THE JOURNAL OF INFECTIOUS DISEASES • VOL. 137, NO. 2 • FEBRUARY 1978
 0022-1899/78/3702-0005$00.75

Central Nervous System Immunity in Mice Infected with Theiler's Virus. I. Local Neutralizing Antibody Response

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Experimental Theiler's mouse encephalomyelitis virus (TMEV) infection in mice is atypical of most other picornavirus infections because virus persists in the host. It was shown previously that low levels of infectious virus are readily detectable in the central nervous system (CNS) despite the presence of substantial titers of serum neutralizing antibody. In this study antibody assays were performed on CNS tissue homogenates, and neutralizing antibody was regularly found in the CNS of TMEV-infected mice. That neutralization by infected CNS extracts was due to antibody was demonstrated by the specificity of neutralization for TMEV and by elimination or marked reduction of neutralization by in vitro treatment with goat antiserum to mouse IgG. In addition, immunofluorescent staining consistently revealed IgG- but not IgM-containing cells in perivascular cuffs and parenchymal lesions of the brains of infected animals. Evidence of local antibody formation in the CNS was found in the actual reversal of the serum-CNS antibody ratio in about one-third of infected mice after three weeks. In contrast, normal mice had a mean serum-CNS antibody ratio of approximately 100:1 after passive transfer of antibody. Possible reasons for the fact that TMEV is not neutralized by antibody and chronic infection is not aborted include the formation of complexes of infectious virus and antibody in the CNS and the production of antibodies with low affinity for TMEV.

Theiler's mouse encephalomyelitis virus (TMEV), which causes widespread asymptomatic enteric infections in mice, produces a biphasic disease of the central nervous system (CNS) when inoculated intracerebrally (ic) into its natural host [1]. In acute infection there are replication of virus and poliomyelitis-like pathologic involvement in the gray matter of the CNS. Surviving animals invariably develop chronic CNS infection and have marked mononuclear cell infiltrates in the white matter of the spinal cord and concomitant demyelination. The chronic lesion in the white matter appears to be immunopathologic since demyelination is prevented by immunosuppression [2].

It is of particular interest that TMEV causes persistent infection of the CNS. In this regard TMEV is atypical of most other picornaviruses. It was shown previously that systemic humoral and cellular immunity to TMEV develop [1, 3], but that these responses are somewhat delayed. Since picornaviruses are not vertically transmitted, they must spread to other susceptible cells by the extracellular route. Therefore, the reason that TMEV is not eliminated by neutralizing antibody, which is present in substantial titers in serum, is unclear. One possible explanation is that sufficient amounts of neutralizing antibody do not reach the CNS; however, there is an increasing body of literature that suggests antibody can be formed locally in the CNS, particularly in infections [4–10]. We felt that a critical first step in understanding the mechanism (s) of viral persistence in this model would be to determine whether neutralizing antibody can be detected in the CNS. Antibody assays were performed on homogenates of CNS tissue because of the small amount of cerebrospinal fluid (CSF) available from individual mice. In this study neutralizing

Received for publication April 22, 1977, and in revised form August 22, 1977.

This work was supported by grant no. R68910C-3 from the National Multiple Sclerosis Society and by grant no. NIH 1 R01 AI 14139-01 from the U.S. Public Health Service.

We thank Kimiko K. Matsutani and Jackie Sloan for technical assistance and Jody Leimbach for secretarial help.

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antibody was regularly found in the CNS of TMEV-infected mice, and evidence is presented that this antibody is synthesized in the CNS. Furthermore, the levels of antibody in CNS and CSF were compared, the temporal course of antibody in the CNS was compared with that in serum, and the presence of IgG- and IgM-containing cells in sections of the CNS was determined by fluorescent antibody staining.

Materials and Methods

Viruses. Stock DA virus, a strain of TMEV, was prepared from the third suckling mouse brain passage as a 10% suspension (wt/vol) of brain in Hanks' balanced salt solution (HBSS). The ic suckling mouse LD_{50} (referred to hereafter as the LD_{50}) was $10^{6.9}$/ml. A stock of GDVII virus, a strain of TMEV serologically related to DA virus, was prepared from the eighth passage in BHK 21 cells. The minute plaque variant of encephalomyocarditis (EMC) virus was obtained from K. Takemoto (National Institutes of Health, Bethesda, Md.), and a stock was prepared from the second passage in L cells. The Indiana strain of vesicular stomatitis virus (VSV) was purchased commercially (American Type Culture Collection, Rockville, Md.), and a stock was prepared from the first passage in L cells.

