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*J Immunol* 2001; 166:2116-2121;
doi: 10.4049/jimmunol.166.3.2116
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Effective Antigen-Specific Immunotherapy in the Marmoset Model of Multiple Sclerosis


Mature T cells initially respond to Ag by activation and expansion, but high and repeated doses of Ag cause programmed cell death and can suppress T cell-mediated diseases in rodents. We evaluated repeated systemic Ag administration in a marmoset model of experimental allergic encephalomyelitis that closely resembles the human disease multiple sclerosis. We found that treatment with MP4, a chimeric, recombinant polypeptide containing human myelin basic protein and human proteolipid protein epitopes, prevented clinical symptoms and did not exacerbate disease. CNS lesions were also reduced as assessed in vivo by magnetic resonance imaging. Thus, specific Ag-directed therapy can be effective and nontoxic in primates. The Journal of Immunology, 2001, 166: 2116–2121.

Multiple sclerosis (MS) is a paralytic disease involving destruction of myelin sheaths surrounding axons in the CNS. MS affects young adults, most often women residing in northern latitudes. The disease exhibits relapsing and remitting symptoms including disturbances in vision, speech, coordination, and cognition as well as weakness, spasticity, and paralysis. Lymphocytic infiltration in the CNS white matter and immune reactions against myelin Ags indicate an autoimmune etiology for MS. Allergic encephalomyelitis was first observed as a side effect of the rabies vaccine prepared from rabbit brains by Pasteur in the 1880s. Rivers and others showed that the CNS inflammation was caused not by the rabies virus but by immune sensitization to the combination of adjuvant and brain tissue contaminating the vaccine. Experimental allergic encephalomyelitis (EAE) models in various animal species, typically rodents, were later developed by immunization with myelin proteins in adjuvant or by the adoptive transfer of myelin-reactive T cells, causing inflammatory damage to the white matter.

Received for publication August 2, 2000. Accepted for publication October 31, 2000.

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4 Abbreviations used in this paper: MS, multiple sclerosis; MBP, myelin basic protein; PLP, proteolipid protein; EAE, experimental allergic encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MAG, myelin-associated glycoprotein; eMOG, rat myelin oligodendrocyte glycoprotein; MT, magnetization transfer; MRI, magnetic resonance imaging; HPRT, hypoxanthine phosphoribosyltransferase.
in the macaque model (31, 32). However, EAE in macaques involves hemorrhagic lesions with neutrophil infiltrates that are not characteristic of MS (7, 30–32). A new nonhuman primate model of EAE in Callithrix jacchus jacchus (common marmoset) has been developed that has clinical and pathological features closely resembling those of MS (7, 8). Disease in marmosets involves predominantly perivascular lymphocytic infiltrates and demyelination. The marmoset model has advantages over rodent EAE models in that a rigorous neurological examination and the evaluation of lesions by high resolution magnetic resonance imaging (MRI) are possible (8, 29, 33). Only a single study of Ag treatment of EAE, which used the low abundance MOG protein, has been conducted in marmosets, with the ominous finding that acute disease was suppressed but severe fatal disease rebounded after treatment cessation (34). However, the previous study did not resolve the important question that remains, whether a defined protein representing the abundant myelin epitopes could have a significant and durable therapeutic effect in primates. Here we show that Ag treatment of marmoset EAE achieves a clinical benefit without severe late toxicity.

Materials and Methods

Animals

Nine C. jacchus jacchus marmosets 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, all males, ranged in age from ~1 year, 8 mo to 2 years, 2 mo 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.

Antigens

MP4 was prepared by metal affinity chromatography and reversed phase HPLC as previously described (20). The recombinant extracellular domain of rat MOG (rMOG), was prepared as described (35).

Induction of EAE

MP4 was emulsified 1:2 in TiterMax adjuvant (Vaxcel, Norcross, GA). Animals received 100 μl intradermal injections containing a total of 1 mg MP4 at four sites on the back. On the day of immunization and again 2 days later, all immunized animals were given an i.v. injection of 5 ml sterile normal saline containing 10⁶ killed Bordetella pertussis organisms. The B. pertussis was kindly provided by Dr. Pat Van Zandt (Wyeth-Lederle Vaccines, Madison, NJ).

