MONOCLONAL ANTIBODY TO THEILER'S MURINE ENCEPHALOMYELITIS VIRUS DEFINES A DETERMINANT ON MYELIN AND OLIGODENDROCYTES, AND AUGMENTS DEMYELINATION IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

By MASAHITO YAMADA, ANDREAS ZURBRIGGEN, AND ROBERT S. FUJINAMI

From the Department of Pathology, University of California, San Diego, La Jolla, California 92093

Theiler's viruses have been known for many years to cause an encephalomyelitis in mice (1, 2), and have been classified in the picornavirus family (3). The DA strain of Theiler's murine encephalomyelitis viruses (TMEV)[1] can cause a biphasic central nervous system (CNS) disease in mice (4): acute polioencephalomyelitis and/or chronic inflammatory demyelinating disease, depending on the dose and strain of the virus and genetic background of the mouse. The former is clearly the result of direct viral cytopathic effects, while the pathogenesis of the latter is under intense exploration.

The CNS disease induced by TMEV is an animal model for human demyelinating diseases, including post-infectious encephalomyelitis and multiple sclerosis (4–6). Both direct viral (7–10) and immune-mediated (11–13) mechanisms affecting oligodendrocytes and/or myelin have been proposed to initiate inflammatory events within the CNS, leading to the chronic demyelination. Immune-mediated mechanisms include an: "innocent bystander" theory; immunologic injury to oligodendrocytes infected with virus; and virus-induced autoimmune events (5, 6, 14, 15). Autoimmune events may be induced by sensitization to myelin antigens that are secondarily released by viral infection of oligodendrocytes, or induced by the presence of a common epitope between virus and myelin components resulting in crossreactive immune response.

We have proposed a mechanism in which viruses may initiate or enhance autoimmune diseases by possessing common or crossreacting determinants (molecular mimicry) with self components (16). In earlier studies, we found that mAbs to viral pro-

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Address correspondence to Robert S. Fujinami, Department of Pathology-M-012, School of Medicine, University of California, San Diego, CA 92093.

[1] *Abbreviations used in this paper:* ABC, avidin-biotin-peroxidase complex; BBB, blood brain barrier; BHK, baby hamster kidney; CNS, central nervous system; EAE, experimental allergic encephalomyelitis; GC, galactocerebroside; HRPO, horseradish peroxidase; MBP, myelin basic protein; PFU, plaque-forming unit; RT, room temperature; TMEV, Theiler's murine encephalomyelitis virus.

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teins could react with host cell components for measles, herpes, and vaccina viruses, i.e., both RNA and DNA viruses (17, 18). These studies demonstrated the existence of common determinants between viruses and cellular- or tissue-specific elements. In addition, we were able to induce an experimental allergic encephalomyelitis (EAE)-like disease by sensitization of animals with a viral peptide having a sequence similarity with the encephalolitogenic (disease-inducing) region of myelin basic protein (MBP) (19). Sensitized animals generated autoantibody that reacted with the host protein, MBP, and PBMC proliferated when cultured in the presence of viral peptide or with MBP. This previous study demonstrated that a virus (viral peptide) that shares a common determinant with a disease-inducing epitope could initiate autoimmune disease.

A similar crossreactive immune response is a possible contributing factor for virus-induced demyelinating diseases of humans. For example, in patients with post-infectious encephalomyelitis after measles virus infection, response of lymphocytes to MBP and lack of evidence of viral invasion of the CNS have been demonstrated (20), suggesting an autoimmune pathogenesis of demyelination. The existence of common immunologic element(s) between virus and MBP or other CNS components is a possible event, although this remains to be determined.

In another report, we have described mAbs to TMEV (DA strain), and discovered that one of these mAbs, H8, reacted both with TMEV viral protein VP-1 and lipid-like moieties, including galactocerebroside (GC) (21). This indicated an overlapping epitope between TMEV VP-1 and GC-like molecules. GC is a major lipid component of myelin (22). Others have demonstrated that immunization of animals with GC or passive transfer of anti-GC antibody to animals could lead to demyelinating disease (23–26). If mAb H8–like antibodies are produced in mice after TMEV infection, they could play an active role in demyelination through an immune-mediated process.

Here, we describe the binding of mAb H8 to oligodendrocytes in brain cell cultures and to myelin structures in CNS tissue sections. We further document an in vivo demyelinating effect of mAb H8 and the presence of antibody(s) with the same specificity as mAb H8 in the sera of mice chronically infected with TMEV. Our results indicate that antibody(s) with such specificities are generated after infection, can enhance demyelination, and contribute to the observed pathology. This is the first report demonstrating that an antibody to virus can augment demyelination of CNS in vivo.

Materials and Methods

Production of mAbs to TMEV and Characterization of mAb H8

The details for production and characterization of mAbs to TMEV were previously reported (21). Briefly, BALB/c mice were inoculated intracerebrally with 5×10^5 plaque-forming units (PFU) of a tissue culture–derived virus, DA strain of TMEV. The DA strain of TMEV was originally obtained from J. Lehrich and B. Arnason (27). After 6 wk, mice were boosted intraperitoneally with 10^7 PFU of virus on days -3, -2, and -1 before fusion. Spleens were removed and cells fused with myeloma cells. After screening, hybridoma-cloned cell lines producing mAbs that reacted with TMEV and/or with CNS antigens, including GC (Sigma Chemical Co., St. Louis, MO) by ELISA (28), were obtained. One of these mAbs, H8, reacted both with GC and TMEV in ELISA. In neutralization assays, mAb H8 effectively neutralized TMEV in vitro. In immunoblotting, mAb H8 strongly reacted with VP-1, the most external capsid protein of TMEV (21).

