Fast Progression of Recombinant Human Myelin/Oligodendrocyte Glycoprotein (MOG)-Induced Experimental Autoimmune Encephalomyelitis in Marmosets Is Associated with the Activation of MOG\textsuperscript{34–56}-Specific Cytotoxic T Cells

Yolanda S. Kap, Paul Smith, S. Anwar Jagessar, Ed Remarque, Erwin Blezer, Gustav J. Strijkers, Jon D. Laman, Rogier Q. Hintzen, Jan Bauer, Herbert P. M. Brok and Bert A. ’t Hart

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Fast Progression of Recombinant Human Myelin/Oligodendrocyte Glycoprotein (MOG)-Induced Experimental Autoimmune Encephalomyelitis in Marmosets Is Associated with the Activation of MOG<sub>34–56</sub>-Specific Cytotoxic T Cells

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The recombinant human (rh) myelin/oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) model in the common marmoset is characterized by 100% disease incidence, a chronic disease course, and a variable time interval between immunization and neurological impairment. We investigated whether monkeys with fast and slow disease progression display different anti-MOG T or B cell responses and analyzed the underlying pathogenic mechanism(s). The results show that fast progressor monkeys display a significantly wider specificity diversification of anti-MOG T cells at necropsy than slow progressors, especially against MOG<sub>34–56</sub> and MOG<sub>74–96</sub>. MOG<sub>34–56</sub> emerged as a critical encephalitogenic peptide, inducing severe neurological disease and multiple lesions with inflammation, demyelination, and axonal injury in the CNS. Although EAE was not observed in MOG<sub>74–96</sub>-immunized monkeys, weak T cell responses against MOG<sub>34–56</sub> and low grade CNS pathology were detected. When these cases received a booster immunization with MOG<sub>34–56</sub> in IFA, full-blown EAE developed. MOG<sub>34–56</sub>-reactive T cells expressed CD3, CD4, or CD8 and CD56, but not CD16. Moreover, MOG<sub>34–56</sub>-specific T cell lines displayed specific cytotoxic activity against peptide-pulsed B cell lines. The phenotype and cytotoxic activity suggest that these cells are NK-CTL. These results support the concept that cytotoxic cells may play a role in the pathogenesis of multiple sclerosis. *The Journal of Immunology, 2008, 180: 1326–1337.*

Multiple sclerosis (MS) is a chronic progressive inflammatory demyelinating disease of the human CNS. The pathological hallmark of MS is the lesion, being in the majority of cases a sharply demarcated demyelinated area in the CNS white matter (WM) that expresses variable degrees of inflammation, axonal injury, gliosis, and remyelination (1, 2). Although the trigger of MS is not known, it is generally believed that lesion formation involves the synergistic action of cellular and humoral autoimmune reactions directed against components of the myelin sheath (3). Antimyelin autoimmune reactions may be induced by viruses that share cross-reactive T and B cell epitopes with myelin Ags, a phenomenon known as molecular mimicry (4).

In response to the requirement for a useful preclinical model for efficacy evaluations of new biopharmaceutical agents for the treatment of MS, we have developed a chronic progressive experimental autoimmune encephalomyelitis (EAE) model in the New World primate species, the common marmoset (Callithrix jacchus). This EAE model has several intriguing aspects that also make it a highly useful disease model for basic research, including the 100% incidence despite the outbred nature, the chronic progressive course (reviewed in Refs. 5 and 6), and the heterogeneous pathology present in white and gray matter that ranges from early active to chronic inactive/remyelinating lesions (6–8). Moreover, using serially applied magnetic resonance (MR) imaging (MRI) sequences, brain lesions can be visualized and characterized in relation to the expression of an overt neurological deficit (9–11).

Several lines of evidence point to a critical role of autoimmune reactions directed against myelin/oligodendrocyte glycoprotein (MOG) in the induction of chronic progressive EAE. Marmosets immunized with a chimeric protein combining myelin basic protein (MBP) and proteolipid protein (PLP) developed clinical EAE only after the spreading of the autoimmune reaction to MOG has taken place (12). Moreover, the development of
chronic progressive EAE in both Biozzi ABH mice (13) and marmosets (S. A. Jagessar, P. A. Smith, E. Blezer, C. Delarasse, D. Pham-Dinh, J. D. Laman, J. Bauer, S. Amor, and B. ‘t Hart, manuscript in preparation) is impaired when the animals were immunized with MOG-deficient mouse myelin.

The marmoset EAE model induced with recombinant human (rh) MOG1–125 is characterized by a 100% disease incidence but a variable clinical course. The high suscpetibility of marmosets to this model maps to an invariant MHC class II molecule, Caja-DRB*W1201, which emerged as a dominant restriction element for the activation of CD4+ T cells specific for the epitope MOG24–36 (14). The monomorphic allele is present and expressed in MOG34–56in CFA developed CNS inflammation and widespread demyelination in the white and gray matter, which may be caused by cytotoxic activity of infiltrated T cells. In support of this, MOG34–56-specific T cell lines (TCL) were found to express markers of NK-CTL (CD3+, CD4+ or CD8+, CD56+, and CD16+) and to lyse peptide-pulsed, autologous as well as allogeneic EBV-transformed B cell lines.

Materials and Methods

Animals

All monkeys included in the current study were purchased from two purpose-bred colonies, one being kept at the Biomedical Primate Research Centre (Rijswijk, The Netherlands) and the second at the German Primate Center (Goettingen, Germany). Individual data of all monkeys used in this study are listed in Table I. Monkeys were included in the study only after a complete physical, hematological, and biochemical checkup had been performed. The reported experiments span a period of 6 years. During the experiments, the monkeys were initially housed individually in spacious cages with a padded shelter provided on the floor and were under intensive veterinary care. Since 2005, pair housing became the standard within the Biomedical Primate Research Centre. The daily diet during the study consisted of commercial food pellets for New World monkeys (Special Diet Services) supplemented with rice, raisins, peanuts, marshmallows, biscuits, fresh fruit, grasshoppers, and maggots. Drinking water was provided ad libitum.

