IMMUNE MEDIATED CLEARANCE OF JHM VIRUS FROM THE CENTRAL NERVOUS SYSTEM

Mark A. Sussman[1], John O. Fleming[2], Hamish Allen[3], and Stephen A. Stohlman[1,2]

Departments of Microbiology[1] and Neurology[2]
University of Southern California
School of Medicine
Los Angeles, CA 90033

Biogen Research Corp.[3]
Cambridge, MA 02142

Introduction

The study of viral pathogenesis has revealed how animals respond to viral infection and the subsequent damage which results. It has become evident that the immune response plays the central role in determining the outcome of viral infection. Many studies have demonstrated the capacity of the immune system to combat viruses with a variety of weapons including interferons, T lymphocytes, natural killer cells, macrophages, antibody, and polymorphonuclear granulocytes (1). The T lymphocytes that respond to viral infection are traditionally recognized as helper, cytotoxic, or supressor cells. In the mouse, each of these groups posess cell surface molecules which confer distinct antigenic phenotypes to these various T cell subsets. Although recent evidence shows there are exceptions to this phenotypic classification of functional activity (2,3), this approach remains useful in examining the population(s) responding to viral infection and their functions in clearance of the infectious agent from the host.

Despite a great body of knowledge which exists regarding systemic viral infection (1) the immune response to infection of the central nervous system (CNS) remains enigmatic. Cellular infiltration into brain parenchyma with perivascular cuffing occurs during many acute virus infections (4,5). Generally, these infiltrates are initially macrophages and mononuclear cells followed by lymphocytes (4,6,7). However, due to the immunologically privileged status of the CNS, conferred by the blood/brain and blood/CSF barriers, the course of viral infection may be altered. The CNS presents other unusual circumstances such as limited extracellular space, and unique cell types such as astrocytes and microglia, which may participate in immune responses.

Of a number of viruses which cause acute and chronic neurologic disease (8), only a few infections result in demyelination of white matter tracts in the brain and spinal cord (9). For our studies of the immune response to CNS viral

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infection, we have used murine hepatitis virus (MHV) type 4, strain JHM (JHMV). When innoculated intracerebrally (i.c.) into susceptible animals, this virus causes acute encephalomyelitis and demyelination due to a cytolytic infection of oligodendroglial cells (10). This pathologic feature provides an interesting system for studying how the immune response can combat virally induced disease within the unique environment of the CNS.

A variety of immune mediators have been implicated in modulating MHV infection. Some of these include antibody (11,12), macrophages (13,14,15,16,17,18), T lymphocytes (19,20,21,22), interferon (23,24,25) and natural killer or "viral killer" cells (24,26,27,28,29). We have previously shown that L3T4$^+$ T cell clones, which mediate JHMV specific delayed type hypersensitivity (DTH), can protect animals from lethal infection (20). However, protection is not accompanied by supression of viral growth in the CNS. In addition, we have recently found that protection requires a virus specific component since non-viral induced DTH responses in the CNS are not protective (Sussman; unpublished data). Despite the variety of viral models which have demonstrated the role of cytotoxic T lymphocytes in reducing virus titer in vivo, the CTL response to coronaviruses has not been identified. In this study we have characterized a cell population which protects from JHMV induced lethal disease and also results clearance of virus from the CNS of infected animals. The implications of these findings and a possible model for immune mediated viral clearance of JHMV from the CNS are discussed.

Materials and Methods

Mice. C57B1/6, C3H/Fe, B10.A (5R), B10.A (2R), B10.MBR and B10. BR mice were purchased from the Jackson Laboratory, Bar Harbor, ME at 5 wks of age. Mice were used as recipients in the adoptive transfer experiments within 8 days of arrival. Prebleeds from representative mice were seronegative for MHV by ELISA (20).

