Determinant Spreading Associated with Demyelination in a Nonhuman Primate Model of Multiple Sclerosis


*J Immunol* 1999; 162:2384-2390; ;
http://www.jimmunol.org/content/162/4/2384

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Determinant Spreading Associated with Demyelination in a Nonhuman Primate Model of Multiple Sclerosis

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Definition of the immune process that causes demyelination in multiple sclerosis is essential to determine the feasibility of Ag-directed immunotherapy. Using the nonhuman primate, Callithrix jacchus jacchus (common marmoset), we show that immunization with myelin basic protein and proteolipid protein determinants results in clinical disease with significant demyelination. Demyelination was associated with spreading to myelin oligodendrocyte glycoprotein (MOG) determinants that generated anti-MOG serum Abs and Ig deposition in central nervous system white matter lesions. These data associate intermolecular “determinant spreading” with clinical autoimmune disease in primates and raise important issues for the pathogenesis and treatment of multiple sclerosis. The Journal of Immunology, 1999, 162: 2384–2390.

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) that causes disabling neurological deficits in young adults and is generally held to have an autoimmune etiology (1). Experimental allergic encephalomyelitis (EAE) is an animal model of demyelinating disease resembling MS that can be antigenically induced in various species, such as rats, mice, guinea pigs, rabbits, and nonhuman primates including the common marmoset, Callithrix jacchus jacchus (2–6). Disease is initiated either by immunization with myelin Ags or by transfer of activated myelin-specific CD4+ T cells (7, 8). Typical EAE lesions in the CNS white matter show perivascular inflammation, variable demyelination, and the relative sparing of axons, although axonal pathology may be evident (6, 9, 10). In marmosets, more subtle clinical evaluation is possible than in rodent models, and the clinicopathological features closely resemble MS (5, 11). Disease progression can also be monitored in living marmosets by magnetic resonance imaging (MRI), making marmoset EAE useful for preclinical testing of MS therapies. Disease outcome depends on the immunizing Ag (11). Demyelination appears to require active immunization with human white matter homogenate or other myelin Ag preparations containing myelin oligodendrocyte glycoprotein (MOG) and may involve both humoral and T cell components. Although MS is believed to be mediated principally by T cells, humoral responses to MOG may also play a role (11–14). Anti-MOG Abs have been observed in proteolipid protein (PLP)-immunized marmosets, but these have not been previously associated with clinical disease (11).

A promising area of investigation is the testing of Ag-specific immunomodulation to block the autoimmune process in MS and related EAE animal models. The value in such an approach is the possibility of highly specific therapy without the side effects of general immunosuppressants. New discoveries in T cell biology reveal that Ag can down-regulate cognate T cells by inducingergy or apoptosis (15–17). For example, we have demonstrated that high-dose Ag administration can ameliorate autoimmune demyelinating disease by diminishing T cell responses to myelin basic protein (MBP) and PLP epitopes (18, 19). The success of this approach depends critically upon defining the Ags that trigger the pathogenetic immune response throughout the natural history of the disease. However, an important effect in autoimmune disease progression may be the occurrence of epitope or determinant “spreading” (20, 21). The early immune response against a tissue Ag can expose “cryptic” epitopes that stimulate additional pathological immune responses (20, 21). In rodent EAE models, determinant spreading within the same myelin protein (intramolecular) as well as between myelin proteins (intermolecular) may cause disease relapses (20–23). Sensitization to cryptic epitopes may result from changes in the peptide availability, enhanced Ag presentation, increased T cell recognition, or other stimulatory effects of cytokines (20, 21). Understanding this process is crucial for guiding the development of Ag-specific immunotherapies against autoimmune diseases but has not been investigated in primate disease.

To understand the contribution of these Ags to demyelinating disease, we immunized marmosets with MP4, a chimeric molecule composed of the human 21.5-kDa isof orm of MBP and ΔPLP4, a recombinant form of human PLP lacking the hydrophobic domains (19, 24). The MP4 molecule contains all known human MBP and PLP epitopes but none from MOG or other myelin Ags. We show that immunization with MBP and PLP epitopes results in intermolecular determinant spreading that generates humoral responses.
against MOG that are associated with clinical disease and CNS demyelination.