Ethics

In accordance with the Dutch law on animal experimentation, all study protocols and experimental procedures were reviewed and approved by the Institute’s Ethics Committee before the experiments could be started.

RhMOG-induced EAE

A recombinant protein encompassing the extracellular domain of human MOG was produced in Escherichia coli and purified as previously described (18). RhMOG-induced EAE was evoked in a total of 23 monkeys by injection into the dorsal skin of a 600-μl stable emulsion containing 100 μg of rhMOG in 300 μl of buffered saline and 300 μl of CFA (Difco Laboratories) under ketamine anesthesia (40 mg/kg; AST Pharma) as described previously (14).

MOG peptide-induced EAE

All MOG peptides for immunization and cell culture were purchased from Prof. A. Ben Nun (Weizmann Institute of Sciences, Rehovot, Israel) or from ABC Biotechnology. EAE was induced with synthetic peptides that represent aa 34 to 56 (MOG34–56) and 74 to 96 (MOG74–96) of the human MOG extracellular domain. The monkeys were immunized with 100 μg of MOG peptide dissolved in 300 μl of buffered saline and 300 μl of CFA as previously described (14). Monkeys that did not develop an overt neurological deficit (score ≥ 2.0) within 28 days received booster immunizations with the same amount of peptide in IFA at the time points indicated in Figs. 2 and 6. Antigen-adjuvant emulsion was prepared by gentle stirring of the PBS/oil mixture at 4°C for at least 1 h.

Clinical scoring

Clinical signs were scored twice daily by trained observers using a previously described semiquantitative scale (19). Briefly, 0 = no clinical signs; 0.5 = apathy, loss of appetite, altered walking pattern without ataxia; 1 = lethargy, anorexia, tail paralysis, tremor; 2 = ataxia, optic disease; 2.5 = paraparesis or monoparasiparesis, sensory loss, brain stem syndrome; 3 = paraplegia or hemiplegia; 4 = quadriparesis; and 5 = spontaneous death attributable to EAE. A score of 2 or higher reflects an overt neurological deficit. Monkeys were sacrificed for ethical reasons once complete paralysis of hind limbs (score ≥ 3.0) was observed or at the predetermined endpoint of clinical scoring.

### Table I. Overview of monkeys included in this study

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Twin Pair</th>
<th>Sex</th>
<th>Age</th>
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<th>Experiment</th>
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<td>MOG34–56 plus MOG34–56</td>
<td>3, B</td>
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</table>

* Twin pairs are indicated by “Twin.”
  * M, Male; F, female.
  * Age in months of the monkeys at the start of the experiment.
the study. Moreover, monkeys were weighed three times per week. As in rodent EAE models, body weight serves as a reliable surrogate disease marker in the marmoset. Body weight data are depicted as a separate disease parameter above the clinical score graphs.

Ex vivo analysis of T cell responses

The maximum blood sample that can be collected in a month from primates at the BPRC should not exceed 1% of the body weight. For an average adult marmoset weighing 350 grams, this equals a maximum monthly blood sample of 3.5 ml. Hence, volumes of up to 1.5 ml at 2-wk intervals were collected into heparinized Vacutainer tubes (Greiner). PBMC were isolated from heparinized venous blood using lymphocyte separation medium (ICN Biomedicals). Moreover, cell suspensions were prepared from aseptically removed axillary (ALN), inguinal (ILN), and cervical lymph nodes (CLN) and spleen. PBMC, lymph node, and spleen cells were cultured in triplicate for the detection of proliferative responses toward rhMOG and a panel of MOG peptides as previously described [14]. In some assays, PLP peptide 139–151 (PLP139–151), recombinant human MBP, and recombinant human β-crystallin (both provided by Dr. J.M. van Noort, TNO-Preventie en Gezondheid, Leiden, The Netherlands) were included. OVA served as control Ag in all studies. All Ags were tested at 5 μg/ml. After 48 h of culture, 0.5 μCi of tritiated thymidine ([3H]Thy) was added per well, and incorporation of the radiolabel was determined after 18 h using a matrix 9600 beta counter (Packard). Results are expressed as the mean stimulation index (SI). SI values above 2.0 were considered to be relevant.

Flow cytometry and CFSE labeling

To determine the phenotype of proliferating cells, 4 × 10⁶ viable mononuclear cells (MNC) from ALN were suspended in 1 ml PBS and incubated for 7 min at room temperature with CFSE (final concentration 1.5 μM; Fluka). The labeled cells were cultured for 7 days with peptides under the standard conditions described above. For flow cytometric analysis we used the following commercially available, labeled mAbs directed against human CD markers: anti-CD3 with PerCP or Alexa Fluor 700 label (BD Biosciences), allophycocyanin-labeled anti-CD4 (DakoCytomation), biotinylated anti-CD8 (SeroTec), anti-CD56 with PE-Cy7 label, anti-CD16-PE, and streptavidin PE-Cy7 or streptavidin PerCP (BD Biosciences). Flow cytometric analysis was performed on a FACSort flow cytometer using FACSDiva software (BD Biosciences). First, viable cells were gated using the live/dead fixable violet viability stain (Invitrogen Life Technologies). Within the viable cell gate, lymphocytes/monocytes were selected using forward and side scatter. Within the lymphocyte/monocyte gate, CD3+ cells were selected. The CD3+ population in the CFSE experiment consisted of CD4+ cells (48–70%) and CD8+ cells (12–18%). Within each gated subpopulation the percentage of cells with CFSE dilution was calculated.

