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*J Immunol* 2001; 167:1803-1808; doi: 10.4049/jimmunol.167.3.1803
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It has been reported recently that the bacterial respiratory pathogen Chlamydia pneumoniae is present in the cerebrospinal fluid of a subset of multiple sclerosis (MS) patients. However, it is not known whether this organism is a causative agent of MS, or merely an opportunistic pathogen that takes advantage of a disease process initiated by some other means. We report identification of a 20-mer peptide from a protein specific to C. pneumoniae which shares a 7-aa motif with a critical epitope of myelin basic protein, a major CNS Ag targeted by the autoimmune response in MS. This bacterial peptide induces a Th1 response accompanied by severe clinical and histological experimental autoimmune encephalomyelitis in Lewis rats, a condition closely reflective of many aspects of MS. Studies with peptide analogues suggest that different populations of encephalitogenic T cells are activated by the C. pneumoniae and myelin basic protein Ags. Mild experimental autoimmune encephalomyelitis was also observed when rats were immunized with sonicated C. pneumoniae in CFA. The Journal of Immunology, 2001, 167: 1803–1808.

Multiple sclerosis (MS) is characterized by the presence of autoreactive T cells that target Ags associated with CNS myelin, including myelin basic protein (MBP) (1, 2). Detailed studies of tissue samples from MS patients reveal demyelination and mononuclear cell infiltration of CNS white matter, oligoclonal Ig in the cerebrospinal fluid, and in many cases axonal degeneration (3–5).

Over the years, numerous reports have attempted to associate infectious agents with MS, including paramyxoviruses, T-lymphotropic viruses, and herpesvirus 6 (6–8). Recently, Sriram et al. (9) examined the cerebrospinal fluid of 17 patients with relapsing-remitting MS, 20 patients with progressive MS, and 27 patients with other neurological diseases. They detected Chlamydia pneumoniae DNA in 97% of the MS patients, vs 18% of other neurological diseases controls, and they isolated C. pneumoniae from the cerebrospinal fluid of 64% of the MS patients tested vs 11% of the controls (9). They also reported that Abs to C. pneumoniae elementary bodies were present in the cerebrospinal fluid of 18 of 20 patients tested (9). These findings were independently confirmed, but in a smaller percentage of MS patients, by Layh-Schnitt et al. (10). However, the data remain controversial, because Hammerschlag et al. (11) were unable to detect C. pneumoniae in a large group of MS brain tissue samples by culture or PCR. Furthermore, the mere presence of C. pneumoniae in the CNS does not prove that the organism triggers MS. Rather, chlamydial infection of the CNS simply could be an opportunistic, secondary event in the disease; even in this circumstance, however, the presence of the organism may exacerbate a pathogenic process initiated by other means.

The etiology of MS remains elusive, but one explanation for disease development postulates that specific antigenic epitopes from an unspecified infectious agent or agents induce(s) a host immune response in which cross-reactivity with myelin triggers disease, a concept referred to as molecular mimicry (12–17). In this scenario, some T cells and/or Abs elicited in response to Ags of the infectious agent also recognize relevant self-Ags in the CNS, thereby initiating the destructive autoimmune process. To date, little direct evidence exists to support the molecular mimicry hypothesis, although some data appear to support an infectious cause for MS (6–8, 16). Further, studies in mice have shown that infection with Theiler’s virus elicits an inflammatory response in the CNS that progresses to chronic experimental autoimmune encephalomyelitis (EAE) (18).

Because of its many clinical and immunopathological similarities to MS, EAE in rodents has become a widely accepted model for study of this human disease. In the genetically susceptible Lewis (LEW) rat, immunization with a specific peptide from MBP (see below) induces an acute episode of paralysis mediated by infiltration of activated CD4+ inflammatory T cells into the CNS, thereby duplicating important aspects characteristic of MS pathology (1, 2).

In the work presented here, we asked whether the well-characterized LEW rat model of MS could be used to investigate a causal relationship between infection with C. pneumoniae and MS.