Virus and Cells. The DS small plaque variant of the neurotropic JHMV strain of MHV was used for all intracerebral (i.c.) innoculations. Mice were innoculated i.c. with a lethal dose of approximately $5x10^3$ plaque forming units of JHMV in a volume of 0.03 ml. Animals succumbing to this infection have acute encephalomyelitis with demyelination. The DL subtype of JHM or JHMV was used for all immunizations of donor animals. One ml of viral supernatant containing approximately $2x10^6$ plaque forming units was injected intraperitoneally (i.p.). Viruses were propogated on DBT cells, a continuous murine astrocytoma, as previously described (30). A cell line transfected with H-2 K^b and D^b genes (2-5) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100U/ml penicillin, 100mcg/ml streptomycin, and hypoxanthine, aminopterin, thymidine mixture purchased from Sigma Chemical Company (St. Louis, MO; #H0262). Cells containing the thymidine kinase gene (TK$^+$) were maintained in the same medium as that for 2-5, except aminopterin was not added.

Adoptive Transfers. Six days after i.p. injection with JHMV spleens were removed aseptically, teased, and the cells washed twice with RPMI 1640 containing 10% FCS. Nylon wool used for cell separation was prepared from non-sterile bulk packages (Fenwal Labs, Deerfield Illinois). Briefly, nylon wool was boiled in Alconox detergent solution for one hr, rinsed, and immersed in 1N HCl solution for one hr. After rinsing to neutrality with distilled water, nylon wool was incubated at 37^{o}C while soaking in distilled water, which was changed daily for one week. Nylon wool was then dried, teased, packaged into syringes, autoclaved, and stored for use as previously described (31). Approximately $2x10^8$ cells were adsorbed onto nylon wool columns at 37^{o}C for 1 hr prepared in 10 ml syringes. The eluted cells were collected by washing the column with three volume equivalents of RPMI containing 10% FCS. FACS analysis showed $\sim$ 85%

of these cells to be Thy 1.2 positive and depleted for both B cells and macrophages. The nylon wool adherent population was removed by allowing the column to run dry and refilling with ice cold Hanks balanced salt solution, after which cells were expelled from the column using the syringe plunger. This procedure was repeated three times for each column. Cells were washed twice and resuspended in RPMI 1640 supplemented with 2% FCS prior to the intravenous (i.v.) transfer to recipient mice.

Antiserum Depletions. Cell suspensions at a final concentration of 10^7 cells/ml were incubated for 45 min at 4°C with a 1:100 dilution of monoclonal antibody anti-Thy-1.2, anti-Ly-1.2, or anti-Ly-2.2 purchased from Cedarlane Laboratories (Ontario, Canada) in Hanks balanced salt solution containing 1% fetal bovine serum. Monoclonal antibody GK 1.5, specific for L3T4, (obtained from the American Type Culture Collection) was also used for depletions. Cells were washed, then resuspended in a 1:10 dilution of Cedarlane Low-Tox M rabbit complement at 37° for 60 min. After incubation, cells were washed 3 times, resuspended to the original viable cell concentration, and transferred i.v. to recipients.

Viral Titer Assay. To determine the virus titer, brains were removed aseptically and placed in 2 ml ice-cold Dulbecco's phosphate buffered saline pH 7.4, to yield a 10% (wt/vol) suspension following manual disruption in 7 ml Tenbrock tissue homogenizers. The homogenates were clarified by centrifugation at 800xg for 7 min at 4°C. Virus in the supernatant was determined by adsorbing 0.2 ml of serial ten fold dilutions onto monolayers of L-2 cells in 24 well plates (#3047, Falcon Platics, Oxnard, California). After 1 hr at 37°C the cells were refed with 1.0 ml of Dubecco's modified minimal essential medium (DMEM) supplemented with 2% FCS and 200U/ml penicillin, 200mcg/ml streptomycin and 0.5 mcg fungizone (Irvine Scientific). Virus titer was determined from duplicate assays and the results expressed as the mean titer of the clarified homogenates from groups of three mice plus or minus one standard deviation of the mean.

Delayed Type Hypersensitivity. Footpad swelling was used as a measure of delayed type hypersensitivity (DTH) as previously described (20). Mice received 10^7 cells i.v. and were subsequently injected in the right footpad with 20 ul of an eliciting dose of antigen and in the left footpad with 20 ul of control antigen. The dorso-ventral thickness was measured 24 hr after challenge. The data are presented as the difference of the right foot minus the left foot plus or minus one standard error of the mean.

