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Suppression of Acute Experimental Allergic Encephalomyelitis in Lewis Rats with a Mycophenolic Acid Derivative

Masahiro Mizobuchi, Yuzo Iwasaki[1], Kazuya Sako and Yutaro Kaneko[2]

Department of Neurology, Nakamura Memorial Hospital, Sapporo 060, [1]*Miyagi National Hospital, Miyagi 989-22, and* [2]*Ajinomoto Co., Inc., Tokyo 104*

Mizobuchi, M., Iwasaki, Y., Sako, K. and Kaneko, Y. *Suppression of Acute Experimental Allergic Encephalomyelitis in Lewis Rats with a Mycophenolic Acid Derivative.* Tohoku J. Exp. Med., 1997, **182** (3), 217-229 —— Oral administration of ethyl O-[N-(*p*-carboxyphenyl-carbamoyl]-mycophenolate (CAM), a derivative of mycophenolic acid (MPA) and an inosine monophosphate dehydrogenase inhibitor, dose-dependently suppressed acute experimental allergic encephalomyelitis in Lewis rats without exerting any serious adverse effects. A daily dose of 50 mg/kg of CAM almost completely abolished both the clinical disease and the inflammation in the CNS. In the CAM-treated rats, a weight loss and fluctuations of peripheral lymphocyte subsets were minimized. The CAM treatment was effective when started at the time of sensitization but ineffective when deferred till day 10. Furthermore, CAM reduced the percentage of CD4+CD45RC− cells in the peripheral blood. The only detectable adverse effect was moderate anemia but it was rapidly improved after withdrawal of the drug. This drug could be a useful adjunct for the long-term immunosuppressive therapy for inflammatory diseases of the CNS. ———— mycophenolic acid; experimental allergic encephalomyelitis; immunosuppressant; T cell subset; multiple sclerosis

Various immunosuppressive regimens have been developed for the treatment of multiple sclerosis (MS) but immunosuppressive therapies are often hampered by serious adverse effects (Mckhann 1990). In the present study, we propose the candidacy of mycophenolic acid (MPA) for a long-term immunosuppressive therapy for inflammatory central nervous system (CNS) diseases such as MS, based on our observations on the efficacy of the drug in acute experimental allergic encephalomyelitis (EAE), an animal model for MS (Arnason 1983).

MPA is an inhibitor of inosine monophosphate (IMP) dehydrogenase and it is known to have diverse biological activities such as antiviral, antifungal,

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Address for reprints: Masahiro Mizobuchi, M.D., Department of Neurology, Nakamura Memorial Hospital, Minami-1, Nishi-14, Chuo-ku, Sapporo 060, Japan.

antitumor and immunosuppressive effects (Mitsui and Suzuki 1969; Ohsugi et al. 1976; Eugui et al. 1991a). A paucity of adverse effects makes this drug attractive in the recent clinical trials for an organ transplantation (Sollinger et al. 1992; Ensley et al. 1993) and rheumatoid arthritis (Goldblum 1993), but the application of the drug for CNS diseases has not yet been reported.

In the present study, we assessed the efficacy of the oral administration of ethyl O-(N-(*p*-carboxyphenyl)-carbamoyl)-mycophenolate (CAM), one of the MPA derivatives and a potent immunosuppressive agent (Takazawa et al. 1995), in acute EAE in Lewis rats by monitoring the clinical score, phenotypes of mononuclear cell in the peripheral blood and cellular infiltrates in CNS lesions, and myelin basic protein (MBP)-specific proliferative response of regional lymph node cells.

Materials and Methods

Induction and clinical assessment of EAE

Eight to nine week-old female Lewis rats were purchased from Charles River Japan, Inc. (Kanagawa). Rats were subcutaneously injected with 0.16 ml of inoculum in the bilateral foot pads. Each inoculum consisted of 8 mg of guinea pig spinal cord homogenate in 0.08 ml of saline emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 10 mg/ml of heat-killed *Mycobacterium tuberculosis* (H37Ra; Difco, Detroit, MI, USA). For 28 days after the immunization, neurological signs were daily scored as follows: 0-normal, 1-flaccid tail, 2-hindlimb weakness, 3-hindlimb paralysis, 4-quadriplegia, 5-moribund or death.