Cells and media. BHK 21 and L 929 cells (International Scientific Industries, Cary, Ill.) were grown in Earle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum and 0.1 mM L-glutamine, 100 μg of streptomycin/ml, and 100 units of penicillin/ml. The same medium containing 2% fetal calf serum was used for maintenance of cell cultures.

Inoculation of animals. Three- to four-week-old SJL/J mice (Jackson Laboratory, Bar Harbor, Me.) were inoculated in the right cerebral hemisphere with 0.02 ml of the DA virus (~1,000 LD_{50}). Control animals were inoculated with an equivalent dilution of normal suckling mouse brain homogenate or MEM.

CNS extracts and CSF. Mice were sacrificed in CO_2, bled from the heart, and perfused with phosphate-buffered saline (PBS). The brains and spinal cords from individual animals were quick-frozen and stored at −70 C. CNS extracts were prepared as 25% homogenates in HBSS or MEM and clarified by centrifugation at 18,000 *g* for 30 min. The clarified supernatants, representing a 1:4 dilution of the CNS, were used for assays of antibody and interferon. In the recording of results of these assays, a titer of 1:16, for example, indicates that the CNS extract as prepared above was diluted 1:4 and produced a 50% reduction in pfu at this dilution. Approximately 5 μl of CSF was obtained from each mouse by the technique described by Carp et al. [11]. Equal volumes of CSF from several mice were combined and stored at −70 C until assayed for antibody. CSF that was grossly contaminated with blood or that was blood-tinged was discarded.

Antisera. Hyperimmune mouse serum was produced in SJL/J mice by four ip injections of 10^8 pfu of live GDVII virus at weekly intervals. Serum was obtained seven to 10 days after the last inoculation, heat-inactivated at 56 C for 30 min, and stored in small aliquots at −20 C. This serum had a titer of neutralizing antibody of 1:4,096. The immune serum for cross-neutralization tests was produced in guinea pigs (Scientific Small Animal Farm, Arlington Heights, Ill.) that were given a single ip injection of virus. These animals were sacrificed seven days later, and the serum was heat-inactivated at 56 C for 30 min and stored in small aliquots at −20 C. Goat antiserum to IgG (7S) (Meloy Laboratories, Elkhart, Ind.) was heat-inactivated at 56 C for 30 min and stored in small aliquots at −20 C. The goat antiserum to mouse IgG gave a single precipitin band when reacted in Ouchterlony double gel diffusion against whole mouse serum.

Fluorescent antibody staining. Infected and control mice were sacrificed in CO_2 and perfused through the left ventricle with PBS. Brains, spinal cords, and spleens were quick-frozen, and sections (4–6 μm) were cut in a cryostat and stained by the direct immunofluorescence technique [12]. The reagents for fluorescent antibody staining were heavy-chain-specific goat antiserum to mouse IgG and IgM conjugated with fluorescein isothiocyanate (Cappel Laboratories, Downington, Pa.) and normal goat serum. Both antisera gave distinct precipitin bands when reacted in Ouchterlony double gel diffusion against whole mouse serum. Sections stained for fluorescent antibody were examined with a Zeiss microscope

(Carl Zeiss, New York, N.Y.) with incident light excitation, KP 490, KP 500, and LP 455 excitation filters, and an LP 520 barrier filter.