Cytotoxicity assay

Autologous and allogeneic peptide-pulsed, 51chromium-labeled, EBV-transformed B cell lines were used as target cells to test the cytotoxic potential of MOG peptide-specific TCl. In brief, 10⁶ B cells were incubated for 60 min at 37°C with 51chromium and pulsed with 100 μg of MOG34–56, or MOG74–96 and subsequently washed thoroughly with buffered saline. Peptide-pulsed B cells were mixed with effector T cells at 1:1, 1:4, and 1:16 ratios in U-well microtiter plates and cultured for 5 days at 37°C. Every 2 or 3 days, half of the culture supernatant was replaced with fresh medium containing 20 U/ml rhIL-2 (Proleukin; Chiron) and split when needed. After 14 to 21 days of culture, part of the cells were transferred into 96-well flat-bottom plates (Greiner) and tested for reactivity with a

Table II. T cell and Ab responses against rhMOG and MOG peptides in rhMOG-immunized marmosets

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Sex</th>
<th>Sacrificed (pd)</th>
<th>EAE Score</th>
<th>T-cell reactivity (MOG Peptides)</th>
<th>Ab Reactivity2</th>
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<td>(n = 23)</td>
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</table>

a Male; F, female.

1 MOG peptides of interest are indicated in bold.

2 Ab reactivity was tested in necropsy serum.

3 For the serum reactivity with MOG peptides (pMOG), only the number of recognized peptides is given.

4 NT, Not tested.

Marmosets with positive MRI, no mild clinical EAE.

Generation of MOG peptide-reactive TCl

At necropsy, cell suspensions were prepared from spleen, ALN, ILN, and CLN. MNC of rhMOG- or MOG peptide-immunized marmosets were stimulated ex vivo with rhMOG, MOG34–56, or MOG74–96, to establish specific TCl. Briefly, MNC (10⁶/well) were seeded into 24-well plates (Greiner) and stimulated with 10 μg/ml rhMOG, MOG34–56, or MOG74–96. Every 2 or 3 days, half of the culture supernatant was replaced with fresh medium containing 20 U/ml rhIL-2 (Proleukin; Chiron) and split when needed. After 14 to 21 days of culture, part of the cells were transferred into 96-well flat-bottom plates (Greiner) and tested for reactivity with a
panel of 23-mer MOG peptides (14). Lethally irradiated (50 gray) EBV-transformed marmoset B cells from stably growing lines maintained in 75-cm² tissue culture flasks (Greiner) were used as APC. Lines of interest were characterized by the expression of T cell-specific cell surface markers by flow cytometry using cross-reactive mAbs raised against human CD markers (20). Isotype controls were kindly provided by J. Miller (Chemi-con International).

B cell responses

Venous blood samples were centrifuged and the plasma supernatants were collected and stored frozen at −20°C until further analysis. Ab binding to myelin proteins (rhMOG, MBP, αβ-crystallin, and HPLC-purified human PLP) or to a panel of overlapping 23-mer MOG peptide sequences was determined using ELISA (21). Bound Ab was detected using polyclonal alkaline phosphatase-conjugated goat-anti-monkey IgM (Rock-land) or rabbit-anti-human IgG (Abcam). Ab specific for discontinuous MOG epitopes are considered particularly pathogenic (22). To distinguish between Ab reactivity against discontinuous and linear epitopes, serum samples were preincubated with a mixture of all overlapping MOG peptides (10 μg/ml for each peptide) for 1 h at 37°C before probing them for reactivity with rhMOG coated onto ELISA plates. As an internal control, the MOG54–76 peptide was left out of the peptide mix used for preincubation because in previous studies this peptide was found to contain dominant B cell epitopes. The results of the Ab assays are expressed as the fold increase of light absorbance at 405 nm compared with the reactivity with OVA as an irrelevant Ag or compared with the reactivity in preimmune sera of the same monkeys.

Postmortem examination

Monkeys selected for necropsy were first deeply sedated by i.m. injection of ketamine (50 mg/kg), and subsequently euthanized by the infusion of pentobarbital sodium (Euthesate; Apharmo). Brain, spinal cord, spleen, ILN, ALN, and CLN were removed. Representative parts of all organs were snap frozen in liquid nitrogen or fixed in 4% buffered formalin. Frozen tissues were stored at −80°C. After at least 7 days of fixation in formalin, the tissues were transferred into buffered saline containing sodium azide for stabilization before MRI (11).

To assess the total lesion load in the brain, MR images were made of formalin-fixed brains as described previously (11, 19). Both frozen and fixed tissues were examined with histological and immunohistochemical techniques as previously described (19, 23, 24).

MRI procedures

Brains of MOG34–56- and MOG74–96-immunized animals were analyzed by MRI. MRI experiments were performed ex vivo on a 6.3 T horizontal bore MRI scanner (Varian). The formalin-fixed brains were submerged in a perfluoropolyether (Fomblin) for susceptibility matching. The following parameters were used in all experiments: field of view, 2.5 × 2.5 cm²; matrix, 128 × 128; slice thickness, 1 mm; number of slices, 20. T2-weighted (T2W) images were collected using a spin echo sequence with the
following parameters: repetition time, 4 s; echo time, 35 ms; number of signal averages, 8. $T_2$ maps were recorded with a multiecho sequence using the following parameters: repetition time, 8 s; echo spacing, 20 ms; echo train length, 8; number of signal averages, 4. Diffusion tensor images were made using a pulsed field gradient spin echo sequence with the following parameters: repetition time, 4 s; echo time, 35 ms; number of signal averages, 8. Diffusion weighting was applied in 10 directions with the following pulsed field gradient parameters: $Δ$, 20 ms; $β$, 10 ms; diffusion gradient ($G_{zz}$), 0; and 120 millitesla/meter (mT/m), resulting in $b$-value of 0 and 1717 s/mm$^2$.