CAM treatment

In total of 55 rats were used and five each of immunized rats were given 50 mg/kg, 30 mg/kg or 10 mg/kg of CAM in 0.5% carboxymethyl cellulose (CMC) solution once a day with a gastric tube. CAM was provided by Ajinomoto Co., Inc. (Tokyo). The drug is known to be absorbed from the small intestine and converted to MPA in mucosal cells (Matsuzawa and Nakase 1984). The non-treated control EAE rats received isovolume of 0.5% CMC solution. Two sets of experiments were performed. In the first experiment, the rats were treated with CAM from day 0, the day of immunization, to day 28 (CAM-1) and in the second experiment from day 10 to day 28 (CAM-2).

Preparation of mononuclear cell (MNC) fractions from the peripheral blood

Blood samples, 0.9 ml per rat, were collected by a cardiac puncture under ether anesthesia between 1 : 00 p.m. and 3 : 00 p.m. on days -1, 4, 9, 11, 14, 17, 20, 28. The hematocrit was measured by the microhematocrit method, and total number of WBC was enumerated with a counting chamber. Differential WBC count was measured with a FACScan (Becton-Dickinson, Sunnyvale, CA, USA)

after lysing RBC with a lysing solution (Becton-Dickinson).

For phenotypic analysis of MNCs, blood samples were mixed with an equal volume of PBS. The mixtures were centrifuged through a Ficoll-Paque gradient (Pharmacia LKB, Uppsala, Sweden) at 400×g for 5 minutes, and then at 1,200×g for 12 minutes. MNCs were recovered from the plasma/Ficoll-Paque interface.

Isolation of cellular infiltrates from the CNS tissue

Cellular infiltrates in the CNS tissue were collected on day 13 from the non-treated rats and the rats treated with 50 mg/kg and on day 15 from the rats treated with 30 mg/kg in CAM-1 experiment. Infiltrating cells were isolated by the method of Clatch et al. (1990) with minor modifications. In brief, after transcardial perfusion with saline, the spinal cord was removed, and each spinal cord was separately processed as follows. The tissue was dissociated through a 212 μm stainless steel screen, and the filtrates were centrifuged at 400×g for 10 minutes. Each sediment was resuspended in 5 ml of 68% Percoll (Pharmacia) in PBS, and 3 ml of 30% Percoll in PBS overlaid on the mixture. After centrifugation at 1,200×g for 30 minutes at 25°C, MNCs were recovered from 30%/68% Percoll interface.

Flow cytometric analysis

MNCs suspended in PBS were aliquoted into 100 μl, 5×10^5 cells each, and were dually labeled with the following monoclonal antibodies in combination of CD4 and CD45RC or CD8 and CD3. Phycoerythrin-conjugated W3/25 for CD4, phycoerythrin-conjugated OX-8 for CD8, FITC-conjugated OX-22 for CD45RC were purchased from Serotec Ltd. (Blackthorn, UK) and FITC-conjugated 1F4 for CD3 from Caltag Lab. (San Francisco, CA, USA). The cells were incubated with the antibodies for 45 minutes at 4°C, and washed with three changes of PBS, and then diluted for the FACScan analysis. Forward and side scatter gates were set for lymphocytes to exclude monocytes and RBC residues, and 10,000 cells were analyzed for each fraction.

MBP-specific proliferative assay

The proliferative response to MBP of MNCs isolated from regional lymph nodes was assayed with the colorimetric MTT (tetrazolium) assay kit (Chemicon Int. Inc., Temecula, CA, USA) (Mosmann 1983). On the day 12, MNCs were isolated from bilateral regional lymph nodes of the rats treated with 50 mg/kg of CAM from day 0. As controls, lymph node cells were collected from the control EAE rats and the rats treated with CFA without spinal cord homogenate. The isolated cells were seeded in 96-well flat bottom plates at a density of 0.7×10^5 viable cells in 0.1 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, 5×10^5 M 2-mercaptoethanol, 2 mM L-glutamine, 100 U of penicillin per ml and 100 μg of streptomycin. The culture was prepared in triplicate. After

incubation for 72 hours at 37°C without or with MBP at concentrations of 50, 10, and 1 μg/ml, 0.01 ml of MTT (5 mg/ml) was added to each well, and further incubated for 4 hours. Before measurement, formazan crystals were solubilized with 0.1 ml acid-isopropanol. The amount of reaction product was measured with MTP-32 microplate reader (Corona Electric, Katsuta) at 575 nm and a reference wavelength of 630 nm. The results were expressed as stimulation index (SI) = $OD_{575-630}$ of stimulated culture/$OD_{575-630}$ of unstimulated culture. Statistical analysis was performed by the Student's t-test for two independent means.

