Brief Definitive Report

Chlamydia pneumoniae Infection of the Central Nervous System Worsens Experimental Allergic Encephalitis

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Abstract

Experimental allergic encephalitis (EAE) is considered by many to be a model for human multiple sclerosis. Intraperitoneal inoculation of mice with *Chlamydia pneumoniae*, after immunization with neural antigens, increased the severity of EAE. Accentuation of EAE required live infectious *C. pneumoniae*, and the severity of the disease was attenuated with antiinfective therapy. After immunization with neural antigens, systemic infection with *C. pneumoniae* led to the dissemination of the organism into the central nervous system (CNS) in mice with accentuated EAE. Inoculation with *Chlamydia trachomatis* did not worsen EAE and infectious organisms were not seen in the CNS. These observations suggest that dissemination of *C. pneumoniae* results in localized infection in CNS tissues in animals with EAE. We propose that infection of the CNS by *C. pneumoniae* can amplify the autoreactive pool of lymphocytes and regulate the expression of an autoimmune disease.

Key words: Chlamydia • autoimmunity • multiple sclerosis • demyelination • bystander activation

Introduction

Chlamydia pneumoniae belongs to a family of intracellular organisms that typically causes a self-limiting respiratory infection (1). More recently, *C. pneumoniae* has been linked to a number of chronic human diseases including those that involve the central nervous system (CNS; reference 2). Infections are known to play a role in the development and progression of a number of autoimmune diseases, and we have suggested that multiple sclerosis (MS) may be linked to infections with *C. pneumoniae* (3–7).

Although the etiology of MS is not known, clinical and pathologic observations suggest a close interplay between an infectious agent(s) and an autoimmune response to myelin antigens in the development of the disease (8, 9). Epidemiologic studies have implicated environmental factors and most likely infectious agents as a necessary element in the development of MS (10). The autoimmune basis for MS stems from similarities between MS and the animal model, EAE (11). In view of the possible association between chlamydial infection and the development of MS, we examined the effect of systemic infection of *C. pneumoniae* on the development and progression of EAE.

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Materials and Methods

Animals and Reagents. Female SJL/J and C57BL/6 mice were purchased from The Jackson Laboratory. Guinea pig myelin basic protein (MBP) and mouse spinal cord homogenate was prepared as described previously and myelin oligodendrocyte protein (MOG) peptide (p35–55: MEVGWYRSPFSRVVH-LYRNGK) was synthesized by Genemed Synthesis, Inc. Fluorophenicol was a gift from Dr. F. DeGraves, University of Auburn, Auburn, AL. IFN-γ was measured by a commercial kit obtained from R&D Systems. Concentrated *C. pneumoniae* elementary bodies were obtained by growing *C. pneumoniae* (VR-1310; American Type Culture Collection) and *C. trachomatis* (HAR13; American Type Culture Collection) on a monolayer of HL cells. The number of infectious forming units of chlamydial bodies were estimated using the HL indicator cell line. The GD-11 strain of *S. flexnerii*, a gift from J. Bright (Vanderbilt Medical Center, Nashville, TN), was grown in soft agar. Heat killed *C. pneumoniae* was prepared by boiling the organism in a water bath for 5 min.

Lymphocyte Proliferation Assay. Lymphocytes isolated from draining lymph node cells were cultured in RPMI 1640 complete medium in a 96-well microtiter plate under the atmosphere of 5% CO_2 and 95% air at 37°C, and the proliferation assay was done as described previously (12).

Induction and Evaluation of EAE. Active and adoptively transferred EAE and the clinical scoring of paralyzed mice was done as described previously (12, 13). To examine the effect of chlamydial infection on EAE, mice were injected with live *C. pneumoniae* or *C. trachomatis* organisms. 0.5×10^6 infectious units (in 0.5 ml of PBS) intraperitoneally, 7 d after receiving the first immunization.

