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Is *Chlamydia pneumoniae* Present in Brain Lesions of Patients with Multiple Sclerosis?

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We investigated the presence of *Chlamydia pneumoniae* in 81 normal and pathological specimens obtained from postmortem brain tissues of patients with multiple sclerosis and with other neurological or nonneurological diseases. The assays used included PCR amplification of all DNA samples in the initial study. Culture and a second PCR amplification of the organism in a subset of 19 brain specimens were also performed in two separate laboratories. All results were negative. Thus, this study on a large number of brain tissues suggests that *C. pneumoniae* is not involved in inflammatory demyelination.

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system. The immune attack primarily affects myelin, the insulation that protects nerve fibers and promotes impulse transmission, and/or oligodendrocytes that produce myelin. The clinical manifestations of the disease are highly variable and include impaired vision; abnormalities in the motor, sensory, and coordination systems; autonomous disturbances; and, occasionally, cognitive dysfunctions. About 85% of patients initially present with a relapsing-remitting course, which transforms into a secondary progressive course in most of these patients in a matter of years. A smaller subgroup of patients presents with a primary progressive form, which usually results in a more rapid accumulation of disability than the other forms of the disease. The etiology of MS is unknown, but numerous infectious agents have been considered as potential causes, including measles virus; herpesviruses, such as Epstein-Barr virus and human herpesvirus 6; human retroviruses (especially human T-cell leukemia virus type 1); JC polyoma virus; and *Borrelia burgdorferi*. Recently, *Chlamydia pneumoniae* has been reported as identified in the cerebrospinal fluid (CSF) of patients with MS by culture and PCR (16, 17). However, subsequent studies have yielded conflicting results (3, 7, 8, 12, 19). Sriram et al. (17) demonstrated the existence of *C. pneumoniae* in the CSF of over 90% of their patients by culture and PCR and by detection of specific immunoglobulins. Treib et al. (19) and Layh-Schmitt et al. (7) identified this organism, also by PCR, in the CSF of approximately 50% of their patients with MS. In contrast, Boman et al. (3) and Poland and Rice (12), also using culture and PCR, did not identify *C. pneumoniae* in the CSF or in the CSF, peripheral blood mononuclear cells, or autopsied brain tissues of their MS patients. To further complicate the issue, Li et al. (8) reported finding *C. pneumoniae* DNA in a high percentage of CSFs from both patients with MS and controls. To obtain further evidence for or against the involvement of *C. pneumoniae* in inflammatory demyelination, we tested the brain tissues of MS patients, nonneurological-disease controls (NNC), and other-neurological-disease controls (ONDC) for the presence of the organism. This report presents a detailed account of our findings in order to underline the importance of methodological issues.

(A part of our observations has been summarized previously [Z. Ke, F. Lu, P. Roblin, M. R. Hammerschlag, and B. Kalman, Letter, Ann. Neurol. **48:**400, 2000].)

Brain tissues of MS patients and controls were obtained from the Rocky Mountain MS Center Tissue Bank, Denver, Colo., and from the National Neurological Research Specimen Bank, Los Angeles, Calif. Altogether, 55 brain specimens from 25 patients with relapsing-remitting, secondary progressive, or primary progressive MS were studied. These samples included 9 triplets of corresponding normal-appearing white matter (NAWM) and chronic active plaque and cortical tissues, 11 pairs of NAWM and chronic active plaque tissues, 1 pair of chronic active plaque and cortex, and 4 solitary cortical tissues. Normal-appearing and pathological tissues were selected by gross examination. Small plaques, microglial proliferation, and perivascular or parenchymal infiltration by mononuclear cells within NAWM specimens were excluded after microscopic examination of cryosections using Luxol Fast blue, oil red O, and EBM11 staining. Plaques were similarly identified by microscopic examination. A chronic active plaque was defined by the presence of inflammatory activity and hypercellularity around regions showing demyelination, oligodendrocyte loss, and some degree of astrogliosis. We obtained 21 specimens from 11 NNC patients whose brains were pathology free (most of them died from traffic accidents, two of them suffered from lung cancer, and one patient was positive for α-1 antitrypsin). Both WM and cortical specimens were available from 10 of these patients, while only WM was received from 1 NNC patient. We obtained solitary cortical or WM tissues from five ONDC patients (one with herpes simplex encephalitis, one with dementia with nephrotic syndrome, one with vascular dementia, and two with acute disseminated encephalomyelitis). Patients ranged between 17 and 58 years of age (with the exception of one younger NNC patient), and brain tissues were frozen within 15 h (2 to 5 h in the case of most MS patients) of clinical death. All tissues were kept at −70°C until used. Cells collected from the synovial fluids of eight patients with rheumatoid arthritis were also included in the study. *C. pneumoniae*-infected HEp-2 cells (CDC/CWL-029; American Type Culture Collection) and *C. pneumoniae* organisms isolated