Determination of the plasma concentration of MPA

The temporal change of the plasma concentration of MPA, a bioactive metabolite of CAM, was determined by the HPLC method. Two, 6 and 24 hours after a single oral administration of 50 mg/kg of CAM, 250 μl each of blood was transcardially obtained from 8 rats under ether anesthesia. A mixture of 0.1 ml of the plasma sample and 0.4 ml of methanol was centrifuged at 3,000 $\times$ g for 5 minutes, and 50 μl of the supernatant was applied to the HPLC system (Hitachi Inc., Tokyo) with a YMC-pack ODS-A A-303 column (YMC Co., Kyoto) at a flow rate of 1.0 ml/min with 0.1% TFA CH_3CN and H_2O (7 : 3) as a mobile phase. The effluent was monitored at a wavelength of 254 nm with a UV-detector (Hitachi Inc.).

Results

Amelioration of clinical disease

Control EAE rats inevitably developed flaccid tail on day 10 or 11 and limb paralysis by day 13 (open circle in Fig. 1). In CAM-1 experiment started on day 0 (upper panel in Fig. 1), a daily dose of 10 mg/kg had little effects on both the onset and clinical score (3.0 vs. 3.4 in controls), but with a dose of 30 mg/kg, an average day of onset was delayed (13.4 vs. 10.2) and the maximum clinical score was reduced to 1.5. With a dose of 50 mg/kg, no rats developed limb paralysis, and only slight weakness of tail was suspected on day 12 (clinical score $<$ 1). If the CAM treatment was deferred till day 10 (CAM-2), clinical manifestations of the disease were indistinguishable from those of control EAE rats (lower panel in Fig. 1).

Control EAE rats lost an average of 16% of body weight by day 15. It was reduced to 9% with a dose of 30 mg/kg, and with a dose of 50 mg/kg, no weight loss was evident (upper panel in Fig. 2). In CAM-2 experiment, however, the weight loss in the rats treated with 50 mg/kg of CAM was similar to that of control EAE rats (lower panel in Fig. 2).

Alterations of hemogram

By day 4, WBC count in control EAE rats was almost double of that on day -1, and they decreased with the development of neurological signs on day 11

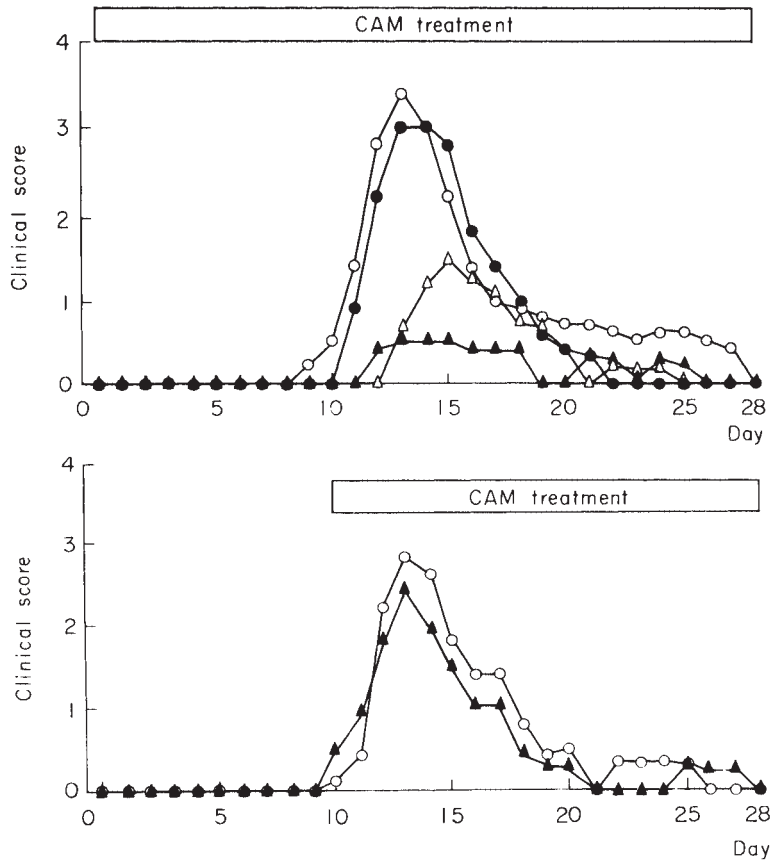

Fig. 1. Clinical score ($n=5$). The closed triangle (▲—▲) indicate the rats treated with 50 mg/kg of CAM, open triangle (△—△) indicate 30 mg/kg, closed circle (●—●) indicate 10 mg/kg and open circle (○—○) indicate controls. Note the dose-dependent amelioration in CAM-1 (upper) and a lack of efficacy in CAM-2 (lower).

to 14. The initial leukocytosis and lymphocytosis, however, were largely abolished in the rats treated with 50 mg/kg in CAM-1 experiment (upper and middle panel in Fig. 3). Similar changes were seen in the rats received 10 mg/kg in CAM-1 experiment (data not shown) and 50 mg/kg in CAM-2 experiment. Moderate anemia developed in the rats received 50 mg/kg of CAM but it was normalized after cessation of the treatment (lower panel in Fig. 3).