Materials and Methods

Peptide synthesis

Software and information provided by NCBI at the National Institutes of Health website (http://www.ncbi.nlm.nih.gov/BLAST/) were used to search for potential peptides unique to C. pneumoniae having high homology with rat MBPβ69–89. A Blast search of the entire chlamydial genome identified a peptide encoded by the Cpn0483 gene that shares a motif with rat MBPβ69–89. Synthetic peptides were prepared using F-moc chemistry in an Applied Biosystems Synergy model 432A peptide synthesizer (Perkin-Elmer, Foster City, CA), according to manufacturer’s instructions. Peptide
structure was confirmed by electrospray mass spectrometry, and purity was determined using HPLC. The peptides used in these experiments were: C. pneumoniae 0483 peptide (Cpn0483) peptide (RFPNHYGCLLPRNPRT EDQN); rat MBP68–86 (YGSLPQKSQRTQDENPV); CpnD→A (RFPNHYGCLLPRNPRT EDQN); MBP→D (YGSLPQK SQRTQDENPV); MBP-D→A (YGSLPQK SQRTQDENPV).

**RT-PCR**

For RT-PCR analyses, pure RNA was prepared as described (19) from Hep-2 cells infected with C. pneumoniae strain TW-183; in vitro infection was done by the standard method. RNA thus prepared was reversed transcribed using the murine leukemia virus enzyme (Life Systems, Gaithersburg, MD) and random hexamers as primers. cDNA was purified from the reaction mixtures by extensive treatment with RNases A, T1, and H, followed by phenol-chloroform extraction and precipitation in ethanol. Amplification of cDNA from infected cultures was done as described for the C. pneumoniae KDO transferase gene and others (20). The primer system used for the mRNA from coding sequence Cpn0483 was 5'-aagactc ctggtagctgcagttcgac-3' and 5'-tgtacga cccgtagttcgtgcagtcg-3'. Amplification using this primer system gives a product of 319 bp. Products were displayed on standard agarose gels and visualized by staining with ethidium bromide. The amplification product was also cloned, and the sequence was determined to verify its authenticity.

**Induction of active and adoptive EAE**

Female LEW rats (8–12 wk old, purchased from Charles River, Raleigh, NC) were immunized s.c. at the hind footpad with the appropriate synthetic peptide, emulsified in CFA (Difco, Detroit, MI) containing 4 mg/ml Mycobacterium butyricum. Because previous work had indicated that 5–50 μg MBP68–86 induces optimal EAE, these concentrations of Cpn0483 peptide were used in the present study. The rats (four to five per group) were observed for clinical signs of EAE, graded as 0 (no disease), 1 (loss of tail tonicity), 2 (hind limb weakness), or 3 (hind limb paralysis) (21). Hematoxylin-eosin-stained spinal cord sections from representative rats were examined for inflammatory cell infiltration and demyelination without knowledge of the group of origin. Some sections were also stained for myelin with Luxol fast blue. Spleen cells from LEW rats with EAE were activated in vitro in the presence of either Cpn0483 or MBP68–86 (10 ng/ml) for 72 h, then transferred i.p. to naive LEW recipients, as previously described (21). Adoptive EAE developed in 5–6 days. Short term T cell lines were prepared as previously described (21).

**In vitro T cell assays**

T cell proliferation assays were performed by isolating splenocytes from peptide-primed rats, adherent cells were removed by culture on plastic petri dishes, and T cells were isolated on T cell columns (Biotec, Edmonton, Canada). The T cells were cultured for 96 h with irradiated (2000 rad) syngeneic thymocytes as APCs and peptide in 96-well flat-bottom microtiter plates. The cultures were pulsed with [3H]thymidine (0.5 μCi/well) 18 h before harvesting cells, and [3H]thymidine incorporation was measured in a Microbeta Plus liquid scintillation counter (1450 Microbeta Plus; Wallac, Gaithersburg, MD). Cultures were run in quadruplicate and each experiment was repeated at least twice. Dose-response studies were performed using various peptides at differing concentrations, and representative results are presented. The stimulation indices were calculated as cpm with peptide/background (cpm of T cells and APC without peptide). Stimulation indices were considered significant only if they exceeded background by at least 3-fold.

**Results**

**Homology between Cpn0483 and MBP 68–86**

In the LEW rat, the dominant encephalitogenic MBP epitope is comprised of aa 68–86 of guinea pig MBP (22, 23). However, EAE is autoimmune because the disease can be induced with self

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EAE Incidence</th>
<th>EAE Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μg Cpn0483</td>
<td>3/5</td>
<td>1.8</td>
</tr>
<tr>
<td>50 μg Cpn0483</td>
<td>14/15</td>
<td>2.3</td>
</tr>
<tr>
<td>50 μg rat 68–86</td>
<td>10/10</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* Mean group severity (maximum, 3.0).