**Image analysis**

First, WM was segmented manually. In the WM, lesions were identified as regions with a $T_2$ value 10% above the normal appearing white matter (NAWM). Average $T_2$, apparent diffusion coefficient, and fractional anisotropy values were determined for lesions and NAWM. Image analysis was done using Mathematica (Wolfram Research Europe).

**Statistics**

The relation between a broad T cell response and fast disease progression (see data in Table II) was analyzed using Kaplan-Meier survival analysis. Statistical significance of differences between groups was calculated using the log rank test. Because four potential contrasts could be chosen for this analysis, $p$ values were considered statistically significant when $<0.05/4 = 0.0125$ (Bonferroni correction).

Results of $T_2$ relaxation time, fractional anisotropy, and apparent diffusion coefficient were analyzed by a two population (independent) $t$ test.

**Results**

**Disease course and autoimmune reactions in rhMOG-immunized monkeys**

Table II gives a summary of data obtained from 23 rhMOG-immunized marmosets. Clinical scores are depicted in Fig. 1, showing that in 100% of the monkeys clinical EAE was induced but that the time interval between EAE induction and the expression of an overt neurological deficit varied considerably. Of the 23 examined monkeys, 20 were sacrificed after they had developed an EAE score of ≥2. Two monkeys were withdrawn from their respective experiments without neurological impairment. One monkey (QK) had experienced serious body weight loss and was sacrificed preterm to avoid a sudden deterioration of the clinical state, as has been observed in rhesus monkeys in which immunization with MOG34–56 induced acute onset EAE (25). Two monkeys (Mi020 and Mi021) were sacrificed with mild signs of EAE (score 0.5) as they reached the end of the experiment. The time interval between EAE induction and the development of hemiplegia/paraplegia (EAE score of 3) varied from 34 to 139 days (mean of 73 days) (Table II). Notably, serial MRI in selected monkeys reveals ample disease activity within the CNS WM during the asymptomatic interval, which apparently does not lead to an overt neurological deficit (10, 26). We have taken advantage of this unique outbred model to test whether monkeys with a fast or slow disease progression rate display different Ab and T cell response patterns.

**Antibodies**

Serum reactivity with rhMOG protein was detected in all monkeys (Table II). The anti-MOG IgG Ab reactivity was directed against linear epitopes within three main regions, namely MOG4–26, MOG24–46, and MOG34–56. Analysis of immune sera that were collected at 2-wk intervals during the entire disease course revealed that the first upcoming Ab reactivity was against MOG54–76. The anti-rhMOG Ab reactivity at necropsy was reduced by >95% when the immune sera were preincubated with a mix of the complete MOG peptide panel before the binding to rhMOG-coated ELISA plates was tested. However, when MOG54–76 was left out of the peptide mix the reduction of serum reactivity with rhMOG was <10% (data not shown). These data indicate that the MOG54–76 peptide contains one or more important recognition sites for an rhMOG-induced Ab. Ab reactivities toward MBP and αB-crystallin were infrequently observed (data not shown). In summary, we observed no obvious association between disease progression and the number of MOG peptides recognized by IgG molecules present in necropsy sera (Table II).

**T cell responses**

RhMOG-induced TCL generated from ILN, ALN, or spleen of slow and fast disease progressors were tested for reactivity with a panel of MOG peptides. In accordance with previously published data (14) a proliferative response against the peptide MOG24–36 was found in all monkeys (Table II). Besides this ubiquitous reactivity with MOG24–36, individual monkeys displayed a variable reactivity with other MOG peptides. When the monkeys were
Characterization of potentially encephalitogenic MOG peptides

To determine the contribution of individual MOG peptide-specific T cell reactivities to the EAE pathogenesis in marmosets, we have taken advantage of a unique biological feature of this model. Marmoset twins develop in utero as stable bone marrow chimeras due to the shared placental blood stream (27). This implies that the T cells of both twins are educated in the same thymic environment to the shared placental blood stream (27). Hence, despite the different genetic background, such nonidentical twin siblings can be regarded as immunologically similar.

Of the three peptides that were most frequently recognized by T cells from fast progressor monkeys, MOG\textsubscript{34–56}, MOG\textsubscript{44–76}, and MOG\textsubscript{76–96} (Table II), we chose MOG\textsubscript{34–56} and MOG\textsubscript{76–96} for the immunization of chimeric twins. Because we were mainly interested in T cell responses, MOG\textsubscript{76–76} was not included for further analyses because this peptide is also a B cell epitope (see above).

MOG\textsubscript{34–56}–induced EAE

A total of 11 monkeys were immunized in three separate experiments with MOG\textsubscript{34–56}/CFA followed by booster immunizations on post sensitization days (psd) 28, 56, and \textasciitilde{}125 with MOG\textsubscript{34–56}/IFA until overt clinical signs of EAE were detectable. The individual EAE scores depicted in Fig. 2 show a heterogeneous clinical picture. One monkey (M0167) developed EAE associated with...
marked weight loss within 30 days after EAE induction. In two monkeys (M0182 and M03017) EAE developed only after psd 145 and one monkey (M02075) was sacrificed at psd 200 with only mild symptoms (score 0.5). In the remaining seven monkeys, overt neurological deficits were first observed around psd 145 and one monkey (M02075), overt neurological disease did not develop during the 200-day observation period. However, mild signs of EAE (score of 0.5) were observed (Fig. 6).

To test whether monkeys immunized with MOG24–96 displayed pathological changes within the CNS WM, high definition T2W images were made of the formalin-fixed brains from all four monkeys. In three monkeys clear abnormalities, albeit of moderate severity, were detected in the brain WM, namely one large periventricular lesion in M02076, two large lesions in M02121, and several small lesions in M02087 (Fig. 3).