Alterations of peripheral lymphocyte subsets

In control EAE rats, concomitant with the development of the clinical disease, the percentage of CD3 cells decreased significantly. The reduction of CD3 cells was coincided with a decrease of CD4 cells and a reciprocal increase of CD8 cells. The reduction of CD4 cells before the peak of the disease was normalized as the disease progressed. The reciprocal increase of CD8 cells was due to an increase in the percentage of CD3−CD8+ cells, possibly natural killer cells, but not of CD3+CD8+ cells.

In the EAE rats treated with 50 mg/kg of CAM from day 0 (CAM-1), the fluctuations of peripheral lymphocytes were largely abolished, but similar fluctua-

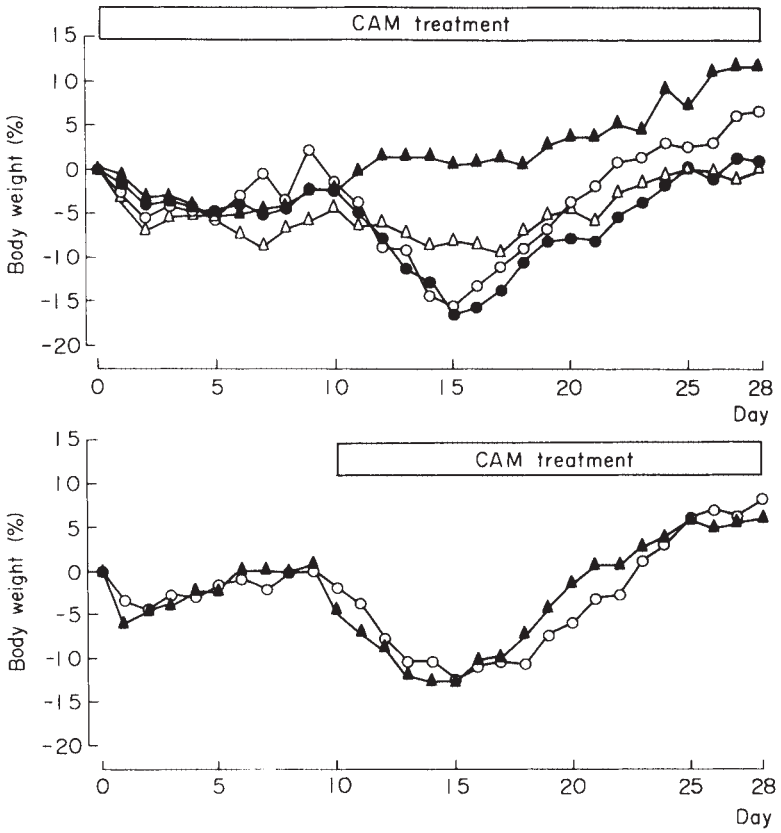

Fig. 2. Body weight ($n=5$). A daily dose is same as Fig. 1. The weight loss in control EAE rats is ameliorated in CAM-treated rats in CAM-1.

tions were seen in the rats treated from day 10 (CAM-2). Furthermore, the percentage of CD45RC− cells in the CD4 subset was gradually decreased from 50% on day -1 to 14% on day 14 in the rats treated with 50 mg/kg of CAM from day 0 (CAM-1). A similar tendency was seen in CAM-2 experiment but it was less remarkable (Fig. 4).

Cellular infiltrates in the CNS tissue

At the height of the clinical disease, approximately 2×10^6 MNCs were recovered from the CNS tissue of control EAE rats, and the majority of these MNCs were lymphocytes. In the CAM-1 experiment, the number of cellular infiltrates was dose-dependently reduced, but it was much less effective in CAM-2 experiment. In the CAM-1 experiment, with a dose of 30 mg/kg, the number of MNCs was reduced to $0.99\pm0.43\times10^6$ vs. $2.06\pm0.49\times10^6$ in controls, and that of lymphocytes was $0.69\pm0.36\times10^6$ vs. $1.79\pm0.45\times10^6$. With a dose of 50 mg/kg, only 0.1×10^6 MNCs were recoverd. In both control and CAM-treated EAE rats, the lymphocytes were dominated by CD4 cells, and more than 95% of the CD4 cells showed CD45RC− phenotype (Fig. 5, Table 1).