Immunohistochemical Localization of Chlamydial Antigens in CNS Tissue of Mice with EAE. The spinal cords were isolated from the mice after perfusion with 4% paraformaldehyde in PBS on day 18, fixed in 10% formalin, and embedded in paraffin. The presence with *C. pneumoniae* antigens in the sections was detected by immunohistochemistry using anti-chlamydial LPS antibody (mAB 807; Chemicon) following the protocol for the M.O.M. mouse blocking kit (Vector Laboratories). Using antigen retrieval techniques the spinal cord sections were incubated with mAB 807 (recognizes all chlamydial LPS), was added at a dilution of 1:750, and placed on a rocking platform overnight at 4°C.) The slides were then washed and biotin conjugated goat anti–mouse antibody was added and the color developed using the Envision kit (DakoCytomation). Anti *Escherichia coli* LPS antibody (Fitzgerald Industries) and isotype matched IgG2a antibodies (Sigma-Aldrich) were used as control antibodies in all the staining procedures.

Isolation of Total RNA and Semiquantitative RT-PCR of Chlamydial Antigens. After perfusion with PBS, spinal cords from mice were collected, and total RNA extracted using TRI Reagent (Sigma-Aldrich) in accordance with manufacturer's protocol. The following sense and antisense oligonucleotide PCR primers were used: *C. trachomatis* 16S RNA, 5′-ATT TGG GCA TCC GAG TAA CG (sense) and 5′ CCA CGC GGT ATT AAC CGT CT, *C. pneumoniae* 16S RNA, 5′-GCT AAT ACC GAA TGT AGT GTA A (sense) and 5′-ATC TAT CCT CTA GAA AGA TAG TT, and GAPDH, 5′-TGA AGG TCG GTG TGA ACG GAT TTG GC (sense) and 5′-CAT GTA GGC CAT GAG GTC CAC CAC. 4 μg of total RNA was reverse transcribed to cDNA using GeneAmp RNA PCR kit with oligo $d(T)_{16}$ primers (Roche). PCR amplification of each cDNA target was performed along with GAPDH, which served as an internal control for RNA quantity. Each PCR reaction contained 5 μl of cDNA, 2 μl of 10× PCR buffer (Perkin Elmer), 1 μl of 25 mM $MgCl_2$, 0.5 μl of each dNTP (10 mM), 0.5 μl of sense and antisense target gene-specific primers (50 pmole/μl), 0.25 μl Ampli-*Taq* DNA polymerase (5 U/μl) (PerkinElmer), and 13.75 μl of nuclease-free H_2O, and was performed in PTC-200 Peltier Thermal Cycler (MJ Research, Inc.). PCR products were resolved on 1.5% agarose in TAE containing 0.5 μg/ml of ethidium bromide and visualized under UV light.

Results

Worsening of EAE in Mice Infected with C. pneumoniae. *C. pneumoniae* infection in mice was induced by intraperitoneal inoculation of infectious elementary bodies after the second immunization with mouse spinal cord homogenate (MSCH). Control mice received equal numbers of infectious *C. trachomatis* or an equal number of colony forming units of *S. flexnerii* (GD-11 strain). As shown in Fig. 1 A, mice immunized with MSCH and injected with live *C. pneumoniae* had a mean maximal disease severity score of 3.1, while those that received PBS, *C. trachomatis* or *S. flexnerii*, had a mean maximal clinical score of 1.8, 2.1, and 1.2, respectively ($P < 0.05$). To determine if enhancement of EAE was specific to the immunogen, MSCH, we examined the effect of *C. pneumoniae* infection in MOGp35–55 induced EAE. The mean clinical score in mice that received live *C. pneumoniae* after immunization with MOG p35–55 was 2.3; in contrast, the mean clinical score in vehicle treated mice was 1.3, suggesting that the effect of *C. pneumoniae* infection on EAE was not specific to the immunizing antigen (Fig. 1 B). A comparison of viable (i.e., infectious) elementary bodies with heat-killed (i.e., noninfec-