Assays of neutralizing antibody. Twofold dilutions of heat-inactivated sera and CNS extracts of CSF were incubated with 50–100 pfu of GDVII virus at 24 C for 60 min for assay of neutralizing antibody to TMEV. Each mixture was assayed in duplicate on L cell monolayers as previously described [3]. In brief, confluent monolayers in 35-mm plastic FB-6TC multiculture dishes (Linbro Chemical Co., Hamden, Conn.) were washed once with MEM and inoculated with mixtures of assay material and virus (0.1 ml per well). After adsorption at 37 C for 60 min, the inoculum was removed, the monolayers were washed once with MEM, and each well was overlaid with 3 ml of medium consisting of 0.95% bacto-agar (Difco Chemical Co., Detroit, Mich.) in MEM containing 1% fetal calf serum. On the third day of incubation at 37 C, 1.5 ml of a second overlay containing 0.01% neutral red was added to each well, and viral plaques were read 6–24 hr later. Neutralizing antibody was considered present if there was a 50% reduction in viral plaques compared with number in a virus-MEM control. A similar plaque reduction assay was used for determination of neutralizing antibodies to EMC virus, except the overlay medium contained 0.9% bacto-agar, and the second overlay with 0.01% neutral red was added to each well after incubation for 24 hr at 37 C.

Assay of interferon. Interferon assays were performed by the plaque-reduction technique using VSV as the challenge virus. Fourfold dilutions of CNS stocks were incubated for 18 hr at 37 C on monolayers of L cells in FB-6TC multiculture dishes. The cells were washed once with MEM, inoculated with 100 pfu of VSV, and incubated for 1 hr at 37 C. Infected monolayers were then washed once with MEM and overlaid with 3 ml of medium consisting of 0.95% bacto-agar in MEM. After 24 hr, 1.5 ml of a second overlay containing 0.01% neutral red was added, and viral plaques were counted 4–8 hr later. The highest dilution of CNS extracts producing a 50% reduction in pfu was taken as the titer of interferon (units/ml). The sensitivity of each assay was monitored by use of a reference mouse interferon standard (G002-904-511) obtained from the National Institutes of Health. This standard consistently had a titer of 1,000 units/ml.

Results

Neutralizing antibody in extracts of CNS. Since brain-derived DA virus does not produce CPE in L cells unless adapted by blind subpassage, CNS tissues from chronically infected mice could be assayed for neutralizing antibody in vitro. The GDVII strain of TMEV, which is closely related to the DA strain and which produces a cytolytic infection in L cells, was used as the TMEV serotype in a standard plaque reduction assay for neutralizing antibody. The brain-derived DA strain does not interfere with replication of GDVII virus in L cells (authors' unpublished observations).

Since it is difficult to obtain adequate amounts of CSF from individual mice, clarified homogenates of CNS were assayed for antibody to TMEV. Thus, CNS extracts from mice inoculated ic with DA virus were obtained and tested for their ability to neutralize GDVII virus in vitro. All animals were thoroughly perfused with PBS to reduce potential contamination of CNS with blood. As shown in table 1, all samples of CNS extract contained substantial titers of neutralizing antibody to TMEV but not to a serologically unrelated picornavirus, EMC virus. In contrast, all CNS extracts from normal mice failed to show neutralizing activity against either virus. This finding demonstrates that the neutralizing activity was specific for TMEV and was not due to

Table 1. Titers of neutralizing antibody in extracts from the central nervous system (CNS) of 10 uninfected mice and 10 mice infected with Theiler's mouse encephalomyelitis virus (TMEV) or encephalomyocarditis (EMC) virus.

	Titer of antibody to*	
Mouse CNS specimen	EMC virus	TMEV
TMEV-infected†	<4	≥16 (16–128)
Uninfected	<4	<4

*Titers expressed as the reciprocal of the final dilution producing a 50% reduction in pfu. Numbers in parentheses indicate range of titers.

†CNS specimens were obtained one to six months after infection.

Table 2. In vitro treatment of Theiler's mouse encephalomyelitis virus-infected extracts of the central nervous system (CNS) of mice with goat antiserum to mouse IgG.

Day after infection CNS specimen obtained	Titer of antibody in extract*		
	Untreated	Treated with NGS†	Treated with antiserum to mouse IgG†
99	64	64	<8
405 (sample 1)	64	64	8
405 (sample 2)	64	64	<8
429	128	⩾128	8

*Titers expressed as the reciprocal of the final dilution producing a 50% reduction in pfu.