Cytotoxicity Assays. To determine cytolytic activity, $5x10^4$ thioglycollate elicited peritoneal exudate cells (PEC) were plated per well in 96 well flat bottom plates (Falcon Plastics, #3072) and allowed to adhere for 2 hr. After three washes to remove non-adherent cells, PEC were labeled with ^{51}Cr overnight in 1% FCS, 200U/ml penicillin, and 200 mcg/ml streptomycin (pen/strep) in DMEM at 37°C in a humidified atmosphere containing 8% CO_2. The next day, cells were washed with serum free DMEM and infected with JHMV for 2 hr. Virus was removed and effector cells were added at appropriate ratios in a final volume of 0.2 ml RPMI 1640 containing 10% FCS and pen/strep. For assays using transfected cells as targets, cells were cultured in $25cm^2$ flasks (Falcon Plastics, #3013). To prepare these targets for the assay, cells were washed once with DMEM, infected with JHMV described above. After 3½ hr, cells were trypsinized from the flask and transferred to bacteriological 100 mm petri dishes (VWR, #25384-070) in 10 ml of DMEM containing 2% FCS and pen/strep. Plates were incubated overnight at 37°C. The next morning cells were removed from the plate by repeated pipetting, centrifuged (75xg, 5 min, 4°C) and the cells labeled with 0.5mC ^{51}Cr for 1 hr at 37°C. Cells were then washed 3 times in DMEM and 1 x 10^4 cells were added to each well in 100 ul of RPMI 1640. Cytopathic effect of virus on infected targets was monitored visually and cytolytic assays were terminated when fusion of targets was

approximately 80%or when targets began to detach from the plate, approximately 8-10 hr later. Samples (0.1 ml) of supernatants were removed after centrifugation (100xg, 5 min, 4°C) and their radioactivity determined. Maximum counts were determined by placing targets in 1N HCl. Spontaneous release never exceeded 25% for all assays. Percent lysis was calculated as follows:

$$100 \times \frac{\text{cpm released with effectors - cpm released spontaneously}}{\text{total radioactivity - cpm released spontaneously}}$$

Results

Injection of naive C57BL/6 mice with JHMV results in the stimulation of JHMV reactive lymphocytes which reach maximum proliferation 6 days post injection (data not shown). Furthermore, i.p. injection is only fully protective if administered 5 or more days prior to the lethal i.c. challenge (data not shown). For these reasons, adoptive transfers of primed splenocytes were carried out day 6 post immunization.

Innoculation of JHMV into susceptible mice results in a rapid increase of viral titer in the brain which peaks 4 to 5 days post injection (Fig. 1). Day 5 post infection was chosen to asses viral titers in this study to demonstrate the maximum effect on viral clearance by transferred cells. After this point titer begins to decline gradually until the animal dies 10-14 days later. We postulated that this decrease in viral titer may be due to the immune response to JHMV, which arrives too late to save the animal from death. Support for this hypothesis is seen in experiments with immunosuppressed mice. No drop in viral titer is observed during late stages of the infection in mice exposed to 800 Rad gamma irradiation 24 hr prior to infection (Fig. 1). On the basis of these results, experiments were conducted to determine the role which the immune system plays in the reduction of CNS brain titers during acute infection with JHMV.

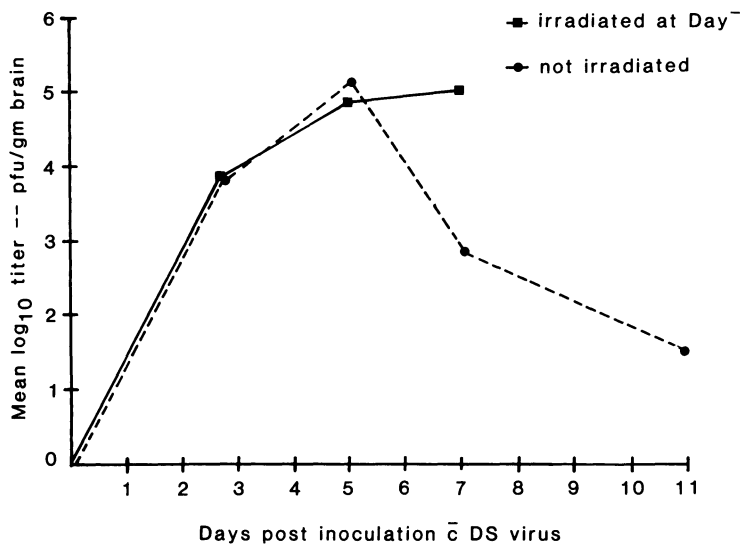

Fig. 1 - Time course of JHMV infection in the CNS of normal mice and mice which received 800 Rad prior to infection

Since immunized mice survived lethal challenge, spleen cell suspensions were transferred to naive recipients to determine if this cell population could reduce viral titers in infected recipient mice. The ability to significantly decrease viral titers in the CNS was clearly present in the cells obtained from immunized mice. Table 1 demonstrates that mice receiving spleen cells from immunized donors have CNS viral titers one thousand-fold lower than control animals which received either no cells or an equivalent number of viable cells from naive mice.