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from the supernatant of infected cells were used as positive controls.

Total DNA was extracted from the 81 brain samples by using the QIAamp tissue and blood kit (Qiagen, Valencia, Calif.). These samples had been used in previous studies which demonstrated a high quality of both mitochondrial and nuclear DNA and a lack of inhibitors of *Taq* DNA polymerase in PCRs. In this study, two sets of PCR amplifications were performed using nested primers. In the first PCR we used the same conditions and primers (specific for the major outer membrane protein [MOMP] gene of *C. pneumoniae*; sense, nucleotides [nt] 1 to 38; antisense, nt 1170 to 1131) as described by Sriram et al. (17). Nine fivefold serial dilutions of DNA from the infected HEp-2 cells (ranging from 0.76 g to 0.19 pg) and from the purified bacteria (ranging from 5×10^5 to 1.3 organisms) were prepared and amplified in 36 cycles. Similarly, 0.2 g of DNA from MS patients and controls was amplified in 36 cycles (17). In a second set of experiments, all the PCR products derived from brain tissues were reamplified with nested primers of the MOMP gene (sense, nt 135 to 154; antisense, 1053 to 1072), in 20 cycles of 95, 56, and 72°C.

Samples of brain tissue were selected from 12 MS, 5 NNC, and 2 ONDC patients of the above cohort and were shipped on dry ice to the Chlamydia Research Laboratory, Department of Pediatrics, Division of Infectious Diseases, SUNY Health Science Center at Brooklyn, Brooklyn, N.Y., for culture of *C. pneumoniae*. The tissue samples were thawed and minced in sterile petri dishes and divided into two equal portions. One portion of each brain tissue sample was further homogenized with tissue grinders in Iscove's Dulbecco modified Eagle medium and sonication. The brain homogenates were then centrifuged at $500 \times g$ for 10 min at 4°C to remove coarse cellular debris. Supernatants were then diluted serially 10-fold to 10^{-5}, and 200 μl of each dilution was inoculated onto 4 wells of HEp-2 cells grown in 96-well microtiter plates (14). Each sample was passaged four times. Culture confirmation was performed by staining with a *C. pneumoniae*-specific, fluorescein-conjugated monoclonal antibody (14).

The second portion was sent to the laboratory of Jens Boman, Department of Virology, Umeå University, Umeå, Sweden, for testing with a second nested PCR using *C. pneumoniae*-specific primers as described by Tong and Sillis (18) (for MOMP gene external PCR, the sense primer was nt 61 to 80 and the antisense primer was nt 373 to 393; for internal PCR, the sense primer was nt 100 to 120 and the antisense primer was nt 286 to 306). These primers target a different part of the MOMP gene than those used in the Philadelphia laboratory. DNA extraction was performed using the QIAamp DNA minikit in accordance with the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). The investigators in Brooklyn and Sweden were blinded as to the identity of the samples.

In the first PCR, we detected the expected 1.2-kb fragment of the MOMP gene by agarose gel electrophoresis in as few as 800 copies of the purified bacteria, corresponding to 120 pg of total DNA of the *C. pneumoniae*-infected cells. All of the specimens, including 81 brain tissue samples of MS, NNC, and ONDC patients as well as synovial cells from eight arthritis patients, were found to be negative. In the second amplification with the nested primers, we were able to detect as few as six bacterial organisms in the positive control, corresponding to 0.96 pg of total DNA extracted from *C. pneumoniae*-infected cells. Nevertheless, all the brain tissue samples were negative.

All of the 19 brain specimens submitted for culture of *C. pneumoniae* were negative after four passages in HEp-2 cells. The second nested PCR assay of the homogenates of these 19 specimens performed at Umeå University showed them all to be negative.