Immunofluorescent Staining of Cultured Mouse Brain Cells with mAb H8 and Antibody to MBP

Mouse Brain Cell Culture. Brains from newborn mice were asceptically removed and mechanically dissociated as previously described (29). The cells were suspended at a concentration of 1.5 × 10^6 cells/ml in RPMI medium 1640 with L-glutamine and 2.0 g/liter sodium bicarbonate (Cell Culture Facility, University of California, San Diego, CA) supplemented with 10% FCS (Flow Laboratories, Inc., McLean, VA) and 1% antibiotics (Fungi-Bact Stock Solution; penicillin G [10,000 U/ml], streptomycin sulfate [10,000 μg/ml], and fungizone [25 μg/ml] in normal saline; Irvine Scientific, Santa Ana, CA). 10 ml of the cell suspension was distributed into 100-mm petri dishes (Falcon tissue culture dish 100 × 20-mm style; Becton Dickinson Labware, Lincoln Park, NJ) containing glass coverslips. The cultures were kept at 37°C in 5% CO_2. After 4 d in culture, the medium was changed every other day. Coverslips from 21-d-old brain cultures were used for immunofluorescent labeling studies.

Immunofluorescent Labeling. Coverslips were incubated with mAb H8 (ascites) (diluted with the culture medium at 1:100) for 2 h at 37°C. As negative controls, coverslips were incubated with only the medium or an irrelevant mouse mAb (ascites). Then, coverslips were fixed with 95% ethanol and 5% acetic acid for 4 min at −20°C, and washed with PBS three times for 5 min each at room temperature (RT). The coverslips were then incubated with FITC-conjugated goat antibody to mouse IgG (dilution 1:40) (CooperBiomedical, Inc., Malvern, PA) for 30 min at 37°C. After a PBS wash, coverslips were mounted in glycerol/PBS solution (glycerol 9/PBS 1) and observed using a fluorescent microscope.

Double Immunofluorescent Labeling. Cells positive for mAb H8 were morphologically similar to oligodendrocytes. Therefore, antibody to MBP was used as a marker of oligodendrocytes (30) in double labeling studies. Coverslips were incubated with a mixture of mAb H8 (ascitic fluid) (dilution 1:100) and rabbit antibody to MBP (Dako Corp., Santa Barbara, CA) (dilution 1:100) for 2 h at 37°C. The coverslips were then fixed with 95% ethanol and 5% acetic acid, as described above. After washing with PBS, coverslips were incubated with a mixture of FITC-conjugated goat antibody to mouse IgG (dilution 1:40) and rhodamine-conjugated goat antibody to rabbit IgG (dilution 1:40) (CooperBiomedical, Inc.) for 30 min at 37°C. The coverslips were washed with PBS, mounted in glycerol/PBS solution, and observed using fluorescent microscopy.

Immunohistochemical Staining of Mouse Brain Sections with mAb H8

Brain tissues were obtained from SJL/J mice and immediately frozen in liquid nitrogen. Cryostat sections were cut (8-μm sections) and air dried. The immunostaining was performed using the avidin-biotin-peroxidase complex (ABC) method (31). Sections were first incubated with isotonic solution (culture medium) containing 5% normal goat serum for 30 min at RT, and then incubated with mAb H8 (ascitic fluid) (1:1,000) for 2 h at 37°C. The sections were fixed in 95% ethanol and 5% acetic acid for 4 min at −20°C. After washing with PBS three times for 5 min each, sections were incubated with 1:40 biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA) for 30 min at RT. After a PBS wash, sections were incubated with ABC (Vectastain ABC kit; Vector Laboratories, Inc.) for 30 min at RT. Sections were washed with PBS and incubated with 0.06% 3,3′-diaminobenzidine (Sigma Chemical Co.) and 0.03% hydrogenperoxide in 10 mM Tris (pH 7.5) for 7 min at RT. After a PBS wash, sections were counterstained with hematoxylin for 30 s.

As a positive control for the reactions with mAb H8, paraffin sections of brains from the nude mice infected with the DA strain of TMEV containing many TMEV-infected cells (32) were used. Deparaffinized sections were incubated with mAb H8 for 2 h at 37°C, with biotinylated horse anti-mouse IgG, and with ABC, and reacted with the diaminobenzidine solution.

In blocking experiments, mAb H8 was preincubated with baby hamster kidney (BHK) cells infected with DA strain of TMEV (10^8 cells) overnight at 4°C. After centrifugation at 5,000 *g* for 5 min, supernatant fluid was used as an antibody preabsorbed with the antigen. mAb H8 preincubated with uninfected BHK cells was also used as a control.

Injection of mAb H8 to Mice with Acute EAE

The experimental design to investigate the effect of mAb H8 on CNS in vivo was derived

from methods described by Schluesener et al. (33) and Linington et al. (34). This is briefly described.