Histological examination confirmed the presence of mild demyelination in frontal regions in the brain of two monkeys that were scored positive with MRI, i.e., M02076 and M02121. Interestingly, very little inflammatory activity was detected within these lesions (data not shown). In monkey 2087 no brain lesions were found, indicating that the abnormalities observed with MRI do not represent areas of inflammatory demyelination.

Experiment B

In the second experiment essentially the same results were obtained after the subsequent immunizations with MOG24–96, namely significant weight loss associated with only mild clinical signs. The mild CNS pathology observed in experiment A was associated with the appearance of low-level T cell reactivity against MOG34–56 (see next paragraph). To amplify this low-level response, all three MOG24–96-immunized monkeys were given a single booster immunization with MOG34–56 in IFA. This induced overt neurological signs in two of the three monkeys within a few weeks (Fig. 6). This observation contrasts with the situation in rhesus monkeys where booster immunization with MOG34–56 in IFA did not induce EAE, although this species is much more

MOG74–96-Induced EAE

To test the encephalitogenic potential of the MOG74–96 peptide, the twin siblings of seven of the 11 MOG34–56-immunized monkeys were immunized with MOG74–96. Two separate experiments, denoted A and B, were done comprising four and three monkeys, respectively (Table I).

Experiment A

In the first experiment (monkeys M02121, M02076, M02079, and M02087), overt neurological disease did not develop during the 200-day observation period. However, mild signs of EAE (score of 0.5) were observed (Fig. 6).

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FIGURE 6. Immunization with MOG34–56/IFA induces full blown EAE in MOG34–56-sensitized marmosets. Seven marmosets, twin siblings of the monkeys in Fig. 2 (see Table I), were immunized with 100 μg of MOG34–56 in CFA. Monkeys without overt signs of EAE (score of <2) at the end of a predetermined episode of ≥28 days received three booster immunizations with 100 μg of MOG74–96 in IFA (arrows). Clinical scores and changes in body weight (percentage compared with day 0) as a surrogate disease parameter of individual monkeys are shown. Because none of the monkeys developed overt neurological deficit within the predetermined observation period of 125 days, three (M03033, M03018, and M03027) received the third booster immunization of 100 μg of MOG34–56 in IFA (arrow with an asterisk), resulting in full blown EAE in two monkeys (M03033 and M03018). Numbers in the panels represent the time points (psd) when overt neurological signs were first observed.

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susceptible to EAE than marmosets (25). In the third monkey (M03027), EAE scores fluctuating between 0.5 and 1.0 were recorded from psd 50 onwards. After the boost with MOG34–56, the disease stabilized at an EAE score of 1. The monkey was finally sacrificed at psd 168 for histological analysis of the CNS.

The T2W postmortem images showed dramatic CNS pathology. Apart from large areas with increased T2W signal intensity, likely due to demyelination, the most remarkable feature was the presence of large “black holes” in the WM (data not shown). Histological analysis demonstrated comparable pathological changes as in MOG34–56-immunized monkeys. Brain and spinal cord contained large demyelinated lesions with infiltrated T cells and activated macrophages and microglia (Fig. 7).

Autoantibodies in MOG peptide-induced EAE cases

Plasma samples prepared from venous blood collected at 7-day intervals from the first immunization and at necropsy were tested with ELISA for the presence of IgM and IgG Abs against rhMOG and MOG peptides. Only total IgG responses were tested, because IgG subclasses are not described for the marmoset. Fig. 8 shows the results for IgG at necropsy, which were representative for IgM and the data of the 7-day interval plasma.

The main Ab response in MOG34–56-immunized monkeys was against MOG24–46. In some monkeys we also found Abs against MOG34–56 and rhMOG. In two MOG34–56–immunized monkeys a diversification of the Ab response to rhMOG and MOG74–96, was found (Fig. 8A). Because this peptide, which is a dominant B cell epitope in rhMOG-induced EAE, has only two overlapping amino acids with the immunizing peptide, it may be that this reactivity is caused by epitope spreading.

The dominant response in MOG74–96–immunized monkeys was against the immunizing peptide; but in the monkeys from experiment B, which were boosted with MOG34–56 in IFA, low-level Ab reactivity against rhMOG and MOG24–46 was also detected (Fig. 8B).

T cell reactivity in MOG peptide-induced EAE

PBMC, isolated at 2-wk intervals after immunization, and MNC from lymphoid organs were cultured with rhMOG and a MOG peptide panel to test proliferation. Moreover, cells of ALN from the twins of experiment 3 (Table I) were CFSE labeled and subsequently stimulated for 7 days with the immunizing peptides for the phenotyping of proliferating cells, visualized by the dilution of the fluorescent dye.

Proliferative responses in MOG34–56–immunized monkeys

In all monkeys, PBMC proliferation was found against MOG34–56 (Fig. 9A). The proliferative response in PBMC remained low, however, with the highest responses were mostly found in ALN and spleen (Fig. 9B), while lower proliferation was found in ILN (data not shown). Only in monkey M02088 could proliferation be detected in CLN (data not shown). Cells of ALN (Fig. 9C), spleen, and ILN (data not shown) also proliferated against rhMOG and peptides overlapping with MOG34–56.