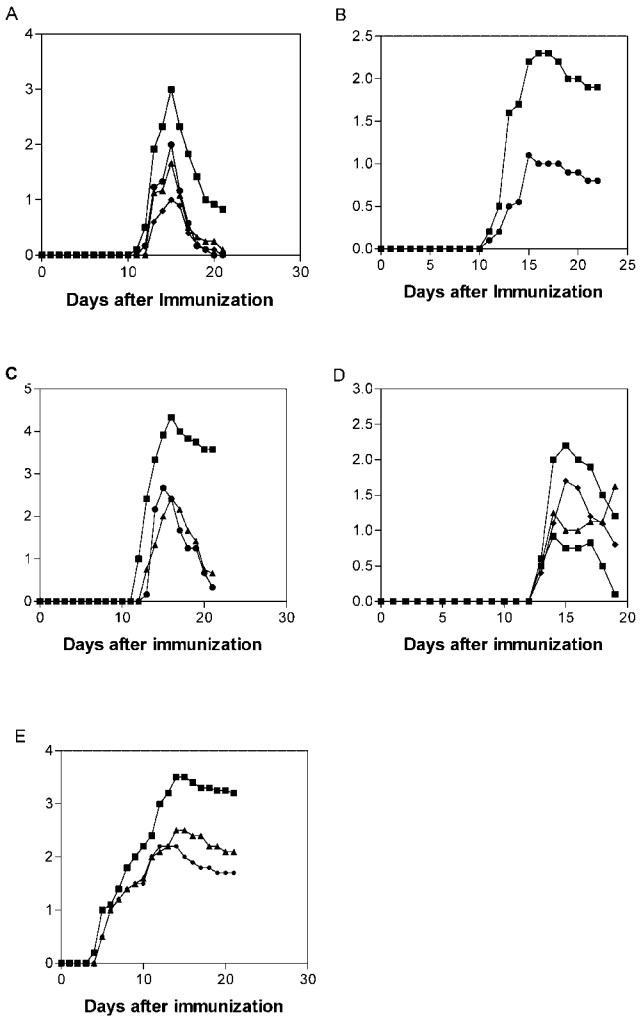

Figure 1. Effect of *C. pneumoniae* infection on the development of EAE. (A) Active EAE in SJL/J female mice was induced by immunization with MSCH in complete Freund's adjuvant. On day 7 animals were injected intraperitoneally with either vehicle (●, $n = 6$), live *C. pneumoniae* (■, $n = 6$) *C. trachomatis* (◆, $n = 6$) (0.5×10^6 IFU) or S. *flexnerii* (▲, $n = 6$) 0.5×10^6 colony forming units (*C. pneumoniae* vs. vehicle/*C. trachomatis*, $P < 0.05$). (B) Active EAE in C57BL/6 female mice was induced by immunization with MOG p35–55. On day 7, animals were injected intraperitoneally with either vehicle (●) or 0.5×10^6 IFU of live *C. pneumoniae* (■) (*C. pneumoniae* vs vehicle, $P < 0.05$; $n = 5$). (C) Active EAE in SJL/J female mice was induced as described in (A), and infected with either vehicle (●) or 0.5×10^6 IFU of live (■) or heat-killed *C. pneumoniae* (▲) (live vs vehicle/heat-killed, $P < 0.01$; $n = 6$). (D) Active EAE in SJL/J female mice was induced as described in (A), and infected with various dose of live *C. pneumoniae*: 0 IFU (●), 5×10^3 IFU (▲), 5×10^4 (◆) and 5×10^5 IFU (■) ($n = 6$). (E) Passive EAE in SJL/J mice following adoptive transfer of MBP-reactive lymphocytes. The animals received either live *C. pneumoniae* (■), heat killed *C. pneumoniae* (▲), or vehicle alone (●), (live vs vehicle/heat-killed, $P < 0.05$; $n = 10$). Concentrated *C. pneumoniae* elementary bodies are obtained by growing *C. pneumoniae* (VR-1310; American Type Culture Collection) and *C. trachomatis* (HAR13; American Type Culture Collection) on a monolayer of HL cells. Y axis represents mean clinical severity scores of EAE.