Induction of Acute EAE. SJL/J female mice, 4-6 wk old, were obtained from The Jackson Laboratory, Bar Harbor, ME, and EAE was induced as described (35). Mice were anesthetized and injected with 100 μl of a mixture of 16 mg mouse spinal cord homogenate in 50 μl of PBS and 50 μg of *Mycobacterium tuberculosis* (Difco Laboratories, Inc., Detroit, MI) in 50 μl of IFA (Difco Laboratories, Inc.). Then, 24 and 72 h later, 10^{10} organisms of washed, killed *Bordetella pertussis* (Michigan Department of Public Health, Lansing, MI) were injected intravenously to each mouse.

Injection of mAb H8. On days 7 and 11 after induction of EAE, 11 mice were given intravenous injections of mAb H8 (ascitic fluid) (IgG 30 μg/body weight).

Control Groups. Three groups of mice were used as controls: (*a*) EAE with intravenous injection of normal mouse Ig (same concentration as mAb H8) (11 mice); (*b*) EAE only without injection of mAb H8 (five mice); and (*c*) intravenous injection of mAb H8 without EAE induction (five mice). Normal mouse Ig was obtained from serum of normal SJL mice and had no reactivity with TMEV antigen, as determined by ELISA. The Ig fraction was obtained by precipitation at 4°C with 50% saturated ammonium sulfate, and the precipitate was dialyzed for 2 d against several changes of PBS. The Ig concentrations of the normal mouse Ig fractions, as well as of mAb H8, were determined by ELISA using normal mouse IgG (ICN Biomedicals, Lisle, IL) as a standard of known Ig concentration.

Clinical Observations. Clinical signs were evaluated by modification of the criteria by Pettinelli et al. (36), graded on a 0-5 scale with increasing severity: 0, no abnormality; 1, a flaccid tail with or without mild hind leg weakness, or mildly unsteady gait without flaccid tail; 2, flaccid tail and moderate hind limb weakness; 3, marked (but not complete) paralysis of the hind limbs with or without mild forelimb weakness; 4, total paralysis of the hind limbs with moderate forelimb weakness; 5, quadriplegia or a premorbid state.

Pathological Evaluations. On day 13 after induction of EAE, mice were perfused with 4% paraformaldehyde in PBS. The brains were post-fixed in the same fixative, cut in the median saggital direction, and embedded in paraffin. Paraffin sections of the brains, 5-μm thick, were stained with hematoxylin and eosin, and with antibody to MBP for detection of myelin. Immunostaining with anti-MBP antibody was performed using the ABC method (31). Deparaffinized sections were incubated with rabbit antiserum to MBP (Dako Corp.) (diluted 1:300) for 2 h at 37°C, followed by biotinylated goat anti-rabbit IgG and ABC, and reacted with the diaminobenzidine/H_2O_2 solution.

The spinal cords were post-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer. At four different levels, including one cervical, two thoracic, and one lumbo-sacral portions, each spinal cord was cut, osmicated, and embedded in resin. Approximately 1-μm thick sections were cut from resin-embedded tissues of the spinal cords at the four levels, and stained with toluidine blue.

Quantification of Demyelination and Inflammation. The degree of demyelination was quantified using toluidine blue sections of the spinal cords, since this method is most suitable in light microscopic observations of demyelination. In each mouse, sizes of both the demyelinated area and area of white matter were measured in four sections of the four different levels of the spinal cord by a computer-assisted morphometric system (C-2 Image Analyzer; Olympus Corporation of America, New Hyde Park, NY). The proportion of the total demyelinated area to total area of the white matter was then calculated for each mouse. Results of each mouse were expressed by 100 × (demyelinated area/area of white matter). For inflammation, a total number of perivascular infiltrates in the brain and spinal cord sections were counted for each mouse.

ELISA of mAb H8 and TMEV-infected and Normal Mouse Sera

Sera were obtained and pooled from normal SJL/J mice (age matched) and from 10 infected SJL/J mice that were intracerebrally inoculated with TMEV (DA) (10^5 PFU) 4-8 wk prior. These mice were 7-8 wk old at the time of inoculation, and pathologically showed chronic inflammatory demyelinating disease of the spinal cord white matter when the sera were obtained 4-8 wk after the inoculation. Ig fractions were isolated by 50% saturated ammonium

sulfate from: (*a*) normal mouse sera; (*b*) TMEV-infected sera; and (*c*) mAb H8 (ascitic fluid). Ig concentrations were determined by ELISA. Ig concentration was adjusted to 4 mg/ml in all the three Ig fractions.

ELISA for Antibody to TMEV (DA). Antibody to TMEV (DA strain) was quantified by ELISA as described by Rice and Fujinami (28). TMEV antigen preparation was made as a cytoplasmic extract of infected cells and processed (37). Briefly, 96-well plates (Linbro/Titertek; Flow Laboratories, Inc.) were coated with 100 μl of TMEV (DA) antigen at 5 μg protein/ml in PBS overnight. After blocking with 10% FCS in PBS, serial dilutions of Ig fractions were applied and incubated for 90 min at RT. After washing, 100 μl of horseradish peroxidase (HRPO)-labeled affinity-purified antibody (goat) to mouse Ig (Kirkegaad & Perry Laboratories, Inc., Gaithersburg, MD) at a 1:1,000 dilution was added, and plates were incubated for 90 min at RT. After washing, 100 μl of the substrate solution consisting of 3 mM H_2O_2 and 2.2 mM *O*-phenylene diamine in 0.1 M sodium citrate buffer (pH 5.0) was added, and plates were incubated for 30 min in the dark. The reaction was stopped by the addition of 100 μl of 1 N HCl, and optical densities (OD_{492}) were measured on a Titertek Multiskan (Flow Laboratories, Inc.).