FIGURE 7. Pathology of MOG34–56–boosted EAE in MOG34–96–immunized monkeys. Pathology present in the spinal cord of monkey M03033 is shown in a–c; pathology in the optic tract is shown in d–j. a, Staining for Luxol fast blue (original staining ×20) reveals demyelinated areas (bordered by blue dotted line) in the spinal cord WM. b, Late active demyelination (original staining ×350) is shown by the presence of PLP-positive degradation products in macrophages. c, Besides demyelination, inflammation of the lesions is characterized by the presence of CD3⁺ lymphocytes (original staining ×150). d, Luxol fast blue staining (original staining ×45) shows large demyelinated lesions (bounded by dotted line) in the optic tract. Magnified views of the rectangular area are shown in e–j. e, Staining for PLP (original staining ×350) reveals the presence of PLP degradation products in macrophages (arrowheads). f, The optic tract lesions contained CD3⁺ lymphocytes (original staining ×150). g, Single macrophages are stained positively for MRP-14 (original staining ×150). h, A Bielschowsky stain for axons (original staining ×150). i and j, Staining for IgM (i, original staining ×300) and complement C9 (j, original staining ×300) shows deposition in the demyelinated areas.

FIGURE 8. Diversification of Ab reactivity in MOG34–56 and MOG74–96–immunized monkeys. Plasma of MOG34–56 A and MOG74–96 (B) immunized monkeys were collected at necropsy and tested with ELISA for IgG reactivity with rhMOG and the panel of 23-mer peptides that covers the extracellular domain of MOG (1–125). Results are expressed as the fold increase of OD relative to preimmune marmoset serum. Neg., <2; +, 2 to <4; ++, 4 to <8; +++, 8 to <16; NT, not tested due to lack of material.
Proliferative responses in MOG 74–96-immunized monkeys

In PBMC from MOG 74–96-immunized monkeys, proliferation against MOG 74–96 was found (Fig. 10A). In addition, from psd 104 proliferation against MOG 34–56 was detectable (Fig. 10A). The response to MOG 74–96 could be amplified by a booster immunization with MOG 34–56 in IFA (Fig. 10B). At necropsy, MNC of MOG 74–96-immunized animals proliferated against MOG 74–96, but not against MOG 34–56 (Fig. 10C). The three monkeys challenged with MOG 34–56 at the third booster displayed a reduced response against MOG 74–96, and increased proliferation against MOG 34–56 (Fig. 10C). Cells of ALN collected at necropsy also proliferated against rhMOG (Fig. 10D).

FIGURE 9. T cell proliferation remains confined to the immunizing peptide MOG 34–56. Eleven marmosets were immunized with MOG 34–56 in CFA (psd 0) followed by booster immunizations with MOG 34–56 in IFA at psd 28, 56, and 119. PBMC collected at different time points and MNC of secondary lymphoid organs collected at necropsy were probed for their proliferative response against the MOG peptide panel. Only responses above an SI of 2 (dotted line) are considered positive. Proliferative responses were found against MOG 34–56. A, PBMC data from two representative monkeys. B, High responses were found in ALN and spleen at necropsy. C, Summary table of proliferative responses of ALN cells from all 11 monkeys against rhMOG and the MOG peptide panel. NT, Not tested.

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Phenotype of MOG 34–56- and MOG 74–96-reactive T cells

MNC from ALN, which contained the highest proliferative response, were stained with the fluorescent dye CFSE and subsequently cultured with the respective peptides. After 7 days, cells were harvested and stained with the fluorescein-labeled monoclonal Ab used for the phenotyping of human MNC subsets that are known to cross-react with marmoset MNC (20). Proliferating cells were identified by the dilution of CFSE. MOG 34–56 stimulation of MNC from MOG 34–56-immunized monkeys induced CFSE dilution in both CD4+ and CD8+ T cell subsets (Fig. 11A). However, the percentage of divided CD4+ cells was about twice as high as that of divided CD8+ cells. Fig. 11B shows the CFSE dilution in MNC cultures of monkeys immunized with MOG 74–96 and boosted with MOG 34–56 (experiment B). Proliferation of cells stimulated with MOG 34–56 or MOG 74–96 was found in both the CD4+ and the CD8+ T cell subsets.

Cytotoxic activity of MOG 34–56- and MOG 74–96-specific TCL

It was very difficult to generate stable TCL from the peptide-immunized monkeys from experiment 3 (Table I), although we used the same method as that successfully applied for the generation of stable TCL from rhMOG-immunized marmosets (14). Most lines collapsed after two or three rounds of restimulation with peptide-pulsed B cell lines. Stable TCL could only be obtained from three monkeys, i.e., the twins M03017 and M03018 and the monkey M03033. Cytolytic
activity toward autologous MOG peptide-pulsed APC by MOG-reactive TCM from MS patients has been described (28). Hence, we hypothesized that MOG peptide-specific T cells from the marmoset might exert cytotoxic activity and kill the peptide-pulsed B cell lines that were used as APC. To test this hypothesis, the phenotype and cytolytic activity of MOG peptide-specific TCL were determined.

MOG34-56- and MOG74-96-specific TCL of M03018 could also kill, respectively, MOG34-56- and MOG74-96-pulsed B cell lines of M03033 (data not shown). No cytotoxicity of the MOG34-56- and MOG74-96-specific TCL against nonpulsed B cells was observed (data not shown).

Cytolytic activity of the three stable lines was tested with peptide-pulsed EBV-transformed autologous and allogeneic B cell lines as target cells. The results in Fig. 11D show the peptide-specific cytotoxicity of MOG34-56- or MOG74-96-induced TCL from M03018 against autologous B cell lines. MOG34-56-specific TCL were cytotoxic for MOG34-56-pulsed B cells and MOG74-96-specific TCL killed MOG74-96-pulsed B cells. MOG34-56- and MOG74-96-specific TCL of M03018 could also kill, respectively, MOG34-56- and MOG74-96-pulsed B cell lines of M03033 (data not shown). No cytotoxicity of the MOG34-56- and MOG74-96-specific TCL against nonpulsed B cells was observed (data not shown).