To determine if histocompatibility between the donor and recipient is necessary to decrease virus titer, cells from C57BL/6 ($H\text{-}2^b$) mice were transferred to C3H/Fe mice ($H\text{-}2^k$). The lack of histocompatibility renders transferred cells incapable of reducing virus titer. In addition, pooled serum from immunized donors had no effect on viral titer in recipients (data not shown). These data suggest that neither non-MHC restricted effectors, such as macrophages or antibody alone can decrease viral titer in the CNS.

In initial experiments to characterize the functional cell population responsible for viral clearance, spleen cell suspensions were separated by nylon wool adherence. The adherent fraction typically contains B cells, macrophages and a small percentage of activated T lymphocytes. When adherent and non-adherent cells were compared to whole spleen for the ability to clear virus, the activity was retained on the column in the adherent cell fraction (Table 2). Surprisingly, the non-adherent T cell enriched population failed to reduce virus titer when transferred into infected recipients.

To determine which population was responsible for decreasing virus titer, spleen cells were depleted of various subpopulations of immune cells and tested for in vivo function. Table 3 shows that the cell surface phenotype of the functional cell responsible for viral clearance is Thy 1.2^+, $L3T4^+$, Lyt 2^-, MAC-1^-. These cell surface markers are typical for a T lymphocyte of the helper/inducer phenotype, and T cell clones with this phenotype have been described by our laboratory which transfer DTH (20). It has been previously suggested that helper/inducer T cell subsets may be functionally distinct from one another, although both appear phenotypically similar by surface marker characterization (32,33). Therefore, it was of interest to determine if the nylon wool adherent fraction also had the ability to transfer DTH responsiveness. To test the functional activity of the nylon wool adherent T cells in vivo, both adherent and

TABLE 1

VIRUS CLEARANCE BY ADOPTIVE TRANSFER OF SPLEEN CELLS

DONOR	IMMUNIZED[1]	RECIPIENT	VIRAL TITER[2] (log $TCID_{50}$/gm)
C57BL/6	-	C57BL/6	6.5±0.5
C57BL/6	+	C57BL/6	1.8±0.4
C3H	-	C3H	7.0±0.3
C3H	+	C3H	0.8±0.3
C57BL/6	+	C3H	7.0±0.4

[1] Immunized 6 days prior to transfer with JHMV i.p.
[2] Determined five days post-injection

non-adherent spleen cell populations were transferred i.v. to naive mice and DTH responsiveness tested by placing JHMV antigen in the footpad. Table 4 shows that the nylon wool adherent cells were unable to transfer DTH responsiveness to recipient mice, although the non-adherent fraction which contains primarily T lymphocytes did posess this activity. Therefore, the T cell subpopulation required for viral clearance has the same cell surface phenotype as that of the JHMV specific T cell clones previously described (20), but an entirely different functional activity with regard to viral clearance and DTH responsiveness in vivo.

TABLE 2

VIRUS CLEARANCE BY NYLON WOOL PURIFIED SPLEEN CELLS

CELLS TRANSFERRED[1]	VIRAL TITER[2] (log $TCID_{50}$/gm)
None	6.3±0.4
Whole spleen	1.6±0.3
Adherent	3.2±0.3
Non Adherent	5.5±0.9

[1] $5x10^7$ cells transferred i.v. per mouse
[2] Determined five days post injection