Materials and Methods

Animals

Callithrix jacchus jacchus were obtained from a colony maintained by the National Institute for Child Health and Human Development at the National Institutes of Health Primate Unit (Poolesville, MD). The animals ranged from 2 to 9 yr of age and were cared for under an approved protocol in accordance with the guidelines established by the National Institutes of Health Animal Care and Use Committee.

Ags

MP4 was prepared by metal affinity chromatography and reversed phase HPLC as previously described (25). Human white matter was generously provided by the Harvard Brain Tissue Resource Center, McClean Hospital (Belmont, MA). The recombinant extracellular domain of rat MOG (rMOG) was prepared as described (25). Human MBP was prepared by the method of Diebler et al. (26).

Induction of EAE

White matter homogent (WMH) was emulsified 1:1 in CFA (Difco, Detroit, MI) containing 3 mg/ml of killed H37 RA Mycobacterium tuberculosi (Difco). MP4 was emulsified 1:2 in TiterMax adjuvant (Vaxcel, Norcross, GA) or in CFA. Animals received 100 µl intradermal injections at four sites on the back. WMH-immunized animals received a total of 100 mg of WMH; MP4-immunized animals received 0.8–1.0 mg. On the day of immunization and again 2 days later, all immunized animals were given 1010 killed of immunization and again 2 days later, all immunized animals were given

Clinical and pathological evaluation of EAE

Marmosets were observed daily, and clinical symptoms were scored as previously described (Table I and Ref. 5). At various times after immunization, animals were euthanized, and the CNS was removed and fixed in Formal-Fix (Shandon, Pittsburgh, PA). The animals were not perfused. Three-millimeter coronal sections of brain and transverse or longitudinal sections of spinal cord were paraffin-embedded, sectioned, and stained using hematoxylin and eosin, Luxol fast blue (LFB), and Bodian’s silver stain techniques (American Histolabs, Gaithersburg, MD). Histopathological evaluation of CNS sections was done in a blinded fashion, and demyelination and inflammation were scored as previously described (5) with minor changes. Briefly, for inflammation: 0, no inflammation present; 1, minimal (1–3 lesions/average section); 2, moderate (3–10 lesions/average section); and 3, extensive. For demyelination: 0, no demyelinating lesions; 1, minimal demyelination (1–3 lesions/average section); 2, moderate demyelination (3–10 lesions/average section); and 3, widespread demyelination with large confluent lesions. Photomicrographs were taken on an Axiophot microscope (Zeiss Thornwood, NY).

Magnetic Resonance Imaging

Scans were performed in the coronal plane with 2-mm interleaved slices on a Signa 1.5 T unit (General Electric, Milwaukee, WI) and included a T2-weighted spin echo pulse sequence SE 2000/20/80 and T1-weighted sequences SE 450/13 with and without a magnetization transfer (MT) pulse, using a 3-inch surface coil. T1-weighted and MT images were performed before and after i.v. administration of the contrast agent gadopentetate dimeglumine (0.3 mmol/kg) (Magnexit, Berlex Laboratories, Cedar Knolls, NJ).

Results

Clinical disease induction with MP4 varies with the type of adjuvant

We immunized four animals using CFA: animals J77 and H67 with 400 µg of MP4, and for comparison, animals H37 and H19 with 100 mg of human WMH. EAE symptoms developed 8–9 days after immunization in the WMH-immunized animals and were typified by paraparesis that progressed to mono- or paraplegia (Table I). These animals were also lethargic and developed anisocoria. The MP4-immunized animals remained without severe clinical symptoms for the entire 222-day observation period (Table I), but showed a significant weight loss at ~4 wk postimmunization. T cell proliferative responses to MP4 occurred in all animals (data not shown). Moreover, at necropsy, we found that all animals exhibited CNS pathology (see below).

