Persistence of Theiler's Virus Infection Following Promotion of Central Nervous System Remyelination

AMY K. PATICK, PH.D., ROGER L. THIEMANN, PETER C. O'BRIEN, PH.D., AND MOSES RODRIGUEZ, M.D.

Abstract. Chronic infection of SJL/J mice with the Daniel's strain of Theiler's virus develop primary demyelination, viral persistence but minimal central nervous system (CNS)-type remyelination. In contrast, treatment of virus-infected mice with sera or immunoglobulin G (IgG) from mice immunized with homogenized spinal cord (SCH) emulsified in incomplete Freund's adjuvant promotes CNS remyelination. We measured levels of infectious virus, virus antigen and virus-specific antibody to determine if treatments which promote CNS remyelination are able to modulate infection. Levels of virus-specific antibody were higher in mice treated with SCH/IgG than control treatment groups and correlated positively with extent of remyelination. Although number of virus antigen-positive cells in spinal cord was less in mice treated with SCH/IgG than mice treated with phosphate buffered saline (PBS)/IgG, there was only a slight negative correlation with extent of demyelination by regression analysis. Titers of infectious virus isolated three to six months following infection were not different among treatment groups. Even though treatment of mice with SCH/IgG reduced number of virus antigen-positive cells and enhanced levels of virus-specific antibody, CNS remyelination can occur despite presence of infectious virus.

Key Words: Central nervous system remyelination; Demyelination; Infectious virus; Oligodendrocyte; Pathogenesis; Theiler's murine encephalomyelitis virus; Viral antigen.

INTRODUCTION

In chronic human demyelinating diseases of the central nervous system (CNS) such as multiple sclerosis (MS), remyelination is sparse and limited (1–4). In contrast, the capacity of the CNS for myelin regeneration has been demonstrated in experimental models after demyelination induced by herpes simplex virus (5–7), demyelinating strains of hepatitis virus (8–12), Ross River virus (13), Cuprizone intoxication (rev. in ref. 14), or autoimmune reaction to myelin (rev. in ref. 15). Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus which induces chronic CNS demyelination in susceptible strains of mice characterized by persistent virus infection and inflammation associated with foci of myelin destruction (16, 17). Demyelination results from direct cytolysis of oligodendrocytes by virus (18–21) and reaction by the immune response to a virus-induced antigen (22–24). The frequent occurrence of spontaneous remyelination in CNS of SJL/J mice following infection with the Daniel's strain of TMEV (DAV) (21, 25–27) mimics the neuropathology observed in MS. We have promoted remyelination of CNS axons in chronically demyelinated mice by systemic injection of either sera or immunoglobulin G (IgG) from mice hyperimmunized with spinal cord homogenate (SCH) emulsified in in-
complete Freund’s adjuvant (IFA) (28, 29). Treatment of mice with SCH/IgG is also associated with an increased proliferation of progenitor glial cells in areas of remyelination (29). The mechanism(s) underlying these phenomena, the significance of the glial cell proliferation, and the antigenic specificities of the anti-SCH serum are not known. In this study, based on observations which indicate that virus infection is necessary for demyelination (30), we tested whether treatments which promote CNS-type remyelination also interfered with virus infection.

**MATERIALS AND METHODS**

**Virus:** The Daniel strain of TMEV (DAV) (31) was used for all experiments. This strain, originally obtained from J.P. Lehrich and associates of the University of Chicago (32), was grown to 5 x 10⁶ plaque forming units (PFU)/ml in baby hamster kidney-21 cells (33).

**Mice Inoculations:** Female SJL/J mice, four to six weeks of age (Jackson Laboratories, Bar Harbor, ME), were injected intracerebrally with 2 x 10⁶ PFU of virus in 10 μl volume. Antisera and IgG samples were prepared as described (28, 29). In brief, donor syngeneic normal SJL/J mice were injected subcutaneously twice a week for one month followed by monthly boosts with 1 mg of SCH/IFA, phosphate buffered saline (PBS)/IFA or PBS alone. IgG was prepared from antisera pools by ammonium sulphate precipitation followed by adsorption to protein A-Sepharose. Chronically infected mice (three to six months) were treated twice weekly for four weeks with intraperitoneal (ip) injections of either 0.5 ml serum (SCH/sera, IFA/sera, PBS/sera) or 1 mg IgG (SCH/IgG, IFA/IgG, PBS/IgG). In the sixth week, sera were collected from all mice by intracardiac puncture and spinal cords were processed for light microscopy or brain and spinal cords were removed for infectious virus determinations.

