Treatment of Encephalomyocarditis Virus-Induced Central Nervous System Demyelination with Monoclonal Anti-T-Cell Antibodies

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Infection of BALB/c mice with the M variant of encephalomyocarditis virus resulted in the development of a paralytic syndrome in 7 to 10 days. The paralysis was maximal during the period of viral clearance; most of the animals recovered from the initial deficit and showed no delayed recurrences. Pathologically, the white matter of brain and spinal cord showed well-demarcated areas of perivascular cuffing, demyelination, and, during recovery, remyelination by oligodendrocytes—all suggestive of postinfectious encephalomyelitis. Depletion of either the CD4 or CD8 subset of T cells in vivo with the appropriate monoclonal antibody, GK1.5 or 2.43, respectively, administered one day (24 h) prior to infection was sufficient to limit the development of the paralytic syndrome by 79% (GK1.5) and 82% (2.43).

Picornaviruses are plus-stranded RNA viruses that have well-known tropisms for specific organ systems in animals and humans (4, 15, 42). Both genetic and environmental factors influence pathogenesis of this viral infection. Variants of the same serotype may demonstrate distinct tropisms for particular tissues; however, even infection of a given organ will not ensure a direct pathological effect on that organ (13, 22, 23, 41). Evidence from certain picornavirus-induced disease states indicates that the tissue injury results from an immune response to infected targets (14, 20).

The M strain of encephalomyocarditis virus (EMC-M) was first isolated during an epidemic of myocarditis affecting Panamanian swine in 1962 (25). This virus has since been extensively studied as a model for viral myocarditis and insulinitis (1, 5). While investigating the diabetogenic properties of this virus, we routinely observed a paralytic syndrome in BALB/c mice 7 to 10 days after viral inoculation. Clinical symptoms were maximal at a time when virus was being cleared from the brain. Although a tropism of EMC-M virus to the central nervous system (CNS) has been described before, the delay in occurrence of disease suggested additional or alternate mechanisms other than direct viremia in pathogenesis.

In the present report we demonstrate that this disease is immune mediated and shows many of the features of postinfectious encephalomyelitis. Clinically, the animals showed various degrees of paralysis, and pathologically, inflammatory demyelinating lesions were seen in the brains and spinal cords. Furthermore, in vivo depletion of particular T-cell subsets results in prevention of disease.

MATERIALS AND METHODS

Animals. Inbred BALB/c female mice were purchased from Cumberland Farm, Clinton, Tenn. Infected animals were housed separately.

Virus. Original virus stock was obtained from J. E. Craighead (Department of Pathology, University of Vermont) as a fourth-passage homogenate of a heart from a CD1 strain mouse and is designated EMC-M2 H4. Virus was passed by

inoculating animals with 60 PFU of EMC-M2 H4 intraperitoneally (i.p.); the animals were sacrificed 3 days later. Their hearts were removed and ground in a motorized homogenizer in phosphate-buffered saline on ice and then centrifuged to remove cellular debris. This passage was designated EMC-M2 H5 and was used for all further experiments.

Cells and media. L929 and Ehrlich-Lettre ascites cells were obtained from the American Type Culture Collection (ATCC CCL-1 and CCL-77, respectively). L929 cells were maintained in Eagle minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 0.1 mM L-glutamine, 50 U of penicillin per ml, 50 µg of streptomycin per ml, and 5% fetal calf serum (Hyclone Laboratories, Logan, Utah). Ehrlich-Lettre ascites carcinoma cells were maintained in NCTC 135 (GIBCO Laboratories) supplemented with 0.1 mM L-glutamine, 50 U of penicillin per ml, 50 µg of streptomycin per ml, and 10% fetal calf serum (Hyclone Laboratories).

Propagation of virus. T-150 flasks containing 20 to 30 ml of the appropriate medium were seeded with either L929 cells or CCL-77 cells and grown until 100% confluent. At this point, cultures were split or washed and inoculated with between 3 and 5 PFU of virus stock per cell. The virus was allowed to adsorb for 30 min., medium was added, and the infected cells were incubated for 48 h at 37°C in 5% CO₂. The supernatant was harvested and frozen at -70°C until needed. Virus grown in this way produced yields of 1×10^7 to 5×10^7 PFU/ml. For use in the enzyme-linked immunosorbent assay technique (31), only CCL-77-prepared virus that was further purified by the technique of Ziola and Scraba (43) was used as antigen. This preparation was aliquoted and stored at -70°C. Inactivated virus was obtained by irradiating the above virus preparations with a General Electric G8T5 UV lamp for 10 min at a distance of 6 in. (15.24 cm).