MBP specific proliferative assay

At a concentration of 1 μg/ml of MBP, the mean SI was 1.54 ± 0.13 for control

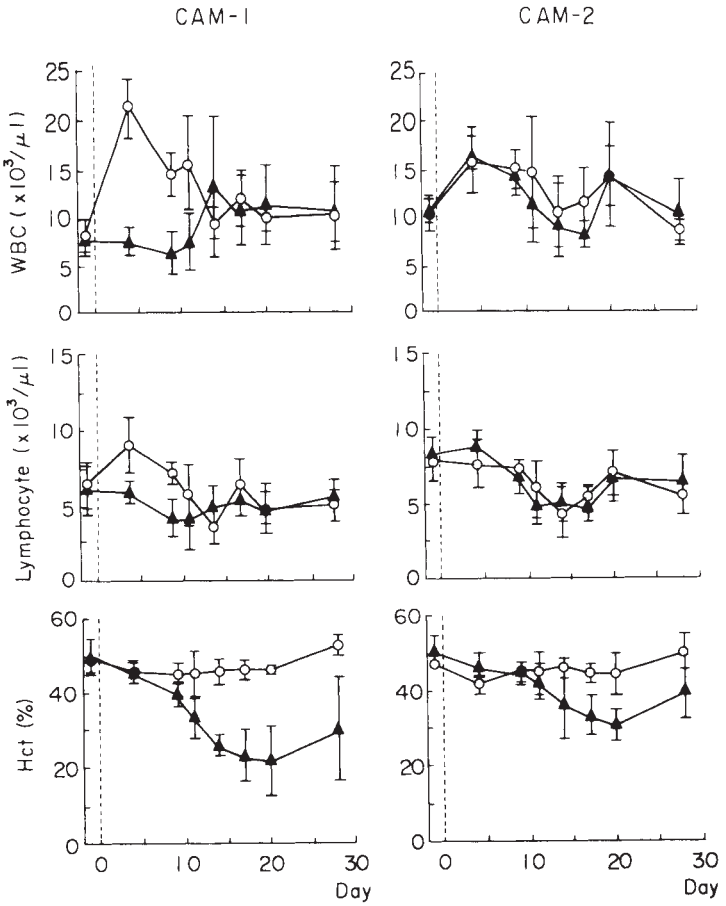

Fig. 3. Profiles of peripheral blood ($n=5$). The closed triangle (▲—▲) represent the rats treated with 50 mg/kg of CAM and open circle (○—○) represent control. Fluctuations of WBC and Lymphocyte counts in control EAE rats are largely abolished in CAM-treated rats in CAM-1.

EAE rats, 1.07 ± 0.00 for the rats treated with CFA alone and 1.07 ± 0.02 for CAM-treated EAE rats. At a concentration of 50 μg/ml, SI was 1.87 ± 0.14 for control EAE rats and 1.29 ± 0.04 for CAM-treated EAE rats. The difference was significant between the control and CAM-treated rats (Fig. 6).

Plasma level of MPA

MPA was a major metabolite of CAM detected by this method. The plasma concentrations of MPA after a single oral administration of 50 mg/kg of CAM were 16.4 ± 7.1 mM (mean$\pm$S.D., $n=8$) at 2 hours, 22.5 ± 9.0 mM at 6 hours, and 2.5 ± 2.0 mM at 24 hours.

Discussion

CAM effectively suppressed acute EAE in Lewis rats. Amelioration of the clinical disease was associated with a marked reduction of the number of cellular infiltrates in the CNS tissue, and both the amelioration of the clinical disease and the reduction of cellular infiltrates in the CNS were achieved dose-dependently. The rats were not only protected from the development of EAE but a weight loss,