**Virus Plaque Assay:** Virus titers in clarified CNS homogenates were determined by plaque assay as described (34). In brief, 0.2 ml of serial dilutions of virus in Dulbecco’s modified Eagles Medium (DMEM) with 1% bovine serum albumin (BSA) were plated in duplicate on confluent monolayers of L2 cells in 12-well plates (Falcon, Cockeysville, MD). Following adsorption for one hour (h) at 37°C, cell cultures were overlaid with 3 ml of DMEM containing 2% newborn calf serum and 0.4% Seaplaque agarose (FMC Bioproducts, Rockland, ME). Two to three days later, agarose was removed and cell cultures were fixed with ethanol/acetic acid/formaldehyde (6:2:1) and stained with 1% crystal violet in 20% ethanol. Plaques were enumerated and results expressed as LOG₁₀ PFU/g CNS. Central nervous system homogenates were prepared from brain and spinal cords aseptically removed from mice sacrificed with ether. A 10% homogenate was prepared in DMEM, sonicated for 2 x 60 second intervals and clarified by centrifugation. Virus preparations were stored at -70°C.

**Virus Neutralization Assay:** Samples of DAV, diluted to contain 50 PFU/0.2 ml, were mixed with an equal volume of two-fold dilutions of heat-inactivated (45 minutes, 56°C) serum from DAV-infected mice, mock-infected mice or medium alone. After incubation at 25°C for one h, virus-serum mixtures were assayed for residual infectious virus by plaque assay. Neutralization titers were expressed as the LOG₂ dilution of serum which reduced virus titer by 50 percent.

**Antibody Determinations:** Anti-DAV titers were determined by indirect ELISA using purified DAV antigen (1 μg per well) as described (23). In brief, plates were blocked with 1% bovine serum albumin (BSA) in PBS for one h. Dilutions of mouse anti-DAV sera (100 μl) or control sera from uninfected mice were added to wells and incubated at 37°C for two h. Plates were washed with PBS containing 0.05% Tween and incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma, St. Louis, MO; 1:1,000 dilution). Plates were reacted with Sigma 104 phosphatase substrate and the reaction terminated with 50 μl of 1 N NaOH. The optical density at 410 nm was read with an MR 600 Microplate reader (Dynatech, Boston, MA) and plotted versus reciprocal dilution of serum.

**Preparation of Tissue for Light and Electron Microscopy:** Mice were anesthetized with 0.2 ml pentobarbital (ip) and perfused by intracardiac puncture with Trump’s fixative (phosphate-buffered 4% formaldehyde with 1.0% glutaraldehyde, pH 7.4). Spinal cords were removed and sectioned coronally into 1 mm thick blocks. Every third block from each mouse was postfixed in 1% osmium tetroxide and embedded in Araldite (Polysciences Inc., Warrington, PA).
<table>
<thead>
<tr>
<th>Treatment†</th>
<th>n</th>
<th>Area of demyelination (mm²)</th>
<th>Area of CNS remyelination (mm²)</th>
<th>Area CNS remyelination/area demyelination (%)</th>
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<tr>
<td>SCH/Sera</td>
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* Morphometric analysis was performed on 1 μM araldite-embedded cross-sections of spinal cord (12 sections/mouse) with a Zeiss interactive digital analysis system and camera lucida attached to a Zeiss photomicroscope; Wilcoxon rank order sum test; data presented as mean ± SD.

† PBS = phosphate-buffered saline; IFA = incomplete Freund’s adjuvant; SCH = spinal cord homogenate; IgG = immunoglobulin G.

\[\dagger\] p < 0.01.

\[\dagger\] p < 0.05.

n = Number of mice.