ELISA for Antibody to GC. Antibody to GC was quantified by ELISA as described by Fujinami et al. (21). Briefly, GC was solubilized in chloroform/methanol (1:1) at a concentration of 1 mg/ml. The solution was further diluted to 5 μg/ml, and 100 μl was then added to 96-well plates (3912 Micro Test III Flexible Assay Plate; Falcon Labware, Oxnard, CA). The chloroform/methanol was removed by evaporation, and the assay was performed as described in ELISA for antibody to TMEV.

Competition ELISA. Antibody with similar specificity as mAb H8 in sera was analyzed by competition ELISA (38). Briefly, mAb H8 was coupled to HRPO by the periodate method (39). Competition assays were performed as follows. 96-well plates were coated with TMEV (DA) or GC antigen as described above. Various dilutions of the unlabeled first (inhibiting) antibodies (Ig fractions) from TMEV (DA)-infected sera, normal mouse sera, or mAb H8 were added to the wells and incubated for 90 min at RT. Then a standard amount of the second antibody, mAb H8, labeled with HRPO, was added to the wells and incubated for 90 min at RT. The plates were then washed, and 100 μl of the substrate solution was added. The enzyme reaction was terminated after 30 min by the addition of 100 μl of 1 N HCl into the wells. OD_{492} were read with a Titertek Multiskan (Flow Laboratories, Inc.). In this system, if the unlabeled first (inhibiting) antibodies recognize the same (or nearby) epitope on TMEV or GC as mAb H8, the bound first antibodies prevent the labeled second antibody (HRPO-conjugated mAb H8) from binding. Blocking of the HRPO-conjugated antibody by mAb H8 itself was defined to be 100% blocking or inhibition. Calculated was percent blocking of HRPO-conjugated mAb H8 by Ig from DA-infected sera or normal sera at the same Ig concentration as mAb H8.

Results

Immunostainings of Brain Cell Cultures and Brain Tissue Sections. In mouse brain cell cultures, small, round cells with or without cellular processes were labeled with mAb H8 (Fig. 1 *a*). These cells were growing on the top of the other large flat cells and appeared morphologically similar to oligodendrocytes. To determine the nature of the positive cells, double-labeling studies were performed. These small round cells were double labeled with mAb H8 and anti-MBP (Fig. 1 *a* and *b*). These data indicate that the mAb $H8^+$ cells were myelin-producing cells, i.e., oligodendrocytes.

In immunohistochemical studies of frozen mouse brain sections, myelin structures in the brain were positively stained with mAb H8 (Fig. 2 *a*). This reaction was inhibited by preincubation of mAb H8 with the TMEV-infected BHK cells (Fig. 2 *b*), but not with uninfected BHK cells. As a positive control, mAb H8 stained TMEV-infected cells in TMEV-infected brain (Fig. 3 *a*). This staining of TMEV

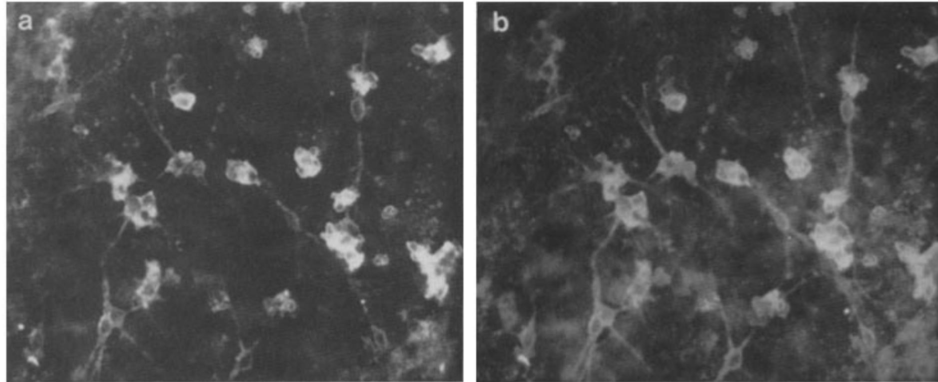

FIGURE 1. 21-d-old brain cell culture from newborn mice. Double immunofluorescent labeling with mAb H8 (demonstrated by FITC [*a*]) and antibody to MBP (by rhodamine [*b*]). Small round cells with or without thin cellular processes are labeled with mAb H8 (*a*). These cells are lying over flat large cells in culture. The cells positive for mAb H8 (*a*) are double labeled with antibody to MBP (*b*) (×230).