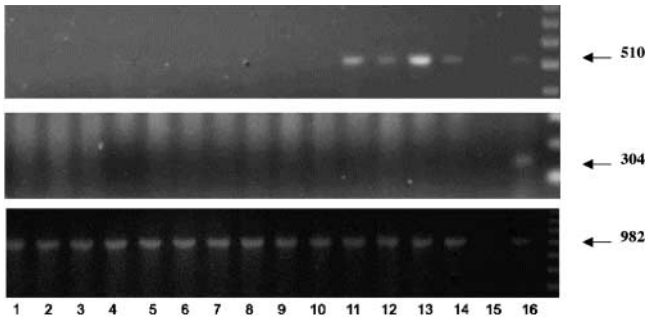

Figure 2. RT-PCR analysis of *C. pneumoniae* gene transcription in the CNS of mice with EAE. Mice were immunized with MSCH and infected with either *C. pneumoniae* or *C. trachomatis* as described in Fig. 1 A. Mice were killed on day 18 and total RNA was isolated from PBS-perfused spinal cords, and then analyzed for the presence of mRNA of *C. pneumoniae* 16S RNA gene by RT-PCR. Lanes 1–5, uninfected mice immunized with MSCH alone; lanes 6–10 mice infected with *C. trachomatis* and immunized with MSCH; lanes 11–14 mice infected with *C. pneumoniae* and immunized with MSCH. Lane 15 internal negative control, lane 16 internal positive control for each of the primers. Arrows indicate the size of PCR product. Top panel, RT-PCR performed with primers for 16SRNA of *C. pneumoniae*, middle panel RT-PCR performed with primers for 16SRNA of *C. trachomatis*, and bottom panel, GAPDH primers.

tious) elementary bodies revealed that worsening of EAE requires viable *C. pneumoniae* organisms (Fig. 1 C). Also, worsening of EAE was dependent upon the actual number of infectious elementary bodies in the intraperitoneal inoculum (Fig. 1 D). To establish whether the effect of *C. pneumoniae* infection on EAE was seen after T cell priming to neural antigens, we examined infection with *C. pneumoniae* after adoptive transfer of MBP reactive T cells. As shown in Fig. 1 E, mean clinical score of mice that received live *C. pneumoniae* and MBP primed lymphocytes was 3.6. In mice that received vehicle or heat killed *C. pneumoniae* the mean clinical scores were 2.6 and 2.4, respectively. The sum of these results show that live *C. pneumoniae* can worsen EAE induced by different neural antigens and also after priming of T cells.

Presence of Metabolically Active Elementary Bodies of C. pneumoniae in CNS of Mice with Worsening of EAE after Infection with Live C. pneumoniae. Three groups of mice (five/group) were immunized with MSCH in CFA and infected with either *C. pneumoniae* or *C. trachomatis* on day 7. The third group of mice was left untreated. 18 d after immunization, the mice were killed, and an RT-PCR analysis of RNA obtained from the spinal cord were performed. As shown in Fig. 2, all four mice from which RNA was available showed an RT-PCR signal for the presence of replicating *C. pneumoniae*. None of the mice infected with *C. trachomatis* showed a signal to primers that were specific for the *C. trachomatis* 16sRNA gene. These studies suggest that *C. pneumoniae* but not *C. trachomatis* is capable of infecting the CNS in mice with EAE.

To further prove that an inflammatory response in the brain was necessary in order to set an infection with *C. pneumoniae*, naive mice were infected with either *C. pneumoniae* or *C. trachomatis* and the presence of chlamydia in the CNS was examined. As shown in Table I, the RT-PCR signal for the 16S gene of *C. pneumoniae* and *C. trachomatis* was noted in the spleen, lung, and lymph nodes of naive mice, infected intraperitoneally with either *C. pneumoniae* or *C. trachomatis*. An RT-PCR signal for the 16S gene was not seen in the CNS in mice infected with either *C. trachomatis or C. pneumoniae* on days 3, 7, and 11 after infection.