FIGURE 11. MOG34-56- and MOG74-96-reactive T cells show an NK-CTL like activity. A and B, Freshly isolated MNC of ALN from EAE-affected marmosets were labeled with the fluorescent probe CFSE and subsequently cultured for 7 days with MOG34-56 or MOG74-96. Harvested cells were stained for CD3, CD4, and CD8 and analyzed by flow cytometry. The percentages of proliferated CD3+CD4+ (light shading) and CD3+CD8+ (dark shading) living cells under three stimulatory conditions are shown: without stimulant, with MOG34-56+ or with MOG74-96+. Data are shown for MOG34-56-immunized monkeys (M03017 and M03032) (A) and monkeys sensitized against MOG74-96 and finally boosted with MOG34-56 (M03018 and M03027) (B). C and D, Short-term TCL (effector cells) from three monkeys (M03017, M03018, and M03033) against MOG34-56 and MOG74-96 were established from MNC of spleen, ALN, and CLN. Shown are the data of M03018. C, Viable CD3+ cells of MOG34-56+ or MOG74-96-specific TCL derived from ALN of M03018 were stained for CD4 and CD56. D, The specific cytotoxicity was tested against 3HCr-labeled autologous EBV-transformed B cell lines (target cells), which were untreated or pulsed with MOG34-56+ or MOG74-96+. Shown is the percentage of killing by MOG34-56-specific TCL (top) and MOG74-96-specific TCL (bottom). TCL were cultured with MOG34-56-pulsed autologous B cell lines (closed symbols) or MOG74-96-pulsed autologous B cell lines (open symbols).

FIGURE 12. Phenotype of MOG34-56-specific TCL. Seven TCL, which were frozen after three rounds of antigenic stimulation and expansion on IL-2, were thawed and restimulated with MOG34-56 presented by autologous EBV-transformed B cells. After 8 days of expansion on IL-2, the phenotype of these lines was determined by FACS analysis. Vital CD3+ lymphocytes were analyzed for CD4 and CD8 expression. This resulted in CD4+, CD8+, and CD4/CD8 double-positive populations. CD16 and CD56 expression was determined in these populations. The percentages of T cell subpopulations in all seven lines is given in the table in A. The dot plots in B depict a representative example, the MOG34-56-specific TCL derived from the ALN of M03017. Numbers in the quadrants represent percentages of the subpopulation described above each dot plot.
To collect phenotypical data from more monkeys, TCL that were stored frozen after three rounds of peptide stimulation and expansion on IL-2 were thawed. Reactivation was performed by a single round of peptide-specific stimulation using EBV-transformed B cells as APC, followed by 8 days of expansion on IL-2. This yielded seven MOG\textsubscript{34–56}-specific TCL derived from five monkeys (M03017, M03018, M03026, M03027, and M03033) and two MOG\textsubscript{34–96}-specific TCL derived from two monkeys (M03017 and M03027). The CD\textsuperscript{3+} population of MOG\textsubscript{34–56}-specific TCL consisted of 30.9% (range 11–58%) CD\textsuperscript{4+} cells, 11.4% (range 3–19%) CD\textsuperscript{8+} cells, and 54.2% (range 37–80%) CD\textsuperscript{4+}CD\textsuperscript{8+} double-positive cells. The high proportions of CD4/CD8 double-positive cells is not an artifact of the freeze/thawing procedure, as this subpopulation is also found in peptide-stimulated cultures of freshly isolated lymph node and spleen cells. CD56 expression was most pronounced on single CD\textsuperscript{8+} T cells (62%; range 33–91%), followed by 45% (range 18–69%) of the double-positive cells and 32% (range 9–58%) of the single CD\textsuperscript{4+} cells. In none of the subpopulations was CD16 expression observed (Fig. 12). The CD\textsuperscript{3+} population of two additional MOG\textsubscript{34–96}-specific TCL consisted of 10.6% (1.8/19.4) CD\textsuperscript{4+} cells, 38.7% (63/413.9) CD\textsuperscript{8+} cells, and 48.3% (32.3/64.2) double-positive cells. All three subpopulations expressed significant CD56 levels, respectively 50.4% (79.5/21.2) of the CD\textsuperscript{4+}, 82.1% (96.9/76.3) of the CD\textsuperscript{8+}, and 72.1% (93.4/50.8) of the CD\textsuperscript{4+}CD\textsuperscript{8+} populations (data not shown).

Discussion

Several aspects of EAE in common marmosets make it a unique experimental model of MS, in particular the outbred nature, the genetic and immunological proximity to humans, and the remarkable neuropathological similarity with the disease in humans (for review, see Ref. 6). The disease typically follows a progressive course that can last from several weeks to more than a year (19). Of the many components of CNS myelin with proven encephalitogenic capacity in mice, MOG appeared to be the most critical for the induction of chronic progressive disease in Biozzi ABH mice and marmosets (13, 29). This prompted us to investigate the MOG-induced autoimmune mechanisms that drive progression of the disease using a rhMOG-induced EAE model.

We have previously shown in a relatively small group of monkeys that the 100% EAE prevalence maps to the invariant MHC class II molecule Caja-DRB*W1201, which is involved in the activation of MOG\textsubscript{34–36} specific encephalitogenic T cells (14). Although the presence of Caja-DRB*W1201 in the MHC repertoire of each individual monkey at the genomic level was already known for several years (15), it was only recently reported that mRNA transcripts of this allele are indeed expressed in the APC of all tested monkeys (16).