Infection of experimental animals. Female BALB/c mice 8 to 12 weeks of age were infected by i.p. injection of virus inoculum. Animals were given 60 PFU in 0.5 ml of phosphate-buffered saline for clinical studies.

Virus infectivity assay. The plaque-forming assay was used to determine amounts of infectious virus. Briefly, L929 monolayers on 60-mm polystyrene dishes (Corning Glass Works, Corning, N.Y.) were infected with appropriate dilu-

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TABLE 1. Treatment of EMC-induced demyelination with rat IgG or monoclonal antibody to T-cell subsets prior to inoculation with virus: summary of four experiments

Treatment ^a	Animals paralyzed (no. per expt)					Maximum grade of paralysis ^b					Histological index		Mean day of onset	Virus titer in brain, day 18 (log 10)	Antibody to EMC, day 18 ($\mu\text{l} \pm \text{SEM}$)
	I	II	III	IV	Total (%)	I	II	III	IV	V	Day 18	Day 25			
Anti-CD4	2/6	3/10	0/6	1/6	6/28 (21)	0	2	4	0	0	6.1	0.5	10.2 $\pm$ 1.6	2.5 $\pm$ 1.1	0.06 $\pm$ 0.04
Anti-CD8	1/6	2/10	0/6	2/6	5/28 (18)	0	4	0	0	1	2.1	0.1	10.4 $\pm$ 2.9	— ^c	0.50 $\pm$ 0.15
Rat IgG	6/6	7/8	6/7	6/6	25/27 (93)	4	5	14	1	1	11.9	5.4	10.2 $\pm$ 2.0	—	0.34 $\pm$ 0.15
Anti-CD4 and Anti-CD8	1/6				1/6	0	1	0	0	0	ND ^d	ND	9	ND	ND

^a BALB/c mice were treated with injections of 1 mg of monoclonal anti-CD4 (GK.15), anti-CD8, rat IgG, or a mixture of anti-CD4 and anti-CD8 antibodies 24 h prior to infection with virus.

^b See text.

^c —, None detected.

^d ND, Not done.

tions of viral supernatants, covered with 0.4% agarose in minimal essential medium, fixed, and stained 48 h later, and plaque numbers were recorded.

Monoclonal antibodies. Purified ascitic fluid preparations of antibodies were used for all in vivo therapy experiments. Anti-CD4 antibody (GK1.5) and anti-CD8 antibody (2.43) were prepared as described previously (32, 33).

Animal evaluation. Beginning 3 days after viral inoculation, the animals were examined daily. The neurological deficits were graded according to the following scale representing clinical advancement of disease: grade I, haunched posture, abnormal gait; grade II, moderate hind-limb paralysis; grade III, complete hind-limb paralysis; grade IV, quadriplegia or inability to move about cage; and grade V, death.

Morphologic studies. Mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, and the spinal cords were processed to provide araldite-embedded or glycol methacrylate-embedded sections. Brains were sectioned coronally and embedded in paraffin. A detailed morphologic analysis of each spinal cord was performed on 15 to 20 coronal spinal cord sections embedded in glycol methacrylate. Araldite sections were stained with toluidine blue; glycol methacrylate sections were stained with a modified erichrome-cresyl violet stain to detect inflammation and demyelination. Pathological scores for each section ranged from 0 to 4. Independent scores were given for inflammatory cells in the meninges or demyelination in the spinal cord white matter. The presence of inflammatory cells or demyelination was determined in the posterior columns, ventral columns, and each lateral column of the mouse spinal cord white matter. The grading was as follows: 4, inflammation or demyelination in all four white matter columns; 3, abnormalities in three columns; 2, abnormalities in two columns; 1, abnormalities in one column; and 0, no inflammatory cells or demyelination. The total pathologic score was determined by adding the individual scores of each spinal cord section, dividing by the possible maximal score, and multiplying by 100. Selected araldite-embedded spinal cord sections were trimmed for electron microscopy. The tissue was osmicated (1% osmium tetroxide) and counterstained with uranyl acetate and lead citrate.