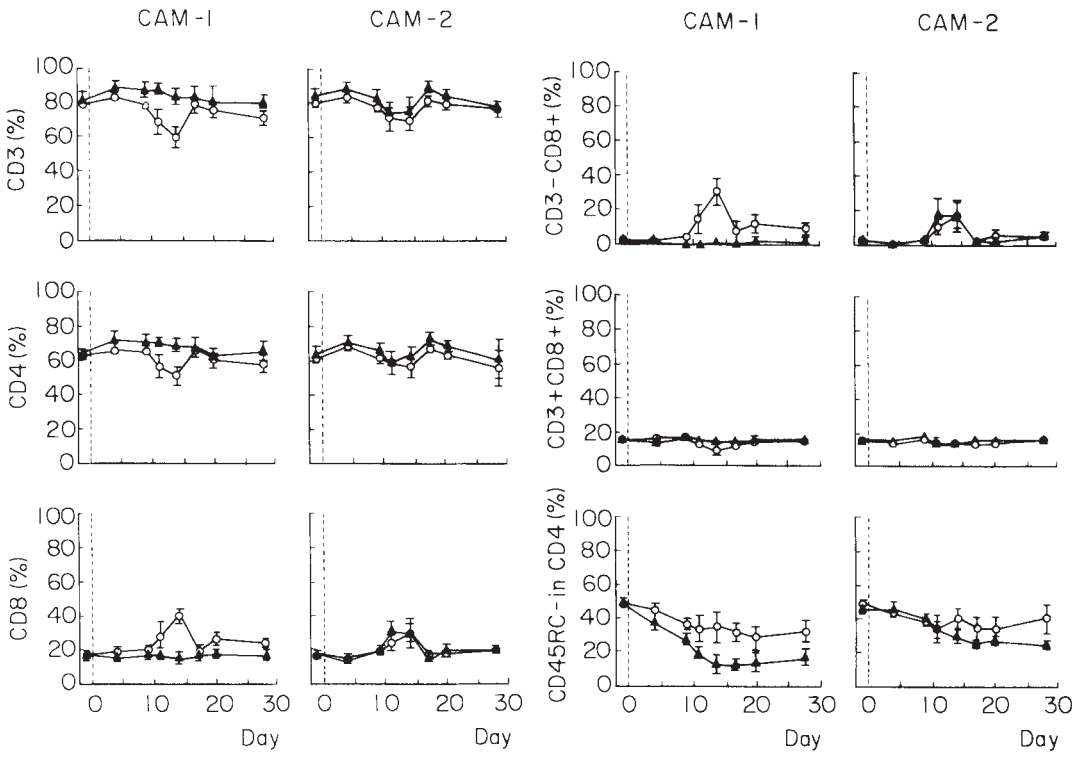

Fig. 4. Peripheral lymphocyte subsets ($n=5$). The closed triangle (▲——▲) represent the rats treated with 50 mg/kg of CAM and open circle (○——○) represent control. Alterations of the percentage of T cell subsets in control EAE rats are less conspicuous in CAM-treated EAE rats, and a steady decline of the percentage of CD4+CD45RC-cells is also evident in the CAM-treated rats.

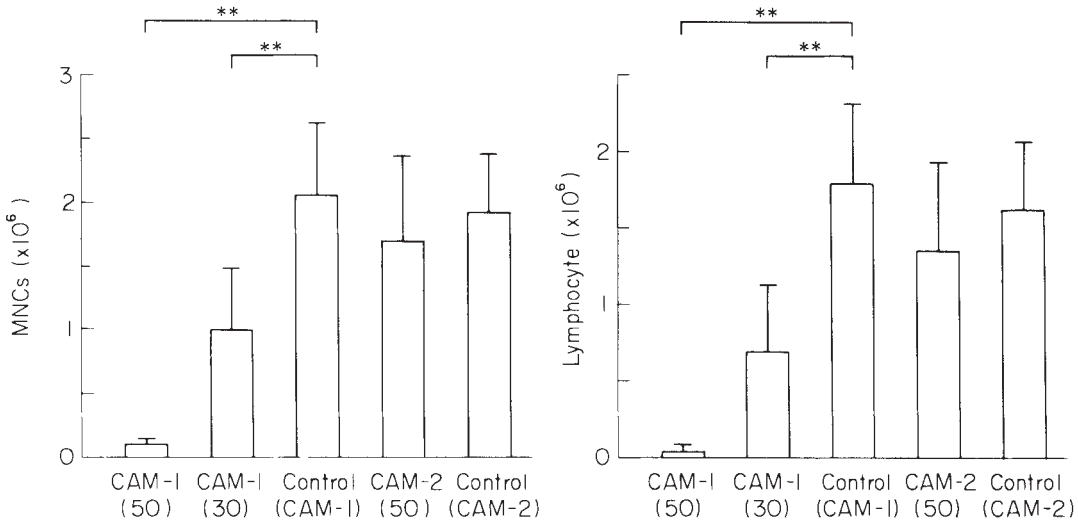

Fig. 5. Mononuclear cells recovered from the spinal cord ($n=5$). The numbers of MNCs and lymphocytes are substantially reduced in the rats treated with 30 mg/kg of CAM and they are hardly isolated from those treated with 50 mg/kg in CAM-1, but the treatment is barely effective in CAM-2.
$^{**}p<0.01$