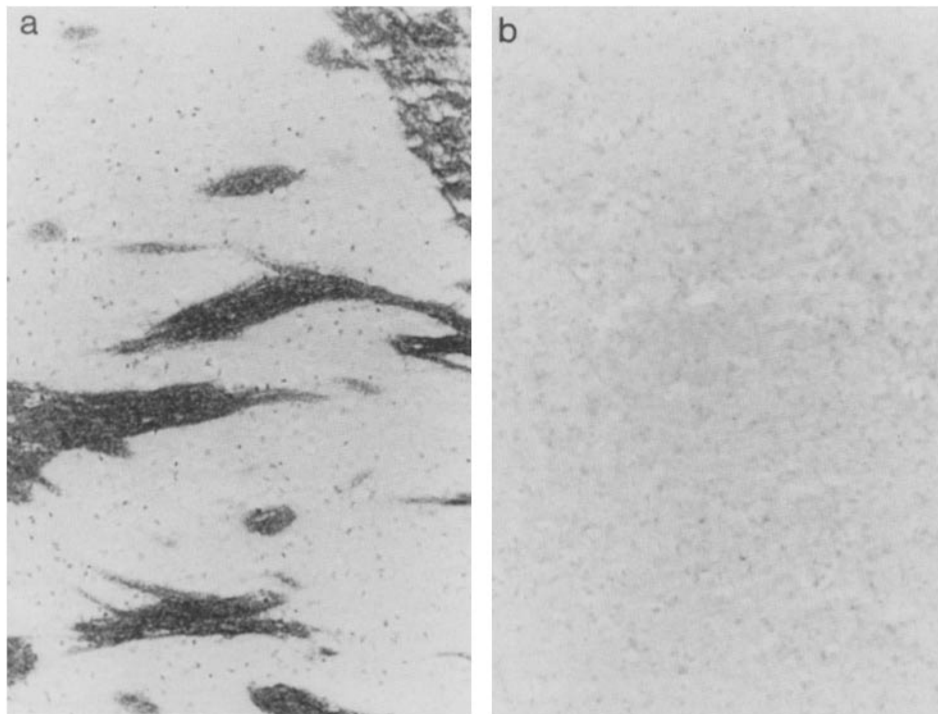

FIGURE 2. Frozen section of mouse brain. mAb H8 immunostains myelin structures in striatum of the brain (*a*). The positive immunoreaction is blocked by preincubation of mAb H8 with TMEV (DA) antigen (*b*) (×124).

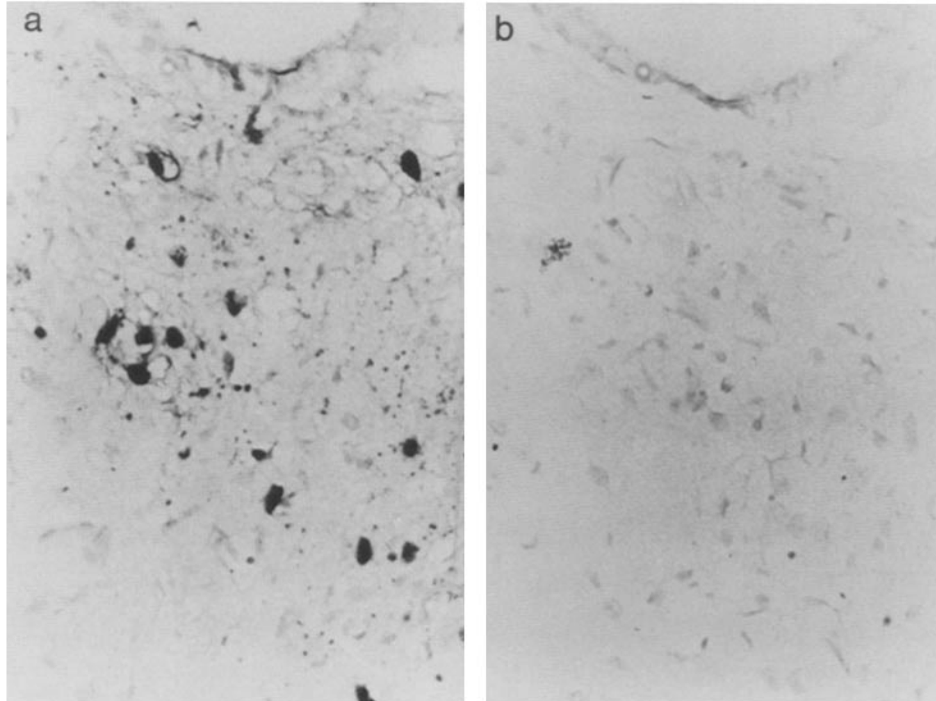

FIGURE 3. TMEV-infected brain. mAb H8 immunostains infected cells (*a*), and the reaction is blocked by preincubation of mAb H8 with TMEV (DA) antigen (*b*) (×250).

antigen was also blocked by preincubation of the mAb with TMEV antigen (Fig. 3 *b*). mAb H8 did not stain myelin structures in paraffin-embedded tissue sections, indicating that the antigen was labile or solubilized by the embedding and/or deparaffinizing procedures.

Modulation of EAE by mAb H8. To investigate the effect of mAb H8 under pathologic conditions, mAb H8 was passively transferred into mice after EAE induction. No clinical disease was observed in mice treated with mAb H8 alone (Table I). Mice given CNS antigen with or without mAb H8 developed clinical signs of EAE. The clinical signs in EAE mice given mAb H8 tended to be more severe than those EAE mice treated with normal mouse Ig or EAE mice without treatment. However, the clinical severity varied from mouse to mouse. Statistical analysis showed no significant difference in clinical score among the three groups (Table I).