RESULTS

Kinetics of development of paralysis and the relation to viral titers in the brains of virally infected animals. To evaluate the kinetics of development of the paralytic syndrome, BALB/c female mice were infected with 60 PFU of the virus i.p. Four

separate experiments involving 28 animals were performed (Table 1). Signs of paralysis were seen as early as 7 days after viral inoculation. The mean day of onset for affected animals was day 10. The initial signs were ruffled fur, weight loss, hunched posture, and an unsteady gait that later evolved into hind-limb paralysis. Tail paresis was a very late event and in many cases never occurred. Recovery from the neurologic syndrome occurred in 10 to 20 days, and by day 30 all mice appeared fully recovered. Continued observation of animals until day 50 did not demonstrate any delayed appearance of clinical symptoms.

To determine the kinetics of clearance of the virus from the brain, four virally infected mice were sacrificed on days 4, 6, 9, 12, 15, 18, and 24, and the quantity of virus was estimated by using the plaque assay (Fig. 1). Viral titers reached a maximum on day 4 and subsequently declined. By day 18, no infectious virus could be detected in the brain. The kinetics of viral clearance from the brain were similar to those reported for the heart and pancreas (25). It should be noted that development of the paralytic syndrome began as virus was cleared from the brain (Fig. 1).

In a separate series of experiments, viral titers were obtained from spinal cords of animals infected with EMC virus. There was no difference in the pattern of viral clearance, and no virus was detected in the spinal cord 18 days after infection (data not shown).

Light microscopy. Animals were sacrificed after recovery from clinical symptoms (days +18 to +25), and the brains

FIG. 1. Kinetics of viral titer in the brain and development of paralytic signs in mice injected with 60 PFU of EMC-M2H5 virus i.p. ●, log 10 PFU of EMC virus in brains of BALB/c mice; ■, day of onset of paralysis.

and spinal cords were analyzed by light and electron microscopy. Histological analysis showed perivascular lymphocytic cuffing in the parenchyma of the brain and more specifically in the cerebellum and periventricular areas. Myelin staining with luxol fast blue showed well-demarcated areas of myelin loss in the brain and at multiple levels in the spinal cord. The areas of primary demyelination in the spinal cord as detected in plastic embedded sections were wedge shaped and extended to the pial surface. At all levels examined in the spinal cord, the anterior horn cells and the anterior roots were spared (Fig. 2A and B and Fig. 3).

Electron microscopy. Plastic embedded sections from EMC virus-infected mice (20 to 25 days postinfection) showed multifocal areas of primary demyelination in the spinal cord. The majority of lesions were localized to the subpial region. On average, approximately 10 to 20% of the sections showed demyelinated lesions. Inflammations in the form of lymphocytes, plasma cells, and macrophages were located primarily along the meninges but also infiltrated into the substance of the white matter. Electron microscopy demonstrated multiple demyelinated axons in association with inflammatory cells. A striking finding was that areas of spinal cord white matter had undergone almost complete remyelination by oligodendrocytes, as characterized by axons with abnormally thin myelin and fewer inflammatory cells. Remyelination by peripheral Schwann cells infiltrating the CNS was not observed. No abnormalities were noted in the gray matter of the spinal cord. No viral particles were detected by electron microscopy (Fig. 4).