TABLE 1. *Phenotypes of lymphocytes isolated from the spinal cord*

Phenotypes of lymphocytes	CAM-1			CAM-2	
	Control (CAM-1)	CAM (30)[a]	CAM (50)[b]	Control (CAM-2)	CAM (50)
	Number ($\times 10^6$) (%)	Number ($\times 10^6$) (%)	Number	Number ($\times 10^6$) (%)	Number ($\times 10^6$) (%)
CD3	1.53±0.43 (84.5±2.9)	0.60±0.34* (86.0± 5.9)	<0.001*	1.42±0.36 (86.6±1.8)	1.18±0.49 (86.6± 4.2)
CD4	1.12±0.35 (61.6±4.4)	0.45±0.26* (64.1± 9.7)	<0.001*	1.12±0.27 (68.8±3.1)	0.97±0.46 (69.6±11.4)
CD3−CD8+	0.02±0.01 (1.3±0.9)	0.01±0.00* (1.5± 0.8)	<0.001*	0.02±0.02 (1.5±0.9)	0.01±0.00 (0.6± 0.2)
CD3+CD8+	0.33±0.09 (18.2±1.7)	0.13±0.08* (18.9± 5.2)	<0.001*	0.26±0.07 (15.9±2.6)	0.21±0.08 (16.6± 7.8)
CD4+CD45RC−	1.10±0.35 (60.6±4.6)	0.44±0.26* (62.9±10.2)	<0.001*	1.10±0.28 (67.4±2.7)	0.95±0.47 (67.8±12.3)
CD4+CD45RC− (in CD4+)	(98.2±0.5)	(98.0± 1.5)		(98.0±1.3)	(97.1± 2.2)

[a]Statistical analysis between Control (CAM-1) and CAM (30)
[b]Statistical analysis between Control (CAM-1) and CAM (50)
*$p<0.05$

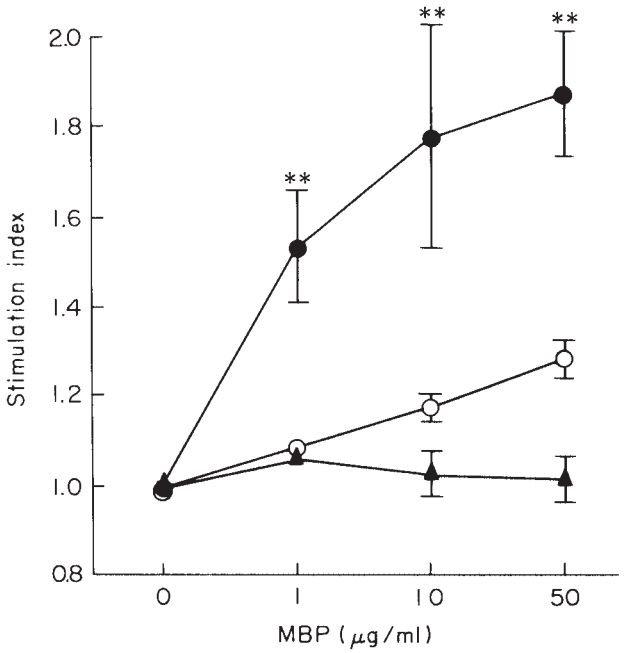

Fig. 6. MBP-specific proliferating assay (triplicate, $n=3$). The closed circle (●—●) indicate EAE rats without CAM treatment as a positive control, open circle (○—○) indicate CAM-treated EAE rats and closed triangle (▲—▲) indicate the rats inoculated CFA alone as a negative control. The cells were harvested on day 12. The MBP-reactive cells are apparently reduced in lymph node cells from CAM-treated rats.
**statistically significant at $p<0.01$.

commonly seen in EAE, was also absent during a four-week CAM treatment, further indicating a paucity of general toxicity of the drug.