We then immunized three animals, D5, E74, and H66, with 800 µg of MP4 in TiterMax adjuvant (TMA) that contains the block
copolymers CRL-8941 in squalene, which is thought to be more immunostimulatory but less toxic than CFA. We found this to be the case in marmosets since TMA prevented the severe skin ulcers that commonly developed with CFA injections but promoted severe disease. Within 6–18 days, we observed EAE symptoms including tail paresis, anisocoria, lethargy, and weight loss (Table I). Animal E74 suffered a seizure 3 days after the onset of lethargic behavior and was found dead the following day. Thus, severe symptomatic EAE can be induced in marmosets by immunization with only MBP and PLP epitopes, and this was facilitated by the synthetic adjuvant TMA.

Inflammatory white matter lesions either with or without demyelination are induced by MP4

Histopathological analysis revealed that lesions induced by MP4 could be either demyelinating, in which staining by LFB is lost widely around the vessel (Fig. 1, b and f), or nondemyelinating, in which axons are stained up to the vessel border (Fig. 1d). Demyelination was associated with intact axons within the affected area as determined by Bodian’s silver stain (data not shown). Both classes of lesions were chiefly perivascular and located in the white matter tracts, although rare cortical lesions were detected. As in MS, little meningeal infiltrate was observed. The histological appearance of the CNS lesions in two MP4-immunized animals, D5 and H66 (Fig. 1, a and c), were comparable to those in the WMH-immunized animal, H19 (for example, Fig. 1e shows a demyelinating lesion from H19). At longer times after immunization, animals more often manifested hypocellular lesions. For example, the analysis on animal D5 (Fig. 1, a and b) was conducted ~4 wk later than for animals H66 or H19 (Figs. 1, c-f). Interestingly, MP4-induced lesions were found most commonly in the white matter tracts adjacent to the corpus callosum, the “wetterwinkel” or “storm center” that is often the focus of CNS pathology in MS (27, 28). By contrast, WMH lesions were more numerous and widespread leading to increased mean inflammation and demyelination scores (Table II). Also, spinal cord lesions were common in WMH disease but not in MP4 disease. These differences were associated with more severe symptoms in WMH-sensitized animals and suggest that a component in the more complex Ag mixture may cause greater disease at earlier time points.

A notable pattern that also emerged was that each animal manifested either a predominant demyelinating or nondemyelinating form of EAE at necropsy. This suggests that, after immunization, lesions in independent CNS locations evolved in a similar manner over time. Of the MP4-immunized animals in this study, four had predominantly demyelinating lesions, whereas one, H66, exhibited nondemyelinating lesions. In a separate study in which marmosets were immunized with MP4 in TMA, we observed inflammatory lesions with no demyelination in two animals and demyelinating lesions in one animal (data not shown). Thus, immunization with MBP and PLP epitopes induced demyelinating disease in five of eight animals analyzed, which was surprising in light of previous work suggesting that immunization against MOG was critical for demyelination (11).

MRI detects both demyelinating and inflammatory lesions

Disease evolution was followed longitudinally in live animals by MRI scans every 2 wk (Table II). In the MP4-immunized animals, MRI abnormalities appeared around the time of clinical disease (Fig. 2). Correlations with microscopic sections revealed that strong contrast enhancement often corresponded to perivascular inflammation and demyelination (Fig. 2A, animal D5). However, discrete MRI “lesions” in animal H66 were due to inflammatory cell infiltrates without demyelination (Fig. 2B). Moreover, MRI changes occurred in H66 at locations where serial sections revealed no histological damage, which could reflect the waxing and waning of inflammatory lesions or the transitory presence of edema as has been described in MS lesions (27, 29). We also documented that both demyelinating and nondemyelinating forms of disease exhibited contrast-enhancing lesions, suggesting that breaches in the blood-brain barrier (BBB) could occur without demyelination (Table II). All but one MP4-immunized marmoset had at least one MRI scan showing contrast enhancement in the white matter. Thus, both demyelinating and nondemyelinating disease were associated with contrast enhancing lesions and changes on T2-weighted images on MRI scans.