In the present study, most Cpn0483-immunized rats developed clinical signs of active EAE that were similar to those induced by MBP68–86. The time course of disease onset was similar for both immunogens (Table II). The disease course from one experiment is shown in Fig. 2, and cumulative results are presented in Table II. Clinical signs in most animals progressed from flaccid tail (grade 1) to complete hind limb paralysis with incontinence (grade 3). At this time, most Cpn0483-immunized rats were sacrificed for studies of T cell proliferative responses; animals that were not

**Expression of Cpn0483 in C. pneumoniae**

The RT-PCR analyses given in Fig. 1 show that Cpn0483 is expressed by the bacterium during normal vegetative growth using Hep-2 cells as host in vitro. Transcripts from the Cpn0483 coding sequence are apparent in samples taken 24 h postinfection, and expression of the gene continues unabated through 72 h postinfection in this system. Uninfected control cells showed no signal in parallel RT-PCR assays, as expected, and RNA prepared from C. pneumoniae elementary bodies was also negative for this transcript.

**Encephalitogenic activity of Cpn0483 peptide in LEW rats**

The MBP68–86 homologue peptide from Cpn0483 was synthesized, and LEW rats were immunized s.c. with 5- or 50-μg doses emulsified in CFA. The animals were followed for 18–21 days postimmunization. Initial clinical signs of EAE began to appear ~12 days postimmunization in groups given either dose, and those signs persisted for 3–6 days. The disease course from one experiment is shown in Fig. 2, and cumulative results are presented in Table II. Clinical signs in most animals progressed from flaccid tail (grade 1) to complete hind limb paralysis with incontinence (grade 3). At this time, most Cpn0483-immunized rats were sacrificed for studies of T cell proliferative responses; animals that were not

![FIGURE 1. RT-PCR analyses demonstrating expression of C. pneumoniae coding sequence Cpn0483 in infected Hep-2 cells in vitro. Lane 1, negative (water) control; lane 2, positive PCR control using purified C. pneumoniae DNA as template; lane 3, negative control using DNA from uninfected Hep-2 cells as template; lane 4, negative control using RNA from C. pneumoniae elementary bodies as template; lanes 5–7, analysis of cDNA made from RNA obtained from Hep-2 cells infected for 24, 48, and 72 h, respectively; lane 8, analysis of RNA from the 48-h-infected cells in the absence of reverse transcription.](image-url)
sacrificed recovered from paralysis. As controls for the Cpn0483 peptide-immunized rats, additional groups of LEW rats (four or five per group) were immunized with 50 \( \mu \)g rat MBP 6–86 peptide, which induced clinical disease with severity comparable to that induced by the \textit{C. pneumoniae}-derived peptide (Fig. 2 and Table II). Thus, the Cpn0483 peptide was encephalitogenic in this animal model.

Extensive perivascular cuffing and parenchymal mononuclear cell infiltration was present in the spinal cords of the Cpn0483-immunized rats (Fig. 3). Both are characteristic pathological findings in the rat model of EAE (1, 2) and are also observed in MS (4). Significant demyelination was not observed in Luxol fast blue-stained sections. This feature is prominent in MS but is not characteristic of acute EAE in LEW rats.

When spleen cells were prepared from Cpn0483-immunized LEW rats with EAE and activated in vitro with the same peptide for 72 h, they transferred clinical disease to six of nine syngeneic recipients. Six of nine recipients of Cpn-primed spleen cells activated with MBP 68–86 also developed clinical EAE. Mononuclear infiltration was present in the spinal cords of recipients with clinical disease, as expected (Fig. 4).

T cell responses to Cpn0483 and MBP 68–86

To further investigate the immunopathology underlying the clinical observations, T cells were isolated from the spleens of rats immunized with Cpn0483 or MBP 68–86, and recall responses were assessed using standard T cell proliferation assays. T cells from rats immunized with the chlamydial peptide responded vigorously to the priming peptide. Moreover, they responded significantly to rat MBP 68–86 (Fig. 5a). In contrast, T cells derived from rats immunized with rat MBP 68–86 proliferated vigorously to the priming peptide but cross-reacted only minimally with the Cpn0483 homologue peptide at relatively high concentrations (Fig. 5b). Immunological specificity was demonstrated by the lack of proliferation in response to an irrelevant nonglymphocytic peptide (MBP 11–30 or MBP 31–50). T cells derived from unimmunized rats showed no proliferative response to any of the peptides tested (not shown).