The drug action of MPA is apparently multifactorial. The CAM treatment completely abolished leukocytosis, largely due to granulocytosis, in acute phase of EAE. This suppression of acute inflammatory response is likely to be mediated by the action of MPA on monocyte lineage cells since it is reported that at a concentration of 0.1 μM, MPA suppresses differentiation of human promonocytes, possibly leading to the down-regulation of cytokine production, and augments the production of IL-1ra, an IL-1 receptor antagonist, in human peripheral monocytes (Waters et al. 1993). In the present study, the plasma level of MPA was higher than 2 mM even at 24 hours after a single oral administration of 50 mg/kg. This would be sufficient to suppress the cell differentiation and the production of cytokines in monocyte lineage cells. The general suppression of acute inflammatory reaction may also affect the generation of MBP-specific lymphocytes in the regional lymph nodes.

The fluctuation in the number of peripheral lymphocytes in control EAE rats was indistinct in the CAM-treated rats. Control EAE rats developed mild lymphopenia concomitant with the development of the clinical disease. This is due to reduction of CD3 cells accompanied by a moderate decrease of CD3+CD4 subset, a slight reduction of CD3+CD8+ cells and a reciprocal increase of CD3−CD8+ cells, NK cells. This lymphopenia is generally thought to be secondary to

the accumulation of lymphocytes in the CNS lesions. If this is the case, the absence of lymphopenia in the CAM-treated EAE rats could be explained by abrogation of inflammation in the CNS.

It would be worth to note that during the entire course of EAE, the numbers of CD3 cells, CD4 cells and CD8 cells were remarkably stable in the rats treated with 50 mg/kg of CAM for 4 weeks from day 0 (CAM-1) where the clinical disease was almost completely abolished. The observation may suggest that CAM selectively suppresses clonal expansions of the cells stimulated with new antigens without disturbing the kinetics of other cell populations. In support of this view, it is suggested that MPA does not interfere early responses of T and B lymphocytes to mitogenic or antigenic stimulation but only blocks the cells at the time of DNA synthesis (Eugui et al. 1991b). This unique drug action seems to be related to the preferential inhibition of inducible type II IMP dehydrogenase by MPA (Carr et al. 1993) since type II IMP dehydrogenase dominates in activated cells and neoplastic cells while type I IMP dehydrogenase is the main species in normal cells (Konno et al. 1991), and it can be up-regulated by mitogenic and antigenic stimulations (Nagai et al. 1992).

The only noticeable change in lymphocyte subsets in the CAM-treated rats was a steady decline of the percentage of CD45RC− population, most conspicuous in the CD4 subset. This phenomenon could be explained by the type specific inhibition of type II IMP dehydrogenase since CD4+CD45RC− cells are thought to be memory/activated T cells (Ericsson et al. 1991) and the decrease of this subpopulation of T cells is likely to be reflecting an inhibition of proliferation of stimulated lymphocytes. A low recovery rate of MBP-reactive cells from the regional lymph nodes in the CAM-treated rats in the absence of a significant loss of total lymph node cells could support this possibility. The reduction of CD4+ CD45RC− cells was also seen in non-immunized rats treated with CAM for more than 2 weeks (data not shown), and it was normalized within 2 weeks after withdrawal of the drug, suggesting non-specificity and reversibility of the phenomenon. Such a non-specific reduction of CD4+CD45RC− cells may be beneficial for the suppression of inflammatory CNS diseases. At least in the CNS lesions of EAE, more than 70% infiltrating CD3 lymphocytes and over 95% of CD4 cells are of this phenotype, and only a small minority of the cells are MBP-reactive and the majority is stimulated by antigens irrelevant to EAE (Cohen et al. 1987).

When the CAM treatment was deferred till day 10, little beneficial effects were observed. Although the treatment appears to be effective only for induction phase, MPA may have a potential to suppress the influx of inflammatory cells into target tissues by blocking the glycosylation of adhesion molecules (Allison et al. 1993) and indeed the inflammation in EAE is abrogated by anti-VLA-4 antibody in this experimental model (Sako et al. 1993). The only adverse effect in the present study was anemia. The lowest hematcrit value in the EAE rats treated

with 50 mg/kg of CAM was nearly half of control EAE rats but the rats rapidly recovered from anemia after withdrawal of the drug. A similar normalization of the anemia was made in non-immunized rats treated with 50 mg/kg of CAM for 3 weeks (data not shown). Thus, the suppression of the hematopoietic system by MPA is reversible.

Unlike other immunosuppressive agents, MPA appears to exert little serious adverse effects, and it seems to be related to the type-specific inhibition of IMP dehydrogenase. Therefore CAM can be a hopeful candidate for the long-term prophylactic treatment of MS. Much work needs to establish an optimal protocol for clinical application of the drug.

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