Histopathologically, in EAE mice with or without antibody treatment, an acute meningoencephalomyelitis was observed. No lesions were observed in mice injected with mAb H8 alone. Lesions in EAE mice were characterized by meningeal and perivascular infiltrates of inflammatory cells consisting of lymphocytes and polymorphonuclear cells associated with rather mild demyelination. Most parenchymal involvement was distributed within the spinal cord, brainstem, and cerebellar white matter. The distribution of inflammatory lesions were similar among the three groups with EAE. Although the number of perivascular infiltrates appeared to be increased

TABLE I

Modulation of Acute EAE by Intravenous Injection of mAb H8

Treatment	No. of mice	Clinical score*	Perivascular infiltrate‡	Demyelinated area§
				%
EAE + H8	11	3.98 ± 1.31	37.1 ± 17.3	5.08 ± 1.81‖
EAE + normal mouse Ig	11	2.64 ± 1.55	24.6 ± 16.6	0.83 ± 0.57
EAE only	5	2.60 ± 1.62	21.8 ± 11.1	0.55 ± 0.24
H8 only	5	0	0	0

Data are mean ± SD.

* Clinical signs graded by a 0–5 scale with increasing severity. See text for details.

‡ The total number of perivascular infiltrates in the brain and spinal cords.

§ Demyelinated area/white matter area. Areas are total of four levels of the spinal cord.

‖ Value of demyelinated area in EAE with mAb H8 is significantly higher than that in EAE with normal mouse Ig ($p < 0.001$) and than that in EAE only ($p < 0.001$) by student's t test. There is no significant difference in the clinical scores or in the numbers of perivascular infiltrate.

in EAE mice receiving mAb H8 vs. EAE mice with normal mouse Ig or without, statistical analysis demonstrated no significant difference among the three groups with EAE (Table I). In contrast, the demyelination in EAE mice treated with mAb H8 was more extensive than that observed in EAE mice injected with normal mouse Ig or without treatment (Figs. 4 and 5, Table I).

Quantification of demyelination was performed by examining cross sections of resin-embedded spinal cord stained with toluidine blue. These materials were more suitable for fine and morphometric evaluation of demyelination rather than paraffin-embedded brain sections immunostained with anti-MBP. In EAE mice injected with normal mouse Ig or without treatment (Fig. 4), loss of myelin was commonly found in and frequently confined to subpial areas of nerve root entry zones of the spinal cord accompanied by infiltration of inflammatory cells. In EAE mice treated with mAb H8 (Fig. 5), the demyelination was widely distributed in subpial areas and often in the deep white matter, as well as lesions around root entry zones. Those lesions were frequently, but not always, associated with inflammation. Quantitative analysis of demyelination clearly demonstrated a significant increase of demyelination in EAE mice receiving mAb H8 (10-fold) compared with EAE mice treated with normal mouse Ig or without ($p < 0.001$) (Table I).

Analysis of TMEV-infected Sera by ELISA. To determine whether antibodies like mAb H8, which has specificity to GC and TMEV and plays a role in demyelination, were present in mice with chronic infection of TMEV (DA), the sera were analyzed by quantitative ELISA. Ig concentrations were first determined from the various sources. Ig concentrations were 2.8 mg/ml in normal sera, 10.0 mg/ml in TMEV-infected sera, and 4.8 mg/ml in mAb H8 ascitic fluid, as determined by ELISA. The Ig fractions were obtained as described in Materials and Methods, and used in the following assays after the Ig concentrations were adjusted to 4 mg/ml.

The results of the ELISAs are shown in Table II. The Ig fraction from normal sera did not contain antibody to TMEV or GC. The Ig fraction from mAb H8 (ascites) had an antibody titer ($\log_2$) of 17.3 to TMEV and 9.2 to GC. Similarly, the

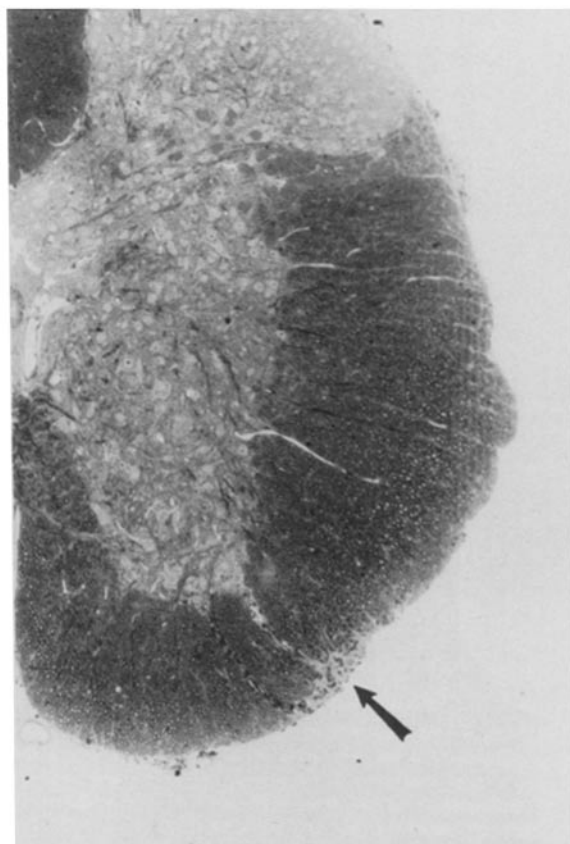

FIGURE 4. Representative spinal cord section of EAE mice treated with normal mouse Ig. Resin-embedded section stained with toluidine blue. A small demyelinated area with inflammatory cells is found to be confined to an entry zone of the ventral root (*arrow*) (×75).