PA). Cross-sections (1 μm thick) from each block were stained with 4% p-phenylenediamine. Selected areas were trimmed and prepared for electron microscopy. The following data were obtained in a non-biased manner from each 1 μm thick spinal cord cross-section using a Zeiss interactive digital analysis system: area of white matter, area of demyelination, percent of total white matter showing demyelination, area of remyelination, and percent of total demyelinated area showing remyelination. For immunoperoxidase studies, extra sections (six to eight per mouse) stored in 0.1 M phosphate buffer were rinsed in 0.1 M Tris buffer with 25 mM hydroxyamine, pH 7.4 at 4°C, treated with 10% dimethyl sulfoxide in the same buffer for one h at 4°C and quick frozen in isopentane chilled in liquid nitrogen. Ten micron cryostat sections were cut and transferred to gelatinized glass slides.

**Immunocytochemistry:** Frozen sections from Trump’s perfused mice were reduced with 1% sodium borohydride in 0.1 M Tris buffer with 25 mM hydroxyamine, pH 7.4 at 4°C and rinsed with 95% alcohol/5% glacial acetic acid. Sections were immunostained with a polyclonal antiserum to purified DAV virions (34) by avidin-biotin immunoperoxidase technique (Vector Laboratories, Burlingame, CA). Slides were developed with a solution of 75 mg of Hanks-Yates reagent [p-phenylene diamine-procatechol (Polysciences, Warrington, PA)] and counterstained with hematoxylin. Virus-antigen positive cells were enumerated in each section from each mouse and the total area of white matter, area of demyelination, and percent of total white matter showing demyelination were morphometrically analyzed as described.

**Statistical Analyses:** Comparisons between groups were based on Student's t-test for normally distributed data (levels of anti-viral antibody), and the Wilcoxon rank sum test was used for non-normal data. Regression analyses were performed to evaluate association between variables, using the method of least squares.

**RESULTS**

**Promotion of Remyelination in Mice Undergoing Theiler’s Virus-Induced Demyelination:** We have shown previously that treatment of chronically infected mice with SCH serum or SCH/IgG statistically increases the total area of CNS remyelination (mm²) and the number of spinal cord lesions with evidence of remyelination when compared to mice receiving control treatments (29). Mice from these treatment groups were selected for subsequent virological analysis (Table 1). Although the area of demyelination examined was similar among all groups, the percent of this area
undergoing CNS-type remyelination was statistically greater in mice treated with SCH/sera(IgG) than mice treated with IFA/sera(IgG) or PBS/sera(IgG). Central nervous system-type remyelination by oligodendrocytes was defined morphologically as abnormally thin myelin lamellae surrounding intact axons (Fig. 1A). In contrast, demyelination was characterized by destruction of the myelin sheaths with preservation of axons (Fig. 1B). Remyelination was most frequently observed at the periphery of active demyelinated lesions.

Anti-DAV Antibody in Mice Undergoing CNS-Type Remyelination: To determine whether administration of SCH/sera(IgG) to chronically infected mice had modulated levels of anti-viral antibody, sera was collected from mice by intracardiac puncture at the end of the treatment protocol and assayed for virus specific antibody by ELISA using purified DAV virions as antigen. Mice treated with either SCH/sera or SCH/IgG had greater levels of antibody than mice treated with either IFA/sera(IgG) or PBS/sera(IgG) (Fig. 2A, B). Statistical significance, however, was observed only between antibody titers from mice treated with SCH/IgG and those treated with PBS/IgG (p = 0.032, 0.035, 0.041 at 1:1,600, 1:3,200 and 1:6,400 dilutions of sera, respectively, by Student’s t-test). The sera and IgG preparations used as treatment for chronically infected mice did not themselves contain detectable anti-viral anti-

**Fig. 2.** Levels of DAV-specific antibody in mice treated with sera (A) or IgG (B) to promote remyelination. Mouse serum immunoglobulin titers against DAV were measured by ELISA with purified DAV as antigen (23). SCH (○); IFA (○); PBS (▲); ▼ = SEM.