Immunohistochemical Analysis for the Presence of C. pneumoniae in CNS of Mice with EAE and Infected with C. pneumoniae. To directly demonstrate the presence of *C. pneumoniae* in the CNS, spinal cords were obtained from mice which were infected with *C. pneumoniae* and induced to develop EAE. Spinal cords were stained with anti-chlamydial antibodies using standard immunohistochemical techniques. Intracellular staining of perivascular mononuclear and CNS parenchymal cells with anti-chlamydial antibodies was seen in at least one section of all four mice examined (Fig. 3, c and d). No staining of elementary bodies were seen in mice immunized with MSCH and inoculated with *C. trachomatis* (Fig. 3 b). The monoclonal antibody 807 recognizes LPS and MOMP antigens of both *C. trachomatis* and *C. pneumoniae*. No staining of chlamydial antigens was seen in mice with EAE and infected with *C. pneumoniae* after incubation with an irrelevant control antibody (anti–*E. coli* LPS antibody, unpublished data). These results corroborate our observations on RT-PCR and offer direct evidence of infection by *C. pneumoniae* of the CNS.

Treatment if EAE Mice Infected with C. pneumoniae with Fluorphenicol. If infection with *C. pneumoniae* directly affects the development of EAE we predicted that antibiotic therapy aimed at *C. pneumoniae* would attenuate the dis-

Table I. *Systemic Dissemination of C. pneumoniae and C. trachomatis after Intraperitoneal Injection of Naive Mice*

	D3 spleen	D3 LN	D3 lung	D3 brain	D7 spleen	D7 LN	D7 lung	D7 brain	D11 spleen	D11 lung	D11 brain
C. trachomatis	2/2	2/2	ND	0/2	2/2	2/2	ND	0/2	1/2	ND	0/2
C. pneumoniae	2/2	ND	2/2	0/2	0/2	ND	2/2	0/2	1/2	2/2	0/2

SJL/J mice were injected intraperitoneally (0.5×10^6) with either live *C. trachomatis* or *C. pneumoniae* organisms. Spleen, lung, mesenteric lymph nodes (LN), and brain were harvested on day 3, 7, and 11. RT-PCR for the presence of infectious organisms was performed using 16S primers for *C. pneumoniae* and *C. trachomatis*, respectively. ND, not done.

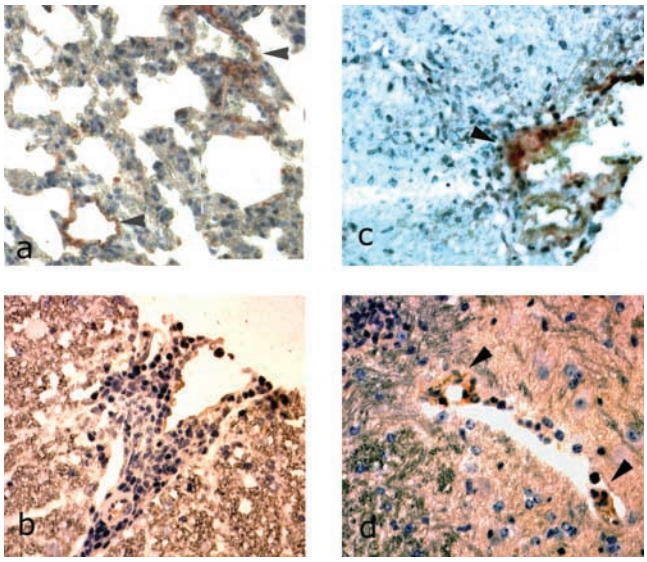

Figure 3. Immunohistochemical detection of chlamydial antigens in the spinal cords of mice with EAE. (a) Presence of chlamydial antigens in lung tissue from Balb/c mice, infected intranasally with *C. pneumoniae* and stained with Mab 807. (b) Representative areas of perivascular infiltration/inflammation in *C. trachomatis*-infected mice with EAE, showing no staining with Mab 807. (c and d) Representative areas of perivascular lymphocytic inflammation of *C. pneumoniae*-infected mice with EAE, indicating staining with Mab 807 (arrows).

ease. Treatment with Fluorophenicol reduced the severity of EAE. The mean maximal clinical score decreased from 2.3 in untreated mice to 1.4 in mice that received Fluorophenicol. The severity of EAE in antibiotic treated mice was similar to mice immunized with MSCH alone. Fluorophenicol did not affect the course of EAE in mice immunized with MSCH that did not receive intraperitoneal inoculation with infectious *C. pneumoniae* elementary bodies suggesting that the effect was unlikely to be due to any immunomodulatory effect of the antibiotic (Fig. 4).