TABLE 3

PHENOTYPE OF THE NYLON WOOL ADHERENT CELL RESPONSIBLE FOR VIRAL CLEARANCE

CELLS TRANSFERRED[1]	TREATMENT	CELLS DEPLETED	VIRAL TITER[3] (log $TCID_{50}$/gm)
-[2]	-	-	6.0±1.1
+	-	-	2.7±0.9
+	Thy 1.2 + C'	T cells	6.2±0.7
+	L3T4 + C'	Helper T cells	6.3±0.5
+	Lyt 2 + C'	Cytotoxic T cells	3.0±1.1
+	Mac 1 + C'	Macrophages	2.2±0.4

[1] $5x10^7$ cells transferred i.v. per mouse
[2] - = none
[3] Determined five days post injection

The helper/inducer T cell subset require histocompatability at Class II genes to function, whereas classical cytotoxic T cells recognize viral antigen in conjunction with Class I on the surface of infected cells. Recombinant inbred strains of mice which differ at specific loci within the H-2 complex were used in adoptive transfer experiments. This allows for the determination of regions within the major histocompatability complex (MHC) critical for donated cells to recognize and clear infectious virus from the recipient mouse. Table 5 shows the haplotype of the recombinant strains and the results of these transfers. The only strain to show a significant drop in viral titer was BIO.A(2R), which shares identity only at the D locus with the donor C57Bl/6 strain. This is a Class I antigen and suggests that the Thy 1^+, $L3T4^+$ adherent cells are not the primary effector cells, and that CTL may be involved in viral clearance.

TABLE 4

ADOPTIVE TRANSFER OF DTH RESPONSIVENESS

Cells Transferred[1]	Footpad Swelling ($mmx10^{-2}$)
None	9.5±7.4
Whole Spleen	29.3±4.7
Non Adherent[2]	42.0±5.0
Adherent[2]	10.0±7.2

[1] 10^8 cells transferred i.v. per mouse
[2] Nylon wool fractions

TABLE 5

CLEARANCE OF VIRUS FROM H-2 RECOMBINANT MOUSE STRAINS

STRAIN	H-2 K I D	NO CELLS[1]	ADHERENT[1,2] CELLS	REDUCTION IN TITER (log)
C57BL/6	b b b	5.5±0.5	1.8±0.4	3.7
B10.A(2R)	k k b	5.3±0.5	3.0±0.6	2.3
B10.A(5R)	b b d	5.7±0.5	4.5±0.8	1.2
B10.MBR	b k q	5.5±0.5	4.0±0.3	1.5
B10.BR	k k k	5.7±0.5	4.6±0.5	1.1

[1] Viral titer expressed as log $TCID_{50}$/gm.
[2] $5x10^7$ Nylon wool adherent cells transferred i.v. per mouse.

To test directly for cytotoxic activity, nylon wool adherent cells were assayed for their ability to lyse infected PEC. No cytotoxicity was observed with cells removed directly from the column (data not shown). However, since our in vivo results showed that substantial viral titer reduction became apparent five days or more after adoptive transfer, nylon wool adherent cells were incubated in the presence of JHMV antigen and Con A stimulated spleen cell supernatants as a source of IL-2 as previously described (20). These conditions support continued proliferation of JHMV specific T lymphocytes, and these cultures were then checked for cytotoxic activity. Table 6 shows the results of these experiments, which demostrate MHC restricted JHMV specific cellular cytotoxicity. PEC from H-2^b C57B1/6 donors were used as targets for initial studies. The antigen stimulated cells were able to specifically lyse infected PEC. Furthermore, this cytotoxicity is MHC Class I restricted, as demonstrated by lytic activity on virus infected cell lines transfected with histocompatability antigens as targets. Thymidine kinase (TK) activity was used as the selectable marker for transfection of class I antigens K^b and D^b into fibroblast cells which lack TK activity. Although these cell lines are less susceptible to JHMV infection (by fluoresence analysis), even at high virus:cell ratios (multiplicity of input) significant cytotoxicity is observed against the infected 2-5 cell line. Only the infected 2-5 transfectant showed substantial lysis in comparison to either uninfected cells, or to histoincompatible TK^+ cells which were infected with JHMV but lack the appropriate H-2 restriction antigens found on H-2^b effector cells.