The cross-reactivity of Cpn0483-primed T cells with MBP 68–86 probably reflects activation of self-MBP-reactive T cells in the host. In contrast, one can speculate that the failure of MBP 68–86-primed T cells to respond significantly to Cpn0483 may reflect the fact that the rats were not previously exposed to this exogenous microbe. Nevertheless, T cells from \textit{C. pneumoniae} peptide-primed rats cross-react with MBP 68–86 consistent with predictions of the molecular mimicry hypothesis.

A short term T cell line from Cpn0483 peptide-immunized rats secreted IFN-\( \gamma \) (7000 pg/ml) when activated for 72 h with chlamydial peptide, as measured using commercial ELISA kits, but these cells did not produce detectable IL-4, confirming that the chlamydial peptide elicited an inflammatory Th1 response. It has been well established that EAE in rodents is mediated by IFN-\( \gamma \)-producing Th1 inflammatory cells (1, 2, 24).

Studies with analogues of Cpn0483 and MBP 68–86

MBP 68–86 and Cpn0483 share a YGxLxxXTxDxN motif (Tables I and II). The aspartic acid (D) residue is reportedly a TCR contact for reactivity of guinea pig MBP 73–86, the minimal encephalitogenic sequence, with LEW rat T cells (24, 25). To determine whether D is also required for Cpn0483-induced EAE in
LEW rats, we prepared the alanine-substituted peptides Cpn0483D and rat MBP 68–86-D and tested them for encephalitogenic activity in LEW rats. We confirmed earlier results that the replacement of D with alanine (A) in MBP68–86 (MBP-D>A) abolished encephalitogenic activity for LEW rats (24). In contrast, the A-substituted Cpn0483 analogue (CpnD>A) elicited severe EAE in these animals (Table III). These findings suggest that different specificity patterns, which presumably reflect activation of different subsets of encephalitogenic T cells, govern the induction of EAE by Cpn0483 and MBP68–86.

Induction of EAE with C. pneumoniae-infected Hep-2 cells

To determine whether the C. pneumoniae 0483 protein could be processed and presented by LEW rat APCs to elicit evidence of EAE, we sonicated C. pneumoniae-infected Hep-2 cells and emulsified the sonicate in CFA. To minimize discomfort to the animals, we reduced the concentration of mycobacteria in the CFA to half the amount normally used in encephalitogenic emulsions. Five rats were immunized with 0.05 ml emulsion containing 175 μg protein (total Hep-2- and C. pneumoniae-derived protein). Five control rats received emulsion containing 175 μg protein from uninfected Hep-2 cells. One of the rats that received the C. pneumoniae emulsion exhibited limp tail consistent with EAE. Focal mononuclear cell infiltrates were present in the spinal cord of this rat (Fig. 6). Neither the remaining four C. pneumoniae-immunized rats nor the five control rats exhibited evidence of EAE. The low incidence of disease is not surprising, given that the sonicate contained the complete range of Hep-2 and C. pneumoniae proteins in relatively low overall dose. Thus, it is unlikely that Cpn0483 protein was present at optimal concentration to induce severe EAE. Furthermore, the CFA contained a suboptimal concentration of mycobacteria.

Discussion

The results presented here demonstrate that a 20-mer amino acid sequence intrinsic to a Chlamydia pneumoniae-specific protein elicits MS-like disease in the LEW rat. Only 7 aa in the Cpn0483 peptide are identical with those of the cognate sequence in rat

Table III. Activity of Cpn0483 and RMBP D>A analogue in Lewis rats

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP68–86</td>
<td>YGSLPQKSRTQDENPV</td>
<td>5/5 (3.0)</td>
</tr>
<tr>
<td>MBP-D&gt;A</td>
<td>YGSLPQKSRTAENPV</td>
<td>0/10</td>
</tr>
<tr>
<td>Cpn0483</td>
<td>RFPNYGCLLPNPTEDDN</td>
<td>5/5 (3.0)</td>
</tr>
<tr>
<td>CpnD&gt;A</td>
<td>RFPNYGCLLPNPTEAQN</td>
<td>9/10 (2.7)</td>
</tr>
</tbody>
</table>

Motif: YGxLxxxxxRxxNx

* Incidence at 50 μg (mean group severity (maximum, 3.0)).