†A 1:4 dilution of CNS extract was incubated with a 1:20 dilution of normal goat serum (NGS) or a 1:20 dilution of goat antiserum to mouse IgG for 60 min at 37 C.

nonspecific inhibitors that might be present in the brain. An additional experiment demonstrated that this neutralizing activity was contained in the antibody fraction of the CNS extracts. CNS extracts from individual chronically infected mice were treated in vitro with goat antiserum to mouse IgG and with normal goat serum. Both sera were diluted 1:20 since they had titers of neutralizing antibody to TMEV of 1:8–1:16. Goat antiserum to mouse IgG but not normal goat serum markedly reduced the titers of neutralizing antibody to TMEV (table 2).

Passive immunization. For determination of the normal serum-CNS ratio of neutralizing antibody to TMEV, mice were given 0.25 ml of undiluted hyperimmune sera iv and sacrificed 24 hr later. As illustrated in table 3, all mice had measurable levels of antibody in CNS after passive immunization; the mean ratio was ~100:1. This ratio is similar to the serum-CNS antibody ratio of other experimental studies in which animals had an intact blood-brain barrier [6, 10]. Thus, in uninfected SJL/J mice a titer of antibody in serum of ⩾1:100 would be necessary for antibody to be detected in the CNS.

Temporal development of neutralizing antibody in the CNS. Mice were sacrificed at various intervals up to one year after infection, and titers of neutralizing antibody in paired sera and CNS extracts from individual animals were determined. In accordance with a prior study of

Table 3. Titers of neutralizing antibody in sera and extracts of the central nervous system (CNS) of mice after passive immunization with sera hyperimmune to Theiler's mouse encephalomyelitis virus.

Animal no.	Experiment no. 1		Experiment no. 2	
	Serum	CNS	Serum	CNS
1	512	8	512	8
2	512	8	512	8
3	512	8	512	8
4	1,024	4	2,048	16
5	1,024	8	...	...

NOTE. Titers are expressed as the reciprocal of the final dilution producing a 50% reduction in pfu.

TMEV infection in outbred Swiss mice [1], neutralizing antibody was detected in sera of SJL/J mice by one week, gradually increased in titer thereafter, and reached maximal levels between four and eight months after infection (table 4). In contrast, neutralizing antibody did not appear in the CNS until three weeks after infection, but subsequently the CNS of most mice contained antibody. Maximal levels of antibody were reached somewhat earlier (about two months) in the CNS than in serum. In all mouse CNS extracts that contained antibody, the normal ratio of serum antibody to CNS antibody of 100:1 was reduced (table 4). Furthermore, in 10 of these animals, titers in the CNS extracts were equal to or exceeded titers in serum, a finding which suggests that antibody was synthesized within the CNS. Such synthesis clearly must have occurred in those mice with higher titers of antibody in the CNS than in serum, since diffusion of antibody from serum across a more permeable blood-brain barrier could not account for this finding. Other evidence was obtained against increased permeability of the blood-brain barrier in this infection. Active immunization of mice with sheep erythrocytes did not show an appreciable difference in hemolysin antibody ratios between chronically infected and normal animals (data not shown).

For further documentation of the reduced antibody ratio, CSF was obtained from some infected mice. Titers of antibody were compared in pooled samples of sera, CSF, and CNS extracts from animals sacrificed three months after infection. As shown in table 5, levels of antibody in the CNS again were equal to or greater than

Table 4. Neutralizing antibody response in mice after intracerebral inoculation of Theiler's mouse encephalomyelitis virus.

Day after inoculation	Animal no. 1	2	3	4
7	4/<4	4/<4	8/<4	...
14	16/<4	8/<4	8/<4	...
21	4/8	8/16	16/8	...
28	16/<4	16/64	64/128	16/16
46	32/<4	256/64	...	...
78	64/128	32/128	32/128	...
131	64/32	64/64	128/32	...
244	256/32	256/32	64/128	...
353	128/64	...	...	...

NOTE. Data are given as titer of antibody in serum/titer of antibody in extracts of the central nervous system. Titers are expressed as the reciprocal of the final dilution producing a 50% reduction in pfu.

titers of antibody in serum, whereas titers of antibody in the CSF were slightly less than those in the sera.