Ab responses

Serum Ab titers were tested in duplicate by ELISA (36). ELISA plates (Pierce, Rockford, IL) were coated overnight with 1 μg/well rMOG or MBP in 0.25 M carbonate buffer (pH 8.6), washed with PBS containing 0.05% Tween 20, and blocked with 1% BSA in the same buffer. After washing, 100 μl of a 1:200 or appropriate dilution of immune sera were added. Plates were developed with 0.2 mg/well o-phenylenediamine dihydrochloride in 0.1 M phosphate-citrate buffer (pH 5.0; Sigma) for 30 min and read at 490 nm. Test wells were prepared in triplicate with medium only, 50 or 100 μg/ml MP4, or 1 μg/ml Con A (Boehringer Mannheim, Indianapolis, IN) and pulsed with 1 μCi (methyl-³H)thymidine (Amersham, Arlington Heights, IL). After 3 days of incubation, plates were harvested on a Tomtec MachIII (Wallac, Gaithersburg, MD), and analyzed using a Betaplate 1205 scintillation counter (Wallac).

Cytokine RT-PCR

PCR were conducted as previously described (34) for primers (5’ to 3’ sequences): HPRT 5’, TGAGCCAGTCAACAGGGGC; HPRT 3’, GCTCT ACTAAGCAGATGGC; IFN-γ 5’, CCTTGTACTGCCCAGGACC; IFN-γ 3’, CGTCTGACTCTTCTCTGCC; IL-10 5’, GGTACTGGTGGTGC CAAGECT; IL-10 3’ (37), CTCTTATGTAGTGATGAGTGCT; TGF-β 5’, GCCCTGGACACCAACTCTGC; TGF-β 3’, GTGCCATTGGCCAG GGCAC. IL-4 5’, TGTCACCGGCACAAGTGCGCA; IL-4 3’, CATGAT CGTCTTATGGCTTCC.

Clinical and pathological evaluation of EAE

Marmosets were observed daily, and clinical symptoms were scored as previously described (Table I) (7). At 105 days after immunization, animals were euthanized, and the brain and spinal cord were removed and fixed in Formal-Fixx (Shandon, Pittsburgh, PA). Sections of 3 mm were prepared in coronal, transverse, or longitudinal orientations using tissue fragments embedded in paraffin. The sections were stained using hematoxylin and eosin, Luxol fast blue, or Bodian’s silver stain techniques (American Histolabs, Gaithersburg, MD). Histopathological sections of CNS were scored in a blinded manner as previously described (7) with minor changes as described in Table I. Typically, seven to nine coronal and transverse 3-mm sections of the entire spinal cord were evaluated. Photomicrographs were taken on an Axiopt microscope (Carl Zeiss, Thornwood, NY).

Magnetic resonance imaging

Scans were performed in the coronal plane with 2-mm interleaved slices on a Sigma 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 (29, 33). T1-weighted and MT images were performed before and after i.v. administration of the contrast agent gadopentetate dimeglumine 0.3 mmol/kg (Magnevist; Berlex Laboratories, Cedar Knolls, NJ). Scans were interpreted in a blinded manner.

Results

High dose i.v. MP4 administration abrogates clinical symptoms

Nine male marmosets were randomly assigned to three treatment groups. Siblings (two sets) were placed in different groups. The groups received 1 ml 5% dextrose in sterile water containing 6 mg MP4 (high dose), 0.6 mg MP4 (low dose), or 0 mg MP4 (sham). The 6-mg high treatment dose was based on a body weight scale-up from a dose that eliminated disease in rodents (14, 18–21). The treatments were administered through an indwelling venous catheter in the tail twice daily at ~10 a.m. and 6 p.m., on days