Table II. Summary of MRI and histopathology

<table>
<thead>
<tr>
<th>Animal</th>
<th>Maximum Inflammation</th>
<th>Mean Inflammation</th>
<th>Maximum Demyelination</th>
<th>Mean Demyelination</th>
<th>Maximum MRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37</td>
<td>3</td>
<td>2.7</td>
<td>3</td>
<td>2.7</td>
<td>ND</td>
</tr>
<tr>
<td>H19</td>
<td>3</td>
<td>3.0</td>
<td>3</td>
<td>2.5</td>
<td>ND</td>
</tr>
<tr>
<td>H77</td>
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<td>1.5</td>
<td>2</td>
<td>0.7</td>
<td>3</td>
</tr>
<tr>
<td>H67</td>
<td>3</td>
<td>1.3</td>
<td>2</td>
<td>0.4</td>
<td>3</td>
</tr>
<tr>
<td>D5</td>
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<td>0.8</td>
<td>2</td>
<td>0.8</td>
<td>3</td>
</tr>
<tr>
<td>E74</td>
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<td>2.2</td>
<td>2</td>
<td>1.4</td>
<td>2</td>
</tr>
<tr>
<td>H66</td>
<td>3</td>
<td>2.6</td>
<td>1</td>
<td>0.2</td>
<td>3</td>
</tr>
</tbody>
</table>

a Maximum score for a single brain slice or two to four spinal cord segments.
b Mean score for all brain and spinal cord segments. Cervical, thoracic, and lumbar spinal cord regions counted as a single slice for purposes of mean calculation.

* Maximum observed MRI score: 0, normal; 1–5 T2 lesions; 2, 5–10 T2 lesions; 3, >10 lesions or focal and diffuse abnormalities; and 4, diffuse white matter abnormalities. Add 0.5 or 1.0 if contrast enhancing lesion indicative of active disease and BBB disruption (0.5 for one lesion; 1 for ≥2 lesions).
The cellular composition of demyelinating and nondemyelinating lesions is different
We used immunocytochemical examination of the cellular infiltrates of white matter lesions to compare demyelinating and nondemyelinating lesions induced by MP4 and WMH (Fig. 3). Necropsies on three animals, H19, E74, and H66, were performed within 4 wk after immunization. All showed lesions consisting of intense perivascular cuffing (PVC) with parenchymal inflammatory infiltrates. However, H19 and E74 had demyelinating lesions, whereas H66 had almost no demyelination. These findings support previous suggestions that intense cellular infiltrates are characteristic of acute, “early” lesions (10, 30). We found the predominant cell type in nondemyelinating lesions to be CD3⁺ T cells (Fig. 3e). In demyelinating lesions induced by WMH or MP4, the major cell type was HAM 56⁺, which are either macrophages or activated microglial cells (31) (Fig. 3, a and k). CD3⁺ T cells were also present (Fig. 3, a and l). CD83, a marker of circulating dendritic cells (32), was expressed by a greater number of cells in demyelinating lesions (Fig. 3, d and l) compared with nondemyelinating lesions (Fig. 3h). In E74, CD83⁺ cells were located in the PVC and invaded the parenchyma in juxtaposition with HAM 56⁺ cells. The larger number of dendritic cells in demyelinating lesions could allow the presentation of newly exposed self Ags in the lesion or in the regional lymph nodes and thereby facilitate epitope spreading.

We also found CD20⁺ B cells, i.e., mature B cells before differentiating into plasma cells (Fig. 3, b, f, and j) (33), but the numbers of CD20⁺ cells (~3–10 cells/lesion) were the same in demyelinating and nondemyelinating lesions. Staining for Ig, however, revealed rings of Ig deposition (Fig. 4, a and c) and “balls” of Ig-stained debris, described previously in MS lesions (34), only at the margins of demyelinating lesions (compare Fig. 4, b and c, arrow). The Ig balls were often found within cells resembling macrophages (data not shown). Multiple sections revealed a strict correlation between Ig deposition and demyelination (data not shown).

Demyelination is associated with determinant spreading leading to a humoral response to MOG
Abs against MOG have been previously associated with demyelinating lesions in EAE and in MS (11–14, 33–35). Therefore, we tested for Abs against MBP and MOG and found that all animals developed significant titers of Abs against MBP. Surprisingly, animals with demyelinating disease also manifested Abs against MOG, indicating determinant spreading of the immune response to MOG (Fig. 5). In this and a follow-up study, animals with nondemyelinating disease did not develop anti-MOG Abs (Fig. 5 and data not shown). Kinetic analyses of serum revealed that anti-MBP Abs developed within 1 wk in all animals, and anti-MOG titers
increased with slower kinetics in animals with demyelinating disease (D5) but not in animals with nondemyelinating disease (H66) (Fig. 6). Unimmunized control animals had no detectable Abs against MBP or MOG.