Ig preparation from the infected sera had a titer of 15.1 to TMEV and 6.2 to GC. To investigate the presence of antibody(s) with the same or similar specificity as mAb H8 in sera, competition ELISAs were performed. Competition of the unlabeled first antibody (Ig from the infected or normal sera) with the labeled second antibody (HRPO-conjugated mAb H8) in binding to TMEV or GC antigen was analyzed as described in Materials and Methods. By competition ELISA for TMEV antigen, percent blocking of HRPO-conjugated mAb H8 by Ig from the infected sera was 32%. Further, competition ELISA for GC antigen revealed 31% blocking of HRPO-conjugated mAb H8 by Ig from the infected sera. No inhibition of binding of the HRPO-conjugated mAb H8 to TMEV or GC antigen could be demonstrated in the Ig fraction from normal serum.

Discussion

This is the first report demonstrating that an antibody to virus can enhance demyelination of CNS in vivo. Our results indicate that antibodies, as those generated by immune response to TMEV, can react with myelin and oligodendrocytes, and contribute to demyelination through an immune process.

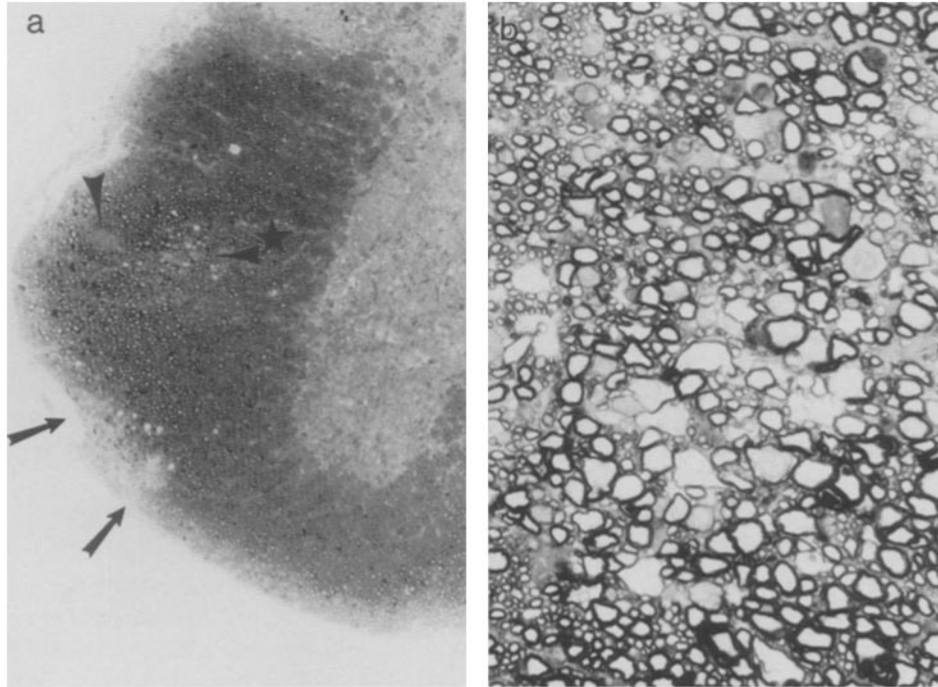

FIGURE 5. Representative spinal cord section of EAE mice treated with mAb H8. Resin-embedded section stained with toluidine blue. Demyelinated areas are found in deep white matter of the lateral funiculus (*arrow heads*), as well as in subpial area around the ventral root entry zone (*arrows*) (*a*). Higher magnification of the lesion indicated by arrow head with asterisk in *a* is shown in *b*. Spongiform changes and loss of myelin are observed (*a*, ×82; *b*, ×500).

Our results of immunohistochemical studies have demonstrated that mAb H8 binds to oligodendrocytes in mouse brain cell cultures and myelin structures in brain sections. Double-immunolabeling studies using brain cell cultures revealed that mAb $H8^+$ cells contained MBP as well, indicating that mAb H8 binds to myelin-producing cells (oligodendrocytes). Immunohistochemistry using frozen brain sections disclosed that mAb H8 reacted with myelin structures at a tissue level. A possible reason why mAb H8 did not stain myelin structures in paraffin-embedded brain sections can be explained since lipid antigens such as GC, with which mAb H8 reacts as well as with TMEV VP-1 (21), are lost during processing for paraffin embedding of tissues and deparaffinization. The molecular background of this crossreaction between TMEV VP-1 and GC is under study. It is possible that there is mimicry in molecular conformation between VP-1 of TMEV and lipid-like structures such as GC, or that the presence of overlapping determinants may be found (21).