| Table I. Summary of clinical and histopathological data |
|----------------|----------------|---------------|-------|
| Animal Treatment Clinical a Onset b Inflammation c Demyelination c |
| J43 | Sham | 2 | 14 | 2 | 0 |
| J81 | Sham | 2 | 7 | 2 | 1 |
| L9 | Sham | 1 | 29 | 1 | 0 |
| J80 | Low dose | 0 | None | 1 | 0 |
| J106 | Low dose | 2 | 24 | 3 | 0 |
| J54 | Low dose | 2 | 42 | 1 | 2 |
| J97 | High dose | 0 | None | 0 | 1 |
| J88 | High dose | 0 | None | 2 | 1 |
| J42 | High dose | 0 | None | 0 | 0 |

a Maximum observed clinical disease score defined as: 0 = normal; 1 = lethargy, anorexia, weight loss; 2 = para- or monoparesis, ataxia, sensory loss, incontinence, anisocoria; and 3 = para- or hemiplegia.

b Onset of clinical symptoms (days postimmunization).

c Inflammation and demyelination were assessed on microscopic sections taken postmortem at the end of the 105-day clinical observation period, and the slides were scored in a blinded manner separately for inflammation and demyelination as follows: 0 = no inflammation present; 1 = minimal (1–3 lesions/average section); 2 = moderate (3–10 lesions/average section); 3 = extensive; 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.
FIGURE 1. Abrogation of clinical symptoms of EAE by high dose MP4 immunotherapy. Histograms of clinical disease scores for the sham treatment group (top), 0.6-mg/dose MP4 treatment group (center), and 6.0-mg/ml treatment group (bottom) as a function of days postimmunization. Individual animal identification numbers are indicated at the right of each treatment panel.

MP4 treatment delays white matter disease evident on MRI

We used serial MRIs performed throughout the 105-day observation period to evaluate white matter disease in real time. MRI changes occurred in all animals with clear differences between the treatment groups (Fig. 2, Table II). First, the onset of MRI changes for the sham-treated animals was at least 2 wk earlier than that of the treatment groups. White matter disease on MRI correlated well with clinical symptoms for the sham and low dose groups. Second, despite the lack of clinical symptoms, animals in the high dose group exhibited MRI changes indicating that the disease process was not completely abolished. Third, severe white matter disease (score, 3) was reached by two of three sham-treated animals, by one of the low dose animals, and in none of the high dose animals. Moreover, only one of three animals in the high dose group reached a score of 2, and the mean MRI score for the high dose group was reduced at all time points relative to the sham-treated animals. Thus, serial MRI imaging demonstrated ameliorative effects of both doses of MP4 treatment in live animals.

Histopathology shows reduced CNS disease in the high dose group

After 105 days, we evaluated pathology in coronal brain and spinal cord sections (Table I). Significant inflammatory infiltrates were found in all animals in both the sham and low dose groups, but only one animal in the high dose group showed perivascular infiltrates in the CNS. Relatively little demyelination was observed in any of the animals, possibly because of the large time interval between the early occurrence of severe clinical/MRI findings and the time of the pathological analyses. Remyelination obscures evidence of early demyelinating disease (1–5).

Decreased proliferation responses and antimyelin Ab production

PBL proliferative responses to the MP4 Ag were also evaluated (Table III). At 18 days postimmunization (9 days post treatment), proliferative responses were seen in all groups, but the mean stimulation index for the sham-treated animals was 21.0 as compared with a mean of 8.3 for the high dose group. Control proliferative responses to Con A did not differ between groups (data not shown). At later time points, significant differences in MP4-specific responses between treated and untreated animals were not observed (data not shown), but were similar to levels shown at 18 days postimmunization.

ELISA was used to measure humoral responses induced to the myelin proteins MBP, PLP, and MOG. Our interest here was to evaluate the effect of treatment on immune responses to the individual marmoset myelin components and not just to the sensitizing Ag MP4. ELISA detected MBP-specific Abs as early as wk 2 postimmunization but anti-MBP titers were decreased in the high dose group (Fig. 3). One sham-treated marmoset, J81, but no other animals showed clear evidence of epitope spreading with markedly elevated anti-MOG titers that we have previously correlated with demyelination in marmosets (29).