Prevention of EMC virus-induced neurological syndrome by in vivo administration of monoclonal anti-CD4 and anti-CD8 antibodies prior to inoculation with virus. (i) **Development of paralytic syndrome.** In vivo administration of monoclonal antibodies directed against the CD4 and CD8 antigens resulted in depletion of the appropriate T-cell subset from the lymph nodes, spleen, and peripheral blood. Depletion was rapid, and repopulation of the depleted T cells was gradual and took place over a period of 2 to 3 months (30). In order to determine the role of the T-cell subsets in the development of the paralytic syndrome, female BALB/c mice were treated with 1 mg (i.p.) of either anti-CD4 (GK1.5), anti-CD8 (2.43), or rat immunoglobulin G (IgG) 24 h prior to inoculation with 60 PFU of EMC-M virus. Four separate experiments involving a total of 28 animals in each treatment group were performed (Table 1). In animals that received rat IgG, 94% of the animals developed paralysis of varying severity, with a mean day of onset of 10.2. In contrast, only 6 of 28 (21%) animals receiving anti-CD4 antibody and 5 of 28 (18%) animals receiving anti-CD8 antibody developed clinical paralysis (chi square, GK1.5 versus rat IgG = 25.48; 2.43 versus rat IgG = 28.02; for both, $P < 0.001$). There was little variation in the disease incidence between the four experiments. In animals that did develop paralysis, there was no apparent difference in clinical severity or recovery pattern. Administration of both anti-CD4 and anti-CD8 antibodies was performed in experiment 1. One of the six animals treated with both antibodies developed mild paralysis. Histological scores obtained from six animals randomly selected from each group were in agreement with the clinical grades. Animals sacrificed on day 18 that had received rat IgG had a mean pathologic score of 11.9, while those that had received anti-CD4 and anti-CD8 antibodies had scores of 6.1 and 2.1, respectively. There was a reduction in severity of the histological scores in all three groups if the day of sacrifice was delayed until day 25 (Table 1; Fig. 2B and C). Animals

not sacrificed were observed until day +50 and did not show any signs of relapse or delayed neurological deficits.

(ii) **Kinetics of viral clearance in normal and T-cell subset-depleted mice.** In order to determine the role of CD4⁺ and CD8⁺ T-cell subsets in the kinetics of EMC viral clearance, BALB/c mice were treated with 1 mg of either anti-CD4 (GK1.5) or anti-CD8 (2.43) monoclonal antibodies 24 h prior to inoculation with 60 PFU of EMC-M i.p. Three animals were sacrificed each day of days 4, 6, 9, 12, 15, 18, and 24, and the quantity of virus was measured. Animals that had received anti-CD8 antibody or rat IgG had complete clearance of the virus by day 18. Animals that received anti-CD4 antibodies showed a minimal delay in the clearance, having a mean of $\log 2.5 \pm 1.1$ PFU on day 18, with no detectable virus in the brain by day 24. There were no significant differences in brain virus titers between the groups on any of the other days tested. (Table 1).

(iii) **Development of an antibody response to EMC virus.** As a means of testing the effectiveness of the anti-T-cell antibodies in producing immunosuppression, serum immunoglobulin against the EMC viral antigen was determined by the enzyme-linked immunosorbent assay method (31). Animals were bled at the time of sacrifice (day 18), and the serum was measured for anti-EMC antibody against known positive-control and negative-control antisera. Values are expressed in terms of microliters of the known positive serum plus or minus the standard error of the mean. Animals receiving either rat IgG, anti-CD8, or anti-CD4 had mean titers of 0.34 ± 0.15 , 0.50 ± 0.15 , and 0.06 ± 0.04 , respectively (Table 1).

DISCUSSION

Monoclonal antibodies to CD4 or CD8 antigens have been used to study the effects of T-cell subset depletion on the immune response in vivo (3, 12). Thus depletion of CD4 cells has been shown to suppress antibody and delayed-type hypersensitivity responses in vivo and prevent a number of autoimmune diseases (2, 24, 28, 36, 37, 40). By using similar techniques, the CD8 population of cells has been shown to be involved in allograft rejection (30), in the rejection of tumors (26), and also in the immune response to parasitic antigens (38). The CD8 population of cells has protective as well as detrimental effects in the regulation of the immune response to a number of viruses. Thus polyclonal or cloned populations of CD8 cells responsive to influenza virus protect mice against lethal infection and limit the replication of the virus in the trachea and lungs (18, 21). Similar observations have also been made after herpes simplex virus infection (27). On the other hand, CD8 cells have been implicated in myocarditis after coxsackievirus (CVB3) infection (20) and in the lethal infection after intracerebral inoculation with lymphocytic choriomeningitis virus (9).