Discussion

Considerable evidence in animal models has shown that Ag-specific therapy for demyelinating disease is both safe and effective (15–17). There appear to be multiple mechanisms by which specific Ag can down-regulate the relevant immune responses and ameliorate disease (15–17). For example, we have shown that repeated high doses of Ag can lead to deletion of activated, cycling encephalitogenic T cells (18). However, the successful application of these advances to human autoimmune diseases, including MS, critically depends on defining the Ags that trigger the pathogenic immune response and assessing the response of Ag-specific effector cells, such as B or T lymphocytes, to therapeutic Ag administration. The occurrence of “determinant spreading” and whether it contributes significantly to disease pathogenesis is potentially a key issue in designing Ag-directed therapeutics. To shed light on demyelinating disease in primates, we studied MS-like encephalitis that was triggered in the nonhuman primate, C. jacchus jacchus, by immunization with a recombinant (MP4) fusion protein containing MBP and PLP determinants in a powerful synthetic adjuvant. In certain animals, this promoted only inflammatory disease, but in others, demyelinated lesions were induced throughout the brain with little involvement of the spinal cord. Demyelination was associated with Ig deposition in the brain and was consistently associated with the development of anti-MOG Abs. Thus, anti-MOG Abs and Ig deposition may be crucial for myelin damage and demyelination. Previous studies by Hauser and colleagues (11–14) have demonstrated that florid demyelinating disease is consistently produced when a humoral response is stimulated in marmosets by immunizing with MOG, and MOG may be important in MS pathogenesis. Therefore, we believe that spreading to MOG determinants that provoked B cell responses was crucial for the demyelinating disease that we have observed. Because we did not initiate disease with MOG, our data demonstrate, for the first time, disease induction by determinant “spreading” in primates. Determinant spreading appeared to be enhanced by TMA, a synthetic adjuvant formulation that contains the metabolizable oil.
squalene, microparticulate silica, and a nonionic block copolymer, CRL89-41 (37, 38). Like similar triblock copolymers, CRL89-41 forms insoluble molecular strands with a large hydrophilic surface area that potently stimulates Ab production (37–39). By inducing TNF-α, granulocyte-macrophage CSF, IFN-γ, and IL-4, this adjuvant might stimulate the maturation of dendritic cells, the most potent inducers of naïve T cell activation (40). We identified CD83⁺ dendritic cells as well as B cells in EAE lesions (32). Thus, the presence of the cellular components of humoral immune responses within the lesions raises the possibility that anti-MOG Ab is produced locally in the inflamed tissue. The proposed autoimmune etiology for MS suggests that myelin Ags are the target of immune attack. However, a sole inciting Ag has not been identified in MS (1, 10). Rather, T cell reactivity has been demonstrated to various epitopes of several myelin Ags, including MBP, PLP, myelin-associated glycoprotein, and MOG, among others (12, 13, 41, 42). Responsiveness to multiple Ags may indicate that the inciting Ag could differ in different individuals, or that intermolecular epitope spreading may occur following initiation by a single Ag or epitope. Our work suggests that the measurement of immune reactivities in established disease may not reflect the inciting Ags, but may be informative about the types of Ag to which tolerance must be induced. In particular, it will be useful to determine whether the detection of anti-MOG Abs can be employed as a prognostic indicator for new treatments for MS.