The experimental modulation of EAE by mAb H8 demonstrated that this antibody can augment demyelination in vivo in mice. Since mice treated with mAb H8 alone did not display demyelination, destruction or alteration of the blood-brain barrier (BBB) by EAE induction, i.e., inflammation, appears to be an important event for mAb H8, which is systemically administered to enter the CNS. This is consistent

TABLE II
ELISA of Sera from TMEV-infected and Normal Mice and of mAb H8 for Antibodies to TMEV and GC and for Antibody with Same Specificity as mAb H8

	Anti-TMEV*	Anti-GC*	Competition with HPRO-conjugated mAb H8 for: TMEV‡	GC‡
			%	%
Normal sera§	0	0	0	0
TMEV-infected sera§	15.1	6.2	32	31
mAb H8§	17.3	9.2	100	100

* Titers (log_2) in ELISA.
‡ Percent blocking of HRPO-conjugated mAb H8. Blocking by mAb H8 itself is defined to be 100%. See text for the details of competition ELISA.
§ Ig fractions were prepared at the same concentration (4 mg/ml) and used for ELISA.

with reports where passive transfer of anti-GC antibody could cause demyelination in CNS when injected intraocularly (25, 26) or injected directly into peripheral nerves (24). Our results indicate that mAb H8, which has both anti-TMEV and anti-GC activities, can induce demyelination in vivo when systemically transferred into mice with EAE. Demyelination induced by the mAb may occur through complement- or cell-mediated processes. Brosnan et al. (25, 40) have postulated that effector cells provided by inflammation are necessary for anti-CNS antibodies to produce demyelination. In our experiments, demyelinated areas in mice with EAE given mAb H8 were frequently associated with infiltration of mononuclear cells. It is possible that mAb H8 can effectively produce demyelination in the presence of inflammatory cells through an antibody-dependent cell-mediated process. Such studies detailing these events have been initiated.

ELISA of sera from mice with chronic TMEV infection has shown that the sera contained antibody(s) to GC as well as to TMEV, and that in competition ELISAs for TMEV or GC antigen with HRPO-labeled mAb H8, a substantial part of the Ig in the infected sera reacted with the same (or nearby) epitope as recognized by mAb H8. It is possible that damage to myelin by cytopathic effects of TMEV infection may result in the generation of antibody to GC. This cannot be entirely ruled out.

Our data indicate that such an antibody(s) in the sera could play a role in demyelination through an immune-mediated process. Barbano and Dal Canto (41) have reported that serum and cells from TMEV-infected mice failed to injure myelinating cultures or to produce in vivo demyelination. In their in vivo passive transfer experiment, mice were injected with splenocytes that were removed from TMEV-infected mice and incubated with MBP or with spinal cord homogenate, and at 10 d after injection, the mice had no apparent disease in the spinal cords. This experimental system is markedly different from ours, where an antibody to TMEV was injected to mice with EAE. In our experimental model, the recipient mice had CNS inflammation with alterations of BBB due to acute EAE and received specific antibodies. In other studies, Rodriguez et al. (42) reported that serum or CSF IgG from TMEV-

infected mice did not bind to myelin or other CNS components. In contrast, Cash et al. (43) has described that Ig secreted by the B cells present in the inflammatory CNS lesions of TMEV-infected animals reacted with nonviral white manner components as well as with the viral capsid proteins, and they suggested that these autoreactive antibodies could have a role in demyelination. In their studies, such nonviral white matter components were present only in infected animals, and are somewhat different from ours. Our data support the presence for autoantibodies and their role in demyelination.

Our results corroborate an autoimmune contribution in the demyelinating aspect of TMEV infection. In TMEV infection, an immune response to TMEV can lead to generation of antibodies to viral components, including VP-1, and myelin components. VP-1 of TMEV contains a highly exposed neutralizing epitope (44). Such an antibody in the sera can work as a neutralizing antibody against the viruses and have an important role to eliminate virus from the animal. We have demonstrated that another neutralizing antibody to TMEV (H7), which reacts with VP-1 but not with GC, can play a dramatic role in the clearance of virus and survival of athymic mice (45). In contrast, antibody(s) of H8 type could in turn contribute to CNS disease. This may occur when the BBB is broken after TMEV infection of the CNS and/or when the antibody is produced intrathecally by infiltrating cells. A part of infiltrating inflammatory cells associated with the viral infection could play an effector role for an antibody-mediated demyelination. Further studies clarifying the role of the antibody-mediated demyelination are ongoing using mAb H8 in TMEV-infected mice, as well as immune sera in EAE mice.

Summary

Theiler's murine encephalomyelitis virus (TMEV) causes a chronic demyelinating disease in mice. The mechanisms underlying the demyelination have not been fully elucidated. We have raised a mAb to TMEV (DA strain), H8, that reacts both with TMEV VP-1 and galactocerebroside (GC). In mouse brain cultures, cells positive for the mAb H8 epitope were double labeled with antibody to myelin basic protein, indicating that those cells were oligodendrocytes. Further, mAb H8 could immunostain myelin structures in frozen sections from mouse brains. When injected intravenously into mice with acute allergic encephalomyelitis, mAb H8 increased by 10-fold the size of demyelinated areas within the spinal cords. This is the first report demonstrating that an antibody to virus can enhance demyelination of a central nervous system disease. Ig fractions from the sera of mice with chronic TMEV infection had antibody(s) to GC, as well as to TMEV, as determined by ELISA. Furthermore, a competition ELISA for TMEV or GC antigen revealed that sera from these infected mice contained antibody(s) with the same specificity as mAb H8. Our results indicate that antibodies generated by immune response to TMEV can react with myelin and oligodendrocytes, and contribute to demyelination through an immune process.

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