Activation of MBP-reactive Lymphocytes after Infection with C. pneumoniae or C. trachomatis. We next examined if worsening of EAE in mice inoculated with *C. pneumoniae* was due to an increase in activation of MBP reactive Th1 cells. We determined the effect of in vivo infection with live *chlamydia* on lymphocyte proliferation and IFN-γ production to MBP in, in vitro cultures. Proliferation counts to MBP, obtained from mice immunized with MBP and infected with *C. pneumoniae* increased from background levels of 4,305 ± 120 to 28,854 ± 1,154 cpm to in the presence of 50 μg/ml of MBP. Background counts in *C. trachomatis* mice was 4,196 ± 1,492 cpm, which increased to 19,308 ± 932 cpm in the presence of MBP. In uninfected mice, proliferative response to MBP increased from 3,916 ± 186 to 15,068 ± 815, in the presence of MBP. The proliferative response to MBP was higher in both *C. pneumoniae* and *C. trachomatis* infected mice when compared with uninfected controls suggesting that concurrent infection with chlamydia can amplify an autoimmune response. A proliferative response to MBP was not seen in

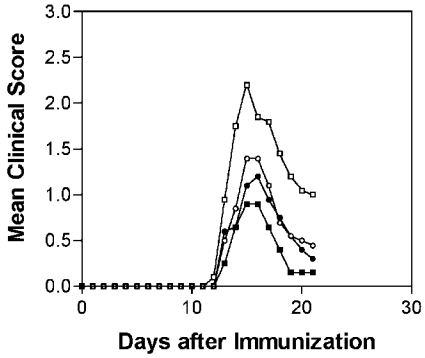

Figure 4. Effect of Fluorophenicol on severity of EAE in mice infected with *C. pneumoniae*. EAE was established as described in Fig. 1 A. Beginning on day 7, till day 18, *C. pneumoniae*-infected EAE mice were either treated with Fluorophenicol (subcutaneous 5 mg/kg daily) (■) or vehicle (□) (treated vs untreated, $P < 0.01$; $n = 12$). Uninfected mice immunized to induce EAE were treated with Fluorophenicol (● subcutaneous 5 mg/kg daily) or untreated (○, $n = 12$).

mice infected with *C. pneumoniae* alone, suggesting a lack of cross reactivity between *C. pneumoniae* antigens and MBP (Fig. 5). IFN-γ levels in lymphocyte culture supernatants were similarly higher in *C. pneumoniae* (1,020 pg/ml) and *C. trachomatis* infected mice (950 pg/ml) when compared with uninfected mice (612 pg/ml; $P < 0.05$, Fig. 5). These observations suggest that unlike its different effects on paralytic EAE, both *C. trachomatis* and *C. pneumoniae* in-

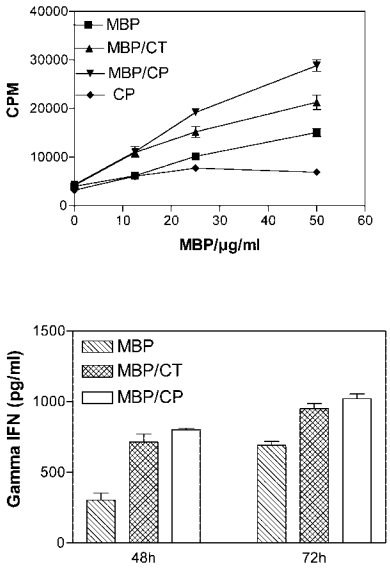

Figure 5. Effect of infection *C. pneumoniae* and *C. trachomatis* on the development of an immune response to MBP. SJL/J female mice were immunized with MBP and infected with chlamydial organisms as described in Fig. 1 A. (Top) Proliferative response of splenocytes to MBP in infected and uninfected mice. The data represents the mean value and SD (standard deviation) of [^{3}H]thymidine uptake (CPM) of triplicate determination at each point. (Bottom) IFN-γ production in culture supernatants in response to MBP. The levels of IFN-γ in supernatants were measured by ELISA at 48 and 72 h, respectively. The data represents the mean and standard value (SD) of triplicate determinations at each time point from a representative three experiments (CT, *C. trachomatis*; CP, *C. pneumoniae*).

fections are capable of enhancing T cell proliferation and IFN-γ production in response to MBP over that seen in uninfected controls.

