fourth ventricle, median eminence, subcomissural organ, and subformricular organ [45, 46]. They all lie close to the ventricular space, and the capillary blood vessels in these regions lack tight junctions and astrocytic foot processes and, hence, do not exhibit the restrictions in trafficking imposed by the blood-brain barrier. Also, the overlying ependyma lack cilia and resemble subependymal glial cells. Studies have shown that, although these regions are small in area, they are sufficient to allow for the migration of compounds that would otherwise be restricted to the vascular compartment [47].

Ependymal cells in MS cases sometimes show features of inflammatory injury. In a series of 129 cases, Adams noted active ependymitis in 11% of MS periventricular lesions, characterized by ependymal granulations [48]. In other regions of the ventricle, chronic ependymal reaction was seen, including flattened ependymal cells and subependymal gliotic nodules [49]. It is therefore conceivable that ependymal and circumventricular organs may be the site of involvement in CNS inflammation, most notably that associated with MS. The absence of the organism in parenchymal regions of active inflammation suggests that infection of CNS tissue may be an epiphenomenon. However, there are a number of chronic diseases in which inflammation is seen in the absence of an abundance of pathogen. In tuberculoid leprosy, granuloma formations are seen in regions in which the bacilli are either absent or few in number [50]. Paucity of organisms is also seen in CNS syphilis and CNS Lyme disease. The inflammatory response may have more

Table 4. Polymerase chain reaction (PCR) assay for Chlamydia pneumoniae major outer membrane protein (MOMP) DNA and parallel immunogold staining of cerebrospinal fluid (CSF) sediments from patients with multiple sclerosis (MS cases) and control individuals with other neurological disease (OND controls).

<table>
<thead>
<tr>
<th>Case/control (diagnosis)</th>
<th>Positive immunogold-labeled cells in 5 grids, no.</th>
<th>CSF PCR for C. pneumoniae MOMP DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS cases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>14</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>−</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>−</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>3</td>
<td>−</td>
</tr>
<tr>
<td>19</td>
<td>7</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>OND controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (paresthesia)</td>
<td>2</td>
<td>−</td>
</tr>
<tr>
<td>2 (fibromyalgia, MS RO)</td>
<td>4</td>
<td>−</td>
</tr>
<tr>
<td>3 (encephalopathy)</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>4 (MS RO)</td>
<td>3</td>
<td>−</td>
</tr>
<tr>
<td>5 (ADEM)</td>
<td>7</td>
<td>+</td>
</tr>
<tr>
<td>6 (encephalopathy)</td>
<td>2</td>
<td>−</td>
</tr>
<tr>
<td>7 (MS RO)</td>
<td>1</td>
<td>−</td>
</tr>
<tr>
<td>8 (APL syndrome)</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>9 (pseudotumor)</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>10 (paresthesia)</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>11 (MS RO)</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>12 (headache)</td>
<td>2</td>
<td>−</td>
</tr>
</tbody>
</table>

**NOTE.** ADEM, acute disseminated encephalomyelitis; APL, antiphospholipid antibody syndrome; CRF, chronic renal failure; MOMP, major outer membrane protein; RO, ruled out.
to do with the nature of the host response to the inciting pathogen than with the number or the proximity of the pathogen to the areas of tissue injury [51, 52].

The studies presented here, along with our previous PCR studies of the presence of Chlamydia pneumoniae DNA in CSF from a subset of MS cases, suggest an association between this pathogen and disease. These studies do not provide evidence of a causal role between C. pneumoniae infection and MS; however, chlamydial antigens may be an infectious “trigger” that initiates or leads to autoimmunity. Molecular mimicry between Chlamydia pneumoniae antigens and myelin antigens has been recognized; immunization of animals with these cross-reactive antigens is sufficient to induce autoimmune disease [53, 54]. Alternatively, the infection may act as a secondary invader in host tissue, accentuating an ongoing inflammatory response [55].

Acknowledgments

We thank Anthony Tharp for technical assistance with the electron microscopy studies. We also thank Camilla Dietz Bergeron, Thomas West, Paul Griffin, Steve Smith, and the family of C. J. Schueler, for their support.

References

22. Owens T. The enigma of multiple sclerosis: inflammation and neuro-