No evidence of immune deviation in MP4-treated marmosets

Previously, MOG treatment of marmoset EAE was suggested to deviate T cells from a Th1 to a Th2 response (34). Cytokine mRNA production was therefore evaluated by semiquantitative RT-PCR using mRNA samples obtained at day 16 (1 wk after the final treatment) from either the sham-treated or the high dose MP4-treated animals (34, 38). Relative mRNA amounts of the Th1 cytokine, IFN-γ, the Th2 cytokines, IL-4 and IL-10, and TGF-β are shown in Fig. 4 as a fraction of HPRT control mRNA. Only IFN-γ
The hyperacute disease observed in the previous marmoset study could pose a very serious drawback to using Ag treatment in human MS (34). The difference between the previous study and our own likely lies in the fact that different Ags were used for treatment. In the prior study, marmosets were immunized with MOG and then treated with an 11-day course of i.p. injections of MOG every other day (34). A hallmark of the disease in that study was severe demyelination possibly due to activated B cells that caused an Ab response against MOG (29, 34, 39). Severe demyelinating disease can also be achieved by immunizing marmosets with total white matter which contains a small fraction of MOG (7, 8). MOG has been consistently shown to provoke strong Ab responses that cause severe demyelination and in the previous study, anti-MOG Ab levels were higher in the treated animals than in the placebo controls at day 21 (29, 34, 42). In contrast, using MBP/PLP epitopes, we found that disease was potently suppressed and that Ab levels against MBP were lower rather than higher in the treated animals than in the placebo controls at day 21 (29, 34, 42). With MBP/PLP treatment, we found that disease was potentely suppressed and that Ab levels against MBP were lower rather than higher in the treated animals than in the placebo controls at day 21 (29, 34, 42).

High dose MP4 treatment eliminated clinical disease, but it was evident from the MRI scans that CNS tissue effects, although reduced, were not completely abolished. However, the mean MRI scores for the treated animals never reached the levels observed in the untreated animals, which peaked at ~8 wk and then fell to

### Table II. MRI scores

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Examination Week 1</th>
<th>Examination Week 2</th>
<th>Examination Week 3</th>
<th>Examination Week 4</th>
<th>Examination Week 5</th>
<th>Examination Week 6</th>
<th>Examination Week 7</th>
<th>Examination Week 8</th>
<th>Examination Week 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>J43 Sham</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2.5</td>
<td>2</td>
</tr>
<tr>
<td>J81 Sham</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>1</td>
<td>1</td>
<td>1.5</td>
<td>1.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L9</td>
<td>0</td>
<td>0.5</td>
<td>ND</td>
<td>2.5</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>J80 Low dose</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>J106 Low dose</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0/1</td>
<td>1</td>
<td>1.5/2</td>
</tr>
<tr>
<td>J54 Low dose</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>J97 High dose</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>J88 High dose</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>J42 High dose</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0/1</td>
<td>0/1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Maximum observed MRI score: 0 = normal; 1 = 1–5 T2 lesions; 2 = 5–10 T2 lesions; 3 = >10 lesions or focal and diffuse abnormalities; 4 = extensive diffuse white matter abnormalities. The scores were increased if contrast-enhancing lesions indicative of active disease and blood brain barrier disruption were detected (0.5 point for one lesion present and 1 point for two or more lesions). Scores separated by a slash (e.g., 0/1) signify examinations in which it was difficult to assign an exact score.