* Native peptide sequence.

FIGURE 6. Hematoxylin-eosin-stained section of spinal cord from LEW rat immunized with C. pneumoniae/Hep-2 cell sonicate in CFA: a, ×100; b, ×400.
MBP_{68–86} constituting a YGxLxxxxRTxN motif (Tables I and II). These residues provide a structural motif that permits interaction of the peptide with MHC class II gene products. This peptide-MHC complex, in turn, interacts with specific TCRs to initiate T cell activation. Although some specific amino acid residues at defined positions in disease-eliciting peptides have been shown to be critical for the induction of EAE, presumably because they interact directly with the TCR (24), it has been demonstrated also that TCRs can recognize sequentially distinct but structurally related peptides (17, 26). The 20-aa chlamydial peptide appears to be as effective as MBP_{68–86} peptide in causing paralysis in this model, and our data demonstrate that different MBP and Cpn0483 residues are critical for disease induction. Using alanine-substituted analogue peptides, the D residue common to the rat MBP and Cpn0483 peptides (Tables I and II) has been shown to be required for proliferative responses of an encephalitogenic LEW rat T cell to a truncated peptide (QKSQRSQDENPV), which corresponds to the core guinea pig MBP peptide (24, 25). We show here that the D residue is necessary for EAE induced with MBP_{68–86} but it is not required for encephalitogenic activity of Cpn0483 (Table III). This indicates that the two peptides differ with respect to their requirements for encephalitogenicity, which may reflect activation of different T cell subsets. This conclusion is consistent with the differences shown in proliferative responses elicited by the respective primed T cells (Fig. 5).

*C. pneumoniae* has been shown to be a highly unusual pathogen during the decade since its identification. During that time, the organism has been associated not only with acute respiratory disease but also with chronic obstructive pulmonary disease, atherosclerosis, temporal arthritis, MS, late onset Alzheimer’s, and other diseases (9, 10, 20, 27); however, the evidence supporting some such associations is still circumstantial. Although the role of *C. pneumoniae* in MS remains controversial (11), the results presented here appear consistent with an infectious etiology for this disease in at least a subset of patients. A number of investigators have postulated, and presented results supporting, such an infectious etiology. It might be significant to relate the epidemiology of *C. pneumoniae* to the incidence and prevalence of MS in areas where an infectious causation has been postulated, e.g., the Faroe Islands (28). Even if this microbe is involved in MS, consideration will have to be given to direct vs indirect effects. In this regard, it has recently been reported that the APOE e4 allele is associated with faster progression to disability in MS (29). Importantly, a recent study showed that 68% of patients with *C. pneumoniae*-associated arthritis possess the APOE e4 allele (19). Thus, one might speculate that exposure of individuals expressing certain genes (e.g., the APOE e4 allele) to the appropriate infectious agent (e.g., *C. pneumoniae*) plays a role in the induction of MS.

Regardless, there are clear differences between EAE in rats and MS. The former is an acute inflammatory disease with scant demyelination (2), whereas demyelination is a prominent feature of MS (3, 4). MBP-reactive T cells in MS patients are predominantly directed toward a sequence contained within residues 84–102 (17, 30, 31), whereas the dominant encephalitogenic epitope for LEW rats is composed of MBP_{68–86} (22, 23). The 84–102 peptide contains the sequence KNIVTPRPPP, and our Blast search also turned up a chlamydial gene, Cpn0442, specifying a protein containing the sequence KNLLPPYPEPP, which conceivably could activate human MBP-reactive T cells. In support of this contention, it has been reported that human papillomavirus 7 contains a VHFFK motif identical with a sequence also present in MBP_{57–99} (16). The viral peptide is capable of selecting papillomavirus-specific SJL mouse T cells that cross-react with MBP_{57–99}, a major encephalitogenic epitope for SJL mice. The papillomavirus-specific T cells proliferate to both the viral and MBP peptides, and are encephalitogenic for SJL mice (16).

Resolution of the controversy concerning whether *C. pneumoniae* actually plays a role in the pathogenesis of MS will require further study. However, the present report reveals that a *C. pneumoniae*-derived peptide is capable of inducing autoimmune CNS disease in a rodent model of the disease.

References