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**Fig. 1.** (A) Electron micrograph of spinal cord section from Theliot’s virus infected SJL/J mouse treated with IgG to spinal cord homogenate (SCH/IgG). Several examples of CNS-type remyelination by oligodendrocytes (o) are evidenced by abnormally thin myelin sheaths relative to axon diameter (asterisks). × 4,100 (B) Electron micrograph of spinal cord section from Theliot’s virus infected SJL/J mouse. An axon (a) undergoing active demyelination in the vicinity of an activated macrophage (m). × 18,000

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Fig. 3. Positive correlation of levels of anti-DAV antibody (optical density) with extent of remyelination in Theiler's virus infected SJL/J mice treated with: SCH/Sera (○), SCH/IgG (●), IFA/Sera (Δ), IFA/IgG (■), PBS/Sera (△), or PBS/IgG (▲). Square root transformation was used on values for area of CNS remyelination/area demyelination (%) to linearize regression model. Linear regression line was calculated by method of least squares and equation solved for \( y = a + bx \).

bodies since an ELISA performed with these components was similar to negative controls (optical density < 0.020 at 410 nm).

Regression analysis was performed to correlate anti-viral antibody levels with extent of remyelination among mice from all treatment groups (Fig. 3). A square root transformation was utilized to reduce skewness. Results from this analysis indicated that levels of anti-viral antibody correlated positively (\( p = 0.030 \)) with extent of remyelination (area, mm²).

To determine if increased levels of anti-viral antibody detected by ELISA also reflected similar changes in levels of neutralizing antibody, similar preparations of sera were analyzed by virus plaque reduction assay. Despite the greater level of anti-viral antibody in mice treated with SCH/sera(IgG) as detected by ELISA, mean titers of DAV neutralizing antibody were similar among all treatment groups; LOG₂[11.36 (SCH/IgG); 11.19 (SCH/sera); 11.76 (PBS/IgG); 10.75 (PBS/sera); 12.76 (IFA/IgG); 10.95 (IFA/sera)].

Viral Antigen Production in Mice Undergoing CNS-Type Remyelination: To determine if treatment of infected mice with SCH/IgG had effected local viral antigen production, coronal spinal cord sections from mice known to have significant remyelination (Table 1) as well as those from mice from control treatment groups were embedded and immunostained for viral antigen by immunoperoxidase technique. Although the mean area of demyelination examined was similar among all treatment groups, the mean number of viral antigen positive cells was less in mice treated with SCH/IgG when compared to those treated with IFA/IgG and the dif-
<table>
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<th>Treatment</th>
<th>n</th>
<th>Area (mm²)</th>
<th>% of White matter</th>
<th>Virus antigen-positive cells (no.)$</th>
<th>Viral antigen cells/area demyelination (no./mm²)</th>
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* Morphometric analysis was performed on 10 μM cross-sections of frozen spinal cord (six to eight sections/mouse) with a Zeiss interactive digital analysis system and camera lucida attached to a Zeiss photomicroscope.
† Wilcoxon rank sum test; data presented as mean ± SD; NS = not significant.
‡ PBS = phosphate-buffered saline; IFA = incomplete Freund's adjuvant; SCH = spinal cord homogenate; IgG = immunoglobulin G.
§ Virus antigen identified by avidin-biotin-immunoperoxidase technique using anti-viral serum (34).
Fig. 5. Positive correlation of local viral antigen production with extent of demyelination in Thelander's virus infected SJL/J mice treated with SCH/IgG (●), IFA/IgG (■) or PBS/IgG (▲). Linear regression line was calculated by method of least squares and equation solved for y = a + bx.

ference was statistically significant when compared to those treated with PBS/IgG (Table 2). However, when the number of viral-antigen positive cells in each mouse was adjusted for area of demyelination, local viral antigen production was found to be similar in groups of mice treated with SCH/IgG and IFA/IgG but still less than in mice treated with PBS/IgG. Viral antigen positive cells were primarily observed in areas of pathology; rarely was an antigen-positive cell observed in an area that lacked inflammatory cells and/or demyelination (Fig. 4A, B, C). The positive correlation of viral antigen with area of demyelination (p = 0.0150) as demonstrated by regression analysis (Fig. 5) further supported the hypothesis that local viral antigen production is necessary for demyelination.