Although other strains of EMC virus have been reported to be neurotropic in mice, the description of a demyelinating syndrome after EMC virus infection is novel. A number of other viruses have well-known demyelinating effects in the CNS (7, 10, 17, 19, 35). Of these, Theiler's virus and the JHM strain of the mouse hepatitis virus have been extensively studied. In contrast to EMC infection, both Theiler's virus and the JHM strain produce a chronic persistent state of cerebral infection (7, 29, 39). Our clinical and pathological studies appear to resemble more closely the paralytic syndrome produced by infection with temperature-sensitive mutants of vesicular stomatitis virus (8). More specifically, in the demyelinating syndrome produced by EMC virus and

FIG. 2. (A) Large area of primary demyelination in the spinal cord of a BALB/c mouse infected i.p. with 60 PFU of EMC-M2H5 virus. There are a few inflammatory cells within this demyelinating lesion. Some of the axons show CNS-type demyelination characterized by abnormally thin myelin. Magnification, $\times 450$. (Araldite-embedded section stained with 1% toluidine blue.) (B) Absence of primary demyelination in an infected BALB/c mouse pretreated with monoclonal antibody to L3T4 (CD4), day 25 postinfection. Magnification, $\times 450$. (C) Absence of primary demyelination in an infected BALB/c mouse pretreated with monoclonal antibody to Lyt2 (CD8), day 25 postinfection. Magnification, $\times 450$.

FIG. 3. (A) Electron microscopy showing three demyelinated axons (A) in the spinal cord of an infected BALB/c mouse 25 days after virus infection. Normally myelinated axons are shown in the bottom of this electron micrograph. The process of an astrocyte (As) is seen in the vicinity of demyelinated axons. Magnification, $\times 13,750$. (B) Multiple remyelinated axons in the spinal cord of a mouse recovering from EMC infection. Almost every one of the over 100 axons shown in this electron micrograph has thin myelin in respect to the diameter of axons. An oligodendrocyte is seen in the midst of these remyelinated axons. Nu, Nucleus. Magnification, $\times 10,000$.

FIG. 4. Kinetics of viral titers in the brains of BALB/c mice that had received 1 mg of anti-CD4, anti-CD8, or rat IgG 24 h prior to receiving 60 PFU of EMC-M2H5 virus. Note the mean titer on day 18 in the anti-CD4-treated group is $\log 2.5 \pm 1.1$ PFU.

temperature-sensitive mutants of vesicular stomatitis virus, the disease is monophasic, occurs during the period of viral clearance, and is immune mediated.

Our present observations on the effects of anti-CD4 and anti-CD8 antibodies in EMC infection differ in some respects from the effects of these monoclonal antibodies on Theiler's virus-induced disease (29, 39). In the latter situation, treatment of animals prior to infection with anti-CD4 antibodies resulted in higher morbidity and mortality. One possible explanation for this difference could be the relative importance of antibody in containing the viral response after Theiler's murine encephalomyelitis virus infection. In EMC virus infection, it is likely that initial viral containment prior to the development of an antibody-mediated humoral response occurs by natural killer cell activity in a manner similar to that seen in coxsackievirus infection (11).

The kinetics of disease development after EMC infection and the relation of viral clearance to development of neurological symptoms suggest that this might represent a form of postinfectious encephalomyelitis. In humans, postinfectious encephalomyelitis is often associated with a number of viral infections, including measles, rubella, and chicken pox (34). Immunological studies in patients recovering from these infections have suggested that they represent an autoimmune response to myelin basic protein and are closely related to the experimental counterpart, experimental allergic encephalomyelitis (16). We have failed to show any autoreactivity to myelin basic protein after EMC infection (data not shown). It is quite likely that autoreactivity to other myelin antigens such as proteolipoprotein may be involved. However, the beneficial effects of CD8⁺ T-cell subset depletion clearly indicate a different immunopathogenic mechanism than experimental allergic encephalomyelitis (32). While immunosuppression with anti-CD4 or anti-CD8 antibodies clearly establishes a role for T cells in the process of demyelination, the fact that clinical signs of disease are observed at a time when virus is present in the CNS prevents us from ruling out a direct role of the virus in the process. Furthermore, the absence of virus particles in the electron micrographs at day 18 does not exclude the persistence of virus (6). In any case, the differential effects of the T-cell subsets indicate that the syndrome of postinfectious encephalomyelitis might be quite variable and that clinical and

histological examination alone cannot establish the immunopathogenesis of the disease.

Our experiments have important implications in understanding the pathogenesis of the most common human demyelinating disease, multiple sclerosis. The various clinical presentations and progression observed in multiple sclerosis could well argue that this is a heterogeneous disease and hence any immunotherapy to patients with this disease must be exercised with great caution.

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