### Discussion

MS is a potentially severe paralytic disease for which no cure is presently known. Much research has focused on new therapeutic approaches to inhibit the immune processes that are believed to initiate CNS damage in MS. The importance of local immune responses in the brain for disease has been demonstrated by the fact that immune modulation by nerve growth factor can ameliorate EAE when administered in the cerebral ventricles (40). Considerable progress has been made in Ag-specific immunomodulation of rodent EAE. For example, Ag-induced T lymphocyte apoptosis can abort the encephalitogenic process (14). Very little has been done to extend these studies to nonhuman primates despite the fact that Kabat and Morgan in the 1940s showed that immunization could induce hemorrhagic EAE and Eylar and Brostoff showed that MBP could ameliorate this form of the encephalitic disease (31, 32). The marmoset model of EAE has clinical and pathological features (7, 41) that more closely resembles those of MS, yet only a single study has previously examined Ag treatment with unsuccessful results (34). It was therefore of great interest to explore whether different Ag preparations could achieve a beneficial effect without toxicity. In particular, it was important to examine abundant myelin Ags, because these might be targets of the encephalitogenic immune responses in MS patients (9–12, 42). Despite the restricted availability of C. jacchus jacchus, the species used for marmoset EAE, we conducted a pilot study to evaluate the feasibility of Ag therapy. The results we have obtained differ markedly from the previous study reporting treatment with repeated doses of MOG (34), and several conclusions can be made.

First and foremost, it was clear that high dose Ag treatment decreased disease as judged by clinical and MRI evaluation. Histopathology also showed that Ag treatment was associated with less lymphocyte infiltration. We also found decreased T cell proliferative responses and Ab production in Ag-treated animals. Taken together, these results are consistent with previous observations in rodent EAE that soluble i.v. Ag decreases rather than enhances cognate immune reactions (14) and suggest that Ag therapy could be safe and effective for T cell-mediated autoimmune diseases. One of the most important outcomes of our study is that we did not find the emergence of severe demyelination or fatalities after Ag administration as previously observed with MOG treatment (34).
levels similar to those of the high dose MP4-treated animals by 15 wk. This lower level of CNS disease intensity on MRI in treated animals is reflected in the absence of clinical symptoms. There is a general correlation in the untreated group between trends in clinical symptoms and MRI data. The appearance of disease symptoms in some cases preceded evidence of CNS disease as demonstrated by MRI. This is likely due to technical limitations in our ability to visualize tiny, early lesions of <0.5 mm (33). In some animals, CNS disease was observed in the absence of clinical symptoms. This is a common observation in EAE and MS and may reflect the fact that most lesions detected by MRI are clinically silent, perhaps because of functional redundancy in the brain or lack of involvement of vital neural pathways to clinically apparent levels (33, 43). A larger lesion load, as was observed in the untreated animals, may increase the likelihood that a lesion will appear in an area in the CNS in which damage can produce an observable clinical outcome. There is also a general correlation between the inflammation demonstrated by histopathology and the MRI and clinical score. Because of the many changes that may have occurred over time including decreased inflammation and remyelination, the histopathology may only dimly reflect the original appearance and number of the lesions, some of which may have been 100 days old. The limitations of histopathology in assessing the efficacy of a CNS immunotherapy highlight the value of MRI in studies of this kind. With these considerations in mind, the clinical, histopathological, and MRI data tell a consistent story: that high dose Ag immunotherapy was effective in reducing the lesion load and eliminating the clinical symptoms of EAE.

Previously, we documented determinant spreading to MOG in three of four animals immunized with MP4 in adjuvant (29). Anti-MOG Abs occurred in only one of the three untreated animals, J81, which was the only animal to show persisting demyelination. This supports the association of determinant spreading to MOG with demyelination in MP4-immunized marmosets (29, 36). We observed no determinant spreading in MP4-treated animals by anti-MOG Ab titers; thus, abolition of immune responses to the abundant myelin Ags might have prevented demyelination by limiting determinant spreading. Additional studies with a greater number of animals are necessary to validate these trends. Nevertheless, our results suggest that the choice of Ag for immunomodulation may be critical for successful treatment and provide new hope for Ag-specific therapy in humans.

Acknowledgments
We thank Dr. Stefan Brocke and Dr. Henry McFarland for helpful discussions and support; Michael Gates and Nicole Belmar for expert technical assistance; Drs. Akiko Iwasaki, Clara Pelfrey, and Steven Wood for a critical reading of the manuscript; and Dr. Andrea Barnes and Dr. Judy Davis for helpful discussions and veterinary care. We also thank Dr. Story Landis and the National Institute of Neurological Disorders and Stroke for generously providing primate housing and care.

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