Correlation of Local Viral Antigen Production with Remyelination and Anti-Viral Antibody: Our results indicated that increasing levels of anti-viral antibody were associated with a greater extent of remyelination (Fig. 3). To relate these parameters with extent of local viral antigen production, regression analyses were performed. Levels of anti-viral antibody correlated negatively with numbers of antigen-positive

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Fig. 4. Viral antigen-positive cells (arrows) in frozen sections of spinal cord from Thelander’s virus infected mice treated with SCH/IgG (A), IFA/IgG (B), or PBS/IgG (C) were detected by an avidin-biotin-immunoperoxidase technique using a polyclonal antiserum to DAV virions. Many cells are found in areas of pathology of infected mice treated with IFA/IgG (B) and PBS/IgG (C); a single cell is found in a lesion from a mouse treated with SCH/IgG (A). × 1,300
Fig. 6. Negative association of local viral antigen production with levels of anti-DAV antibody (optical density) (A) and extent of CNS remyelination (B) in Theller's virus infected SJL/J mice treated with: SCH/IgG (●), IFA/IgG (■), or PBS/IgG (▲). Linear regression lines were calculated by method of least squares and equation solved for $y = a + bx$. 
Fig. 7. Levels of infectious virus in brain and spinal cord homogenates were determined by virus plaque assay. Detection limit of the assay was $1.7 \log_{10}$ plaque forming units (PFU)/g CNS.

cells; this was, however, not statistically significant ($p = 0.392$) (Fig. 6). In addition, although increased numbers of antigen-positive cells were associated with smaller areas of remyelination, this was also not statistically significant ($p = 0.163$) (Fig. 6). These results suggest that increased CNS remyelination by oligodendrocytes is associated with increased anti-viral antibody production and parallels decrease in number of viral antigen positive cells.

Infectious Virus in Mice Treated with Serum to Promote Remyelination: Previous data have indicated that isolation of infectious virus (virus persistence) correlates with susceptibility to demyelination while viral clearance is associated with resistance (35). To determine if remyelination was accompanied by a decrease in the levels of infectious virus from CNS, groups of chronically infected SJL/J mice were treated with SCH/sera, IFA/sera or PBS/sera by standard protocol. One month later, brain and spinal cords were removed and infectious virus determined by plaque assay (Fig. 7). Although infectious virus was detected in CNS from only four of eight mice treated with SCH/sera, this frequency was similar to that observed in mice treated with PBS/sera (five of eight). The number of mice with persistent virus were, however, fewer than untreated mice (six of seven) or mice treated with IFA/sera (seven of eight).

DISCUSSION

Infection of SJL/J mice with DAV results in chronic demyelination, persistent viral infection and minimal spontaneous CNS-type remyelination by oligodendrocytes. These experiments demonstrate promotion of CNS-type remyelination in SJL/J mice by treatment with serum from syngeneic mice immunized with SCH. Understanding the mechanisms which control or limit CNS remyelination may allow
manipulation of these phenomena to design therapeutic strategies to stimulate myelin regeneration.

Central nervous system remyelination has been extensively described in several human and experimental demyelinating conditions (rev. in ref. 36). It is apparent from published studies that under the appropriate conditions remyelination rapidly follows demyelination depending on the availability and regenerative capacity of oligodendrocytes (4, 10, 37, 38). More importantly, the extent of CNS remyelination depends on the persistence of the demyelinating stimuli. In situations where demyelination is acute and proceeds rapidly, e.g. short term treatment with Cuprizone followed by normal diet (39), or acute infection by a variety of viruses (5–9, 11–13), CNS remyelination is extensive and complete. However, in those situations where disease is chronic or progressive in nature, e.g. long term diet of Cuprizone (37), chronic relapsing experimental autoimmune encephalomyelitis (EAE) (15), chronic infection induced by some viruses (10, 21, 25–27), or multiple sclerosis in humans (1–4), CNS remyelination is limited. The capacity of CNS for myelin repair is, however, amenable to manipulation; administration of galactocerebroside (GC) and myelin basic protein (MBP) emulsified in IFA to guinea pigs suffering from chronic EAE produced clinical improvement and remyelination suggesting that chronic progressive demyelination was arrested and the autoimmune reaction was cleared within the CNS (15, 40). Based on these findings, we hypothesized that treatment of chronically DAV-infected mice with components that promote CNS remyelination may also modulate the virus infection.