Discussion

This study shows the effect of live *C. pneumoniae* infection on the amplification of the autoimmune disease after immunization with three different neural antigens (MSCH, MBP, and MOG). The accentuation of EAE was seen in the setting of direct infection of the CNS by *C. pneumoniae*. A causal association between *C. pneumoniae* infection and accentuated EAE can be inferred not only from the direct presence of replicating organism in the CNS, but also from the attenuation of EAE after therapy with Fluorophenicol. We believe that infection of *C. pneumoniae* in the CNS is a requisite for worsening of EAE. Systemic infection with *C. trachomatis* enhanced the in vitro proliferative response to MBP that were higher than controls. Unlike *C. pneumoniae*, *C. trachomatis* did not infect the CNS in mice, which we believe is important to cause worsening of EAE.

One mechanism by which infections can potentially induce autoimmune disease is through molecular mimicry. After immunization with chlamydial peptides that show homology with MBP, rats developed severe EAE (14). In view of the link between chlamydial antigens and heart disease, sequence homology between heart myosin and *C. pneumoniae* antigens were screened and mice were immunized with antigens that showed homology with myocardial antigens (15). These two studies showed that cross reactive epitopes between chlamydial and self antigens are capable of inducing different forms of autoimmune disease.

Our study did not show evidence of molecular mimicry between *C. pneumoniae* and neural antigens in SJL mice. Mice infected with *C. pneumoniae* alone did not show a lymphocyte proliferative response to MBP. Although we cannot fully exclude the expansion of autoreactive T cells that cross-react with *C. pneumoniae*, we think this to be unlikely. Animals showed worsening of EAE induced by three different classes of encephalitogenic antigens, MBP, MOG, and MSCH. It is unlikely that molecular mimicry is present between chlamydial antigens and three different neural antigens.

Infectious agents are well known to rapidly expand the pool of immune cells that recognize the invading pathogen. An increase in population of T cells that recognize other antigens including those that react to self-proteins may occur consequent to the secretion of cytokines and may be sufficient to cause disease (16). In mouse hepatitis model of CNS infection, prominent demyelination mediated by CD8 T cells was seen in mice in the absence of cognate antigen in the CNS, suggesting that demyelination did not require the presence of antigen-specific T cells (17). In the mouse keratitis model induced by HSV-1, both antigenic mimicry and bystander activation are thought to be responsible for tissue injury. In trying to reconcile the views of molecular mimicry and bystander activation in autoimmunity, it was proposed that these two processes may not necessarily be exclusive and may depend upon the circulating levels of autoreactive cells (18).

There has been considerable interest in the role of infectious agents in the development of MS. We have proposed that chlamydial infections should be considered as a potential candidate agent in MS (2). Our current studies suggest that *C. pneumoniae* can infect the CNS in mice. A number of case reports have suggested that *C. pneumoniae* can cause acute CNS infections in humans (19, 20). Persistence of *C. pneumoniae* in the CNS is likely to provide an environment which can lead to the activation of autoreactive T cells and contribute to the pathogenesis of a chronic disease such as MS. The lack of worsening of EAE in mice receiving intraperitoneal inoculations of infectious *C. trachomatis* elementary bodies suggests that direct infection of CNS tissues is necessary to enhance EAE. *C. pneumoniae* and *C. trachomatis* disseminate to lymphoid organs and lung after parental administration of the pathogen (21–23). We did not observe dissemination of *C. pneumoniae* to the CNS in naive mice. We propose that infection of the CNS is a necessary for accentuation of EAE which may be facilitated in the presence of an ongoing CNS inflammation. We suggest that a similar scenario may occur in MS, in which a ubiquitous pathogen may amplify an autoimmune response. We predict that if an infectious agent can persist and amplify an immune response, it can modify the expression of a T cell–mediated autoimmune disease in an organ specific manner. A direct interplay between an infectious agent and autoimmunity is also likely to have immediate therapeutic implications.

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