With regard to the MOG-induced autoimmune mechanisms involved in the progression of rhMOG-induced EAE, we report here several novel findings that in part contrast with published data. The current results show that monkeys displaying a relatively broad reactivity of their lymph node T cells with a MOG peptide panel developed a neurological deficit significantly earlier than monkeys with a more restricted reactivity profile. By contrast, we observed no association between the reactivity profile of immune sera with the MOG peptide panel and the rate of EAE progression. Hence, we conclude that the different time spans between EAE induction and the expression of neurological signs is associated with a diversification of proliferative T cell responses against MOG epitopes beyond MOG\textsubscript{34–36}. Our interpretation of this finding is that fast progressor monkeys may be (genetically predisposed) high responders to peptides processed from rhMOG and presented to encephalitogenic T cells present in the normal repertoire. We have chosen MOG\textsubscript{34–56} and MOG\textsubscript{24–96} for further examination because these peptides were detected in most fast progressor monkeys. Moreover, strong T cell reactivity to these peptides is found in rhMOG-induced EAE models (14, 30).

Ten of 11 monkeys immunized with MOG\textsubscript{34–56}/CFA developed overt clinical EAE, although the susceptibility of individual monkeys varied as reflected by the number of booster immunizations needed for EAE induction. In our previous study we reported that in monkeys immunized with peptide MOG\textsubscript{14–36}, only CD\textsuperscript{4+} Th1 cells were induced together with mild inflammatory CNS pathology (14). Our current data show that in MOG\textsubscript{34–56}-immunized monkeys, CD\textsuperscript{4+} as well as CD\textsuperscript{8+} T cells are activated and that many inflammatory/demyelinating lesions are formed in the WM and, in some monkeys, also in the gray matter. Moreover, MOG\textsubscript{34–56} induced TCL phenotypically resembled NK-CTL and displayed specific cytotoxic activity toward peptide-pulsed B cell lines. The observation that essentially the complete clinical and pathological picture of rhMOG-induced EAE was reproduced in monkeys immunized with a single 23-mer peptide contrasts with data from Genain and colleagues showing that the development of full blown EAE depends on anti-MOG Abs recognizing discontinuous epitopes and that full-blown EAE cannot be induced with MOG peptides (31).

Monkeys sensitized against the peptide MOG\textsubscript{24–96} did not show overt neurological signs, although we observed clinical EAE scores up to 1 as well as low-level CNS inflammation and demyelination by both MRI and histology. Furthermore, we detected low-level T cell proliferation against MOG\textsubscript{34–56}. These data indicate that mild EAE may have been induced by in vivo activation of MOG\textsubscript{34–56}-reactive T cells. This assumption is supported by the observation that a single booster immunization of the monkeys with MOG\textsubscript{34–56} in IFA induced overt neurological signs within a few weeks in two of three monkeys. This observation cannot be explained by cross-reactivity between the two peptides, as neither TCL nor immune sera from MOG\textsubscript{34–56}-immunized monkeys cross-reacted with MOG\textsubscript{74–96} and vice versa. Hence, we conclude that in the MOG\textsubscript{34–56}-immunized monkeys MOG\textsubscript{34–56}-reactive T cells had been recruited from the resting state, indicating that limited epitope spreading had taken place.

We like to propose as the underlying mechanism that MOG\textsubscript{34–56} reactive (memory) T cells present in the naive repertoire are activated by APC carrying myelin Ags from the lesions to draining lymph nodes. This concept is supported by previously reported findings. First, the ubiquitous autoreactivity against MOG\textsubscript{14–36} induces CNS infiltration of T cells and macrophages, which may trigger initial CNS WM damage (14, 17). Second, myelin-loaded APC can be found localized in the CLN and spleen of EAE-affected monkeys (32). We have not (yet) been able to directly demonstrate the induction of autoreactive T cells by myelin-loaded APC present within the CLN. However, the localization in T cell areas of the lymph nodes creates the conditions needed for such a functional interaction. Third, data from the rhesus monkey EAE model show that MOG\textsubscript{34–56}-reactive T cells present in the naive repertoire can be activated by immunization with a 23-mer peptide derived from the major capsid protein (UL86) of human CMV (CMV UL86\textsubscript{981–1003}) (25). The CMV of common marmosets has not been isolated and characterized yet. However, assuming that CMV-induced memory T cells do occur in the natural repertoire of common marmosets, as is the case in humans as well as in rhesus monkeys, it is tempting to speculate that T cells present in lymph nodes and spleen may be activated by myelin-loaded APC draining from the EAE-affected CNS (32).

The mechanisms underlying the dramatic CNS pathology upon immunization with MOG\textsubscript{34–56} remain to be fully elucidated. Our data
demonstrate that the induction of NK-T cell-like cytotoxic activity represents at least one of the pathogenic mechanisms. The cytotoxicity assay shown in Fig. 11 was performed with a mixture of CD56+ cells, namely CD4+*, CD8−/H11001*, and CD4−/H11001* and CD4+CD8−/H11001* T cells. The phenotype of the cells (CD3+/H11001+/H11001* and/or CD8+/H11001+/H11001*) as well as the capacity to lyse peptide-pulsed, autologous, and allogeneic B cell lines are suggestive of an NK-T cell-like activity. Interestingly, a similar activity has been observed for anti-MOG T cell responses in MS patients (28).

The possible involvement of such cells in the pathogenesis of MS has been reported by several groups (33, 34). We are currently exploring further the different cell subsets involved and the cytotoxic effector mechanisms, both ex vivo and in situ.

In conclusion, we report an association between disease progression in the rhMOG-induced EAE model in marmosets with the diversification of the anti-MOG T cell response. In addition to a T cell response against MOGΔ1–40, which is present in all monkeys and is the presumed trigger of the disease, T cell responses against MOGΔ1–47 and MOGΔ13–94 are detectable in monkeys with a rapid disease progression. MOGΔ1–47 displays potent encephalitogenic activity leading to inflammation and tissue destruction in the CNS, most likely via the activation of cytotoxic T cells with a NK-CTL-like phenotype. In our view the marmoset EAE model offers a unique experimental setting to further unravel the pathogenic mechanisms and to develop novel therapeutic approaches targeting these mechanisms, including the cytotoxic activity.

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Disclosures

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References