Disease induction in marmosets allowed us to compare findings in live animals obtained with MRI to histopathological lesions observed after necropsy. An intense perivascular inflammatory infiltrate is characteristic of the acute lesion in MS and EAE (10, 30) and is the earliest known event in the development of these lesions. The formation of PVC is associated with disruption of the BBB as demonstrated by contrast-enhancement of T1-weighted and MT MRI (43–45). Vasogenic edema commonly develops following BBB disruption (29). Both inflammation and edema appear as hyperintense areas on T2-weighted and proton density MRI. The lesions shown in Fig. 3 all demonstrate the intense PVC characteristic of acute lesions. Although BBB disruption is associated with inflammation in acute lesions (43–45), a failure to disrupt the BBB might prevent demyelinating factors such as Ab or complement from entering the lesions, thereby preventing demyelination. Contrast enhancement on the T1-weighted images, indicating a BBB disruption, was associated with both demyelinating and nondemyelinating lesion types (Fig. 2, A and B). Certain MRI findings were associated with no histopathological changes, indicating that they might stem from edema or other transitory changes, which suggests that MRI may reveal subtle physiological changes that are important for disease (29). Also, it would not be possible to detect every lesion on MRI given the limited resolution and partial volume effects from the 2-mm slices. Though conventional MRI cannot demonstrate myelin or myelin breakdown products (46), proton magnetic resonance spectroscopy has been used to monitor myelin breakdown and has associated demyelination in the brains of MS patients to areas of contrast enhancement. This suggests that demyelination is also an early event in lesion development, closely following PVC formation and the opening of the BBB.

Of particular concern for clinical therapy is the prevention of demyelinating lesions. These lesions are accompanied by serious damage to the myelin sheath and nerve conduction dysfunction, and they may be a cause of irreversible nerve degeneration or death (6). Immunocytochemical analysis suggested important differences between demyelinating and nondemyelinating disease. We observed that nondemyelinated lesions had a large excess of T cells, fewer macrophages, and no Ig. By contrast, demyelinated lesions exhibited few T cells, many macrophages, and circumferential depositions of Ig. The occurrence of Ig deposits only in demyelinating disease is likely to be a manifestation of the humoral response against MOG that we found only in animals that had demyelinating disease. All these data are consistent with the model that lesions in EAE and MS are believed to result from a multistep process initiated by myelin Ag-specific Th1-type T cells that infiltrate the perivascular white matter (1, 7). These T cells may catalyze the disease by disrupting the BBB that allows macrophages, B cells, other cell types, and potentially pathogenic Abs to enter the lesion (1, 4, 11, 30, 47–49). Our findings also reveal aspects of the kinetics of these lesions, in that we found that, like MS, infiltrates comprising mostly T cells typify early lesions, whereas in older lesions, macrophages predominate (30).

A spectrum of stages of lesion development from early acute lesions to inactive chronic lesions can be observed by histopathology in an MS brain (10, 30). PVC have been observed in otherwise normal white matter of MS patients, suggesting that PVC formation is an early event in the evolution of the MS lesion and that it can occur in the absence of demyelination (30). Adams (30) suggests that these nondemyelinating MS lesions could represent an aborted disease process. Disease induction in marmoset EAE by immunization may tend to synchronize lesion development, resulting in a predominance of one lesion stage. The animals in which the inflammatory, nondemyelinating type of lesion predominate may represent an early stage in lesion development. We suggest that these lesions are arrested at this early stage and hypothesize that this could be due to a checkpoint in demyelinating disease progression similar to that proposed by Mathis and colleagues (50) to describe the evolution of diabetes in the nonobese diabetic mouse (NOD). The early stage of NOD disease is insulitis that consists of an invasion of the islets with T cells that do not damage the pancreatic islets. In male NOD mice, the disease rarely progresses beyond the checkpoint, and overt diabetes is uncommon. In females, factors such as macrophage recruitment, alterations in regulatory cytokines, the Th1/Th2 balance, or epitope spreading may lead to destruction of the islet cells and diabetes (50). Our data suggests that while MOG may be an important factor in the demyelination process, other, more abundant myelin...
Ags, MBP and PLP, can act as the inciting Ags of perivascular inflammation that may progress after determinant spreading. Our model of MP4 immunization in marmosets to produce either inflammatory or demyelinating disease may allow us to uncover the critical elements that promote progression of disease and how these can be arrested therapeutically.

Acknowledgments

We thank Drs. Stefan Brocke, Henry McFarland, and Richard Asofsky for helpful discussions; Ms. Nicole Belmar for expert technical assistance; the photography department at the National Institutes of Health for their fine work; and Drs. Andrea Barnes, Judy Davis, Randy Elkins, Mark Haynes, Georgina Miller, and Charles Watson for helpful discussions and veterinary care.

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