Although *C. pneumoniae* is an accepted cause of respiratory disease, studies have also implicated the organism as a cause of a number of chronic diseases. The presence of this organism has been identified in foam cells, macrophages and extracellular matrix in arteriosclerotic plaques (2), and synovial cells of some patients with arthritis (15), predominantly by nonculture methods including PCR and immunohistochemical staining. Since the original report by Sriram et al. (16), there has been interest in the possible role of *C. pneumoniae* in neurological diseases. Although one study reported identifying the organism in pericytes, microglia, and astrocytes in brains of patients with Alzheimer's disease (AD) by culture and PCR (1), three subsequent studies from the United States and Europe, using the same or similar methods, did not find *C. pneumoniae* in brains of AD patients (5, 11, 13). The experience with AD is strikingly similar to that discussed here for MS (3, 7, 8, 12, 16, 17, 19; Ke et al., Letter.)

The most obvious explanation for this lack of consistency lies in the methodological approach. It is important to emphasize that there are no standardized PCR assays for the detection of *C. pneumoniae* in respiratory specimens or tissue. Analytical sensitivity does not predict the ability of an assay to detect *C. pneumoniae* in clinical specimens, as was demonstrated recently in a study of peripheral blood mononuclear cells (10). However, our negative PCR results did not appear to be related to technical issues. The quality of both nuclear and cytoplasmic DNA in the samples was excellent. Previous amplifications of mitochondrial DNA in most of the specimens demonstrated that there were no inhibitors of *Taq* polymerase. In order to determine if PCR inhibition was the explanation for the negative PCR results for CSF samples of patients with MS (3), we performed spiking experiments, i.e., we added low concentrations of *C. pneumoniae* bacteria in parallel to pure water and to CSF collected from patients with MS and then performed DNA extraction. These experiments showed that potential PCR inhibitors in the CSF are efficiently removed using the QIAamp DNA extraction procedure, since the levels of sensitivity were identical when *C. pneumoniae* organisms were diluted in water and in CSF. The DNA extraction method used in this and a previous study on MS (3) seems to be useful for the isolation of DNA from microorganisms in body fluids in order to inactivate nucleases, remove nonspecific inhibitors, and concentrate nucleic acids (4, 9). Further, only weakly positive PCR controls were used in the study in order to minimize the risk of contamination and to ensure that the sensitivity of the PCR always was high. In addition, the cultures for this study were performed in the laboratory that has developed the most commonly used technique worldwide for culturing *C. pneumoniae*. This culture technique has been rigorously validated and has been proven to be of high sensitivity and specificity (14).

If *C. pneumoniae* is involved in the pathogenesis of MS, it is very likely that serological analyses should demonstrate reduced serum/CSF ratios of *C. pneumoniae*-specific antibodies (6) due to local intrathecal production of immunoglobulins as a result of the central nervous system infection supposedly caused by *C. pneumoniae*. Such analyses were not performed in the present study. However, using a validated *C. pneumoniae*-specific microimmunofluorescence antibody test in an earlier study, it was not possible to demonstrate local intrathecal production of antibodies to *C. pneumoniae* in patients with MS or with ONDs (3).

In conclusion, our studies on brain tissues do not confirm the recent identification of *C. pneumoniae* in the CSF of MS pa-

tients (7, 16, 17, 19). Nevertheless, these contrasting findings in the brain and CSF are not exclusive of other possibilities. *C. pneumoniae* may get through the blood-CSF barrier or can be carried by infected mononuclear cells into the CSF without the infection of central nervous system cells per se. However, at least one published study also failed to identify *C. pneumoniae* in the CSF of patients with MS using validated, sensitive PCR and culture techniques (3). Based on our studies, we suggest that either the infection level of *C. pneumoniae* in MS brains is below the sensitivity of applied techniques or the organism is not present in the tissues studied. To our knowledge, no histological or molecular study has demonstrated *C. pneumoniae*-like organisms in the brains of patients with MS. These conflicting observations, appearing in increasing numbers not only in the MS literature but also in the literature of AD, rheumatoid arthritis, and atherosclerosis, reflect the existence of methodological difficulties which urgently require a solution.

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