By ELISA, we observed an elevation of anti-viral antibodies in serum from chronically infected mice treated with SCH/IgG when compared to mice from control treatment groups. The difference in anti-viral antibodies between the treatment groups may not be biologically significant since in general this represented less than a four-fold change in titers. Data from plaque reduction assays indicated that there was no significant difference in titers of neutralizing antibody among mice of different treatment groups. This difference may be explained by either discrepancies between the sensitivities of the two assays or by the possibility that the antibodies measured by ELISA represent species that recognize non-neutralizing epitopes. The humoral immune response is protective in DAV-induced demyelinating disease; mice depleted of complement components by treatment with cobra venom factor (41) or of B cells by immunosuppressive regimens (42) have more severe demyelination when compared to control mice. Whether the increase in anti-viral antibody titers observed after treatment with SCH/sera(IgG) represents a nonspecific activation of the immune system that is not important in remyelination or if the antibodies play a vital role in inhibiting virus or blocking a detrimental immune response thereby allowing for remyelination remains to be determined.

Relevant to this last proposal is the observation that increasing amounts of anti-viral antibody were positively correlated with a greater extent of CNS remyelination and also negatively associated with the number of viral antigen-positive cells. The number of viral antigen-positive cells was less in mice treated with SCH/IgG and was negatively correlated with extent of remyelination. However, the low number of viral antigen positive cells precludes firm conclusions. These results suggest that treatments which promote CNS remyelination modulate certain aspects of TMEV infection.

However, we found no difference in levels of infectious virus from CNS of mice among different treatment groups. Discrepancies between infectious virus and viral antigen have been previously described (43, 44). Although infectious virus is a reliable
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measure of the disease process, local viral antigen production reflects the pathology more accurately. Infectious virus was present in four of eight mice treated with SCH/sera. Since the entire CNS is utilized in this assay it was impossible in these mice to morphologically assess the extent of remyelination. Although the extent of CNS remyelination has been shown to vary among mice treated with SCH/sera, the majority of these mice developed significantly greater extent of remyelination when compared to mice receiving control treatments (28).

The araldite embedding technique of osmicated spinal cord sections utilized to preserve myelin and allow quantitative morphological assessment of CNS remyelination did not conserve the immunogenicity of viral protein. Thus, we were unable to determine if local viral antigen production could occur together with remyelination in the same lesion. However, the extent of remyelination that occurs in mice treated with SCH (Table 1), e.g. 52% of demyelinated area with evidence of remyelination, together with the strong spatial correlation between local viral antigen production and pathologic lesions provide inferral evidence that active demyelination and viral antigen production may occur together with remyelination in the same area of pathology. Identification of the specific cell types that contain virus antigen may provide insight into the differences in pathology observed among mice in various treatment groups. Previous immunoelectron microscopic studies in vivo (19, 28) have demonstrated viral antigen within oligodendrocytes during chronic infection. Data derived from in vitro (20) experiments suggest that DAV lytically infects oligodendrocytes and neurons but persists in astrocytes and macrophages.

Our results demonstrating increased anti-viral antibody, decreased number of viral antigen producing cells together with their association with remyelination indicate that treatments which promote remyelination are able to modulate the virus infection. However, the presence of both local viral antigen and infectious virus in CNS of SCH/sera(IgG)-treated mice undergoing CNS remyelination suggest that new myelin synthesis does proceed despite active virus infection. This data suggests that inhibition of detrimental immune response or direct stimulation of appropriate glial progenitor cells should be considered as alternative mechanisms for the promotion of CNS remyelination.

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