TABLE 6

JHM VIRUS-SPECIFIC CYTOTOXICITY

TARGET	H-2	INFECTED	% SPECIFIC LYSIS[2]
PEC	b	-	0
PEC	b	+	38.0±4.1
TK^+	k	-	0
TK^+	k	+	4.3±1.3
2-5	$k+(K^bD^b)^3$	-	0
2-5	$k+(K^bD^b)^3$	+	21.0±2.2

[1] Antigen stimulated nylon wool adherent cells
[2] Effector : Target ratio = 2:1
[3] Transfected genes expressing K^b and D^b

Discussion

Previous studies have attempted to define the effect which individual components of immunity have upon JHMV infection. Recently evidence has accumulated suggesting that MHV infection of mice stimulates a multifaceted immune response. In adoptive transfer experiments, animals lethally infected with JHMV can be protected from death with cloned T lymphocytes of a helper/inducer phenotype (20). Survival can also be mediated by passive immunization of mice with neutralizing monoclonal to JHMV (11,12). However, neither DTH-inducer T cells or humoral immunity alone results in the elimination of infectious virus or viral antigen. Reduced virus in the CNS can be demonstrated in mice which have been immunized by i.p. injection with the neurotropic JHMV strain four or more days prior to lethal i.c. challenge. These

animals are protected from death, show little or no histological evidence of disease, and have eliminated virus from the CNS when assayed for infectious virus in brain homogenates (unpublished observations). In this report we have shown that this capacity can be adoptively transferred to naive recipients with spleen cells from immunized donors. The phenotype of the cell population which participates in reducing viral titer is that of a DTH inducer/helper T cell, although in functional assays it is not capable of transferring DTH to recipient mice as has been previously described for JHMV specific T cell clones (20).

Helper/inducer T lymphocytes are not thought to participate directly in clearance of virus during infection, but rather to aid in the expansion of CTL and antibody producing cells which together are capable of either specific recognition of infected cells or neutralization of infectious virus. It is not surprising that the functional T lymphocyte in these studies is retained on nylon wool columns, as activated T cells have been previously found to adhere to nylon wool (31). Therefore, the T lymphocytes adhering to nylon wool are likely to not be the primary effector cell resulting in viral clearance, but rather a necessary element in a cellular network which results in the amplification of a cell population(s) with direct anti-viral activity.

Resistance to MHV infection shows a clear correlation to age of the mice, which suggests a susceptibility to infection related to lack of immune competence in young mice (34,35,36). Replication of JHMV in macrophages has been studied extensively, yet a clear correllation between macrophages susceptibility to infection in vitro and viral replication in vivo has not been found (14,15,16,17). In the SJL/J mouse model, macrophages transfer resistance to lethal challenge with JHMV and decrease viral production in vitro (13,14). Similarly, investigation of the role which natural killer (NK) cells play in MHV infecton has not provided clear answers for MHV immunity in vivo (27,28). Natural cytotoxicity against cells infected with the A59 strain of MHV has been reported to be mediated by "viral killer" cells with the surface phenotype of B lymphocytes. Their significance in vivo has not been determined as yet (29). Thus far, the only clear demonstration of lymphocytes responsible for conferring resistance to MHV infection in vivo comes from adoptive transfer experiments, which have previously demonstrated the importance of T cells in survival from lethal MHV infection (19,20,21,22).

In spite of the pivotal role which cytotoxic T lymphocytes play in response to other viral infections their importance in immunity to JHMV has not been substantiated. The data in this report are consistent with an immune response to JHMV infection involving the participation of virus specific CTL's. In experiments using recombinant mouse strains, histocompatability at the D locus of the MHC was required for clearance of virus from the CNS. This implicates CTL in the virus clearance because of their restriction to Class I loci. Further evidence to suggest the presence of CTL in the nylon wool adherent lymphocyte population is found when cells are tested for JHMV-specific cytotoxicity. Cultured cells isolated from the adherent fraction kill JHMV-infected cells in an H-2 restricted fashion, recognizing Class I determinants on the 2-5 cell line. This cytotoxicity was specific for both Class I histocompatability antigens and viral infection. These characteristic are typical of CTL mediated killing. Therefore, it seems likely that a population of JHMV-specific CTL's are present in the lymphocyte fraction responsible for protection from lethal infection. Experiments are underway to isolate and characterize cells which posseses JHMV-specific cytotoxic activity. As one of the last components of MHV immunity to be isolated, it will be interesting to determine the functional activity of CTL in vivo during infection and the role they may play in protection from lethal disease and the establishment of chronic demyelination.

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