Immunoglobulin-containing cells in the CNS. An initial comparison of sections, stained for fluorescent antibody, from PBS-perfused mice and unperfused mice revealed intense linear staining with goat antiserum to mouse IgG of the dura mater, leptomeninges, and blood vessel endothelium in the unperfused mice. Sections stained with IgM generally did not show any immunofluorescent staining. Perfusion with PBS eliminated the staining of endothelium, markedly reduced that in the leptomeninges, but did not affect the staining of the dura. Because of this "background" staining with IgG in unperfused normal mice, all infected animals were thoroughly perfused with PBS.

Ten infected mice were sacrificed at various representative times after ic inoculation of TMEV, and multiple sections of CNS were examined. Whereas cells containing IgM were not seen, IgG-containing mononuclear cells were frequently seen in all infected mice. Most IgG-positive cells had a thin cytoplasmic rim of bright fluorescence; however, some cells had more abundant staining in the cytoplasm and an eccentric nucleus typical of plasma cells. Such cells were found in perivascular sites, in parenchymal lesions in grey and white matter, and in the leptomeninges. There were substantially more IgG-staining cells during the chronic phase of the infection, yet the majority of mononuclear cells in perivascular cuffs and in parenchymal lesions did not stain.

Table 5. Titers of neutralizing antibody in sera, cerebrospinal fluid (CSF), and extracts of the central nervous system (CNS) of mice after intracerebral inoculation of Theiler's mouse encephalomyelitis virus.

Specimen	Day after inoculation 82	99
Serum	256	128
CSF	128	64
CNS	512	128

NOTE. Titers are expressed as the reciprocal of the final dilution producing a 50% reduction in pfu.

Local CNS interferon. Since interferon is important in host defense against certain viral infections, local production of interferon was examined in TMEV infection. Interferon was assayed in CNS extracts of individual mice at different times after ic inoculation. Since concentrated suspensions of CNS were found to be toxic to monolayers of L cells during overnight incubation, specimens were tested at a dilution of $\geq$1:20. Only two of 16 specimens of CNS from infected mice had measurable levels of interferon; both were positive at a 1:20 dilution but negative at 1:80. The CNS suspensions from uninfected mice were consistently negative for interferon. These results indicate that interferon is not present in substantial amounts in the CNS during this chronic infection.

Discussion

In this study substantial levels of neutralizing antibody were demonstrated in the CNS of mice chronically infected with TMEV. Although the subject of local production of antibody in the CNS remains somewhat controversial, this work has shown that the neutralizing antibody present in the CNS of TMEV-infected mice is locally produced. We feel this finding may be important to a basic understanding of the mechanism(s) of persistence of TMEV in vivo.

Although not commonly used, there is a precedent for antibody assay of tissue homogenates [4–7], and this approach was necessary in these studies because of the paucity of mouse CSF. That

neutralization by infected CNS extracts was indeed due to antibody was demonstrated by the specificity of neutralization for TMEV (table 1) and by the elimination or marked reduction of neutralization by in vitro treatment with goat antiserum to mouse IgG (table 2). By three weeks after infection, all infected mice had reduced, and some had reversed, ratios of serum antibody to CNS antibody (table 4) compared with those in passive transfer experiments. Although reduced ratios of antibody suggest that antibody was synthesized in the CNS in TMEV infection, leakage of antibody produced in peripheral lymphoid tissues across a more permeable blood-brain barrier could have occurred from cytolytic infection of blood vessels. However, previous studies failed to show evidence of viral replication in endothelial cells in the CNS by fluorescent antibody staining [1] or morphologic change of blood vessels by electron microscopy [13]. More conclusive proof of local formation of antibody in the CNS was evident from the actual reversal of the serum-CNS antibody ratio in about one-third of TMEV-infected mice after three weeks (table 4). In these animals antibody must have been produced in the CNS because diffusion of humoral antibody into the CNS could not account for this finding and because there is no sound evidence for selective transport of antibody from serum into the CNS. Reversal of the serum-CNS antibody ratio has also been found in poliovirus infections of monkeys [7] and rabies virus infection of mice [4]; in both instances the virus was inoculated ic.

The concept of local synthesis of antibody in the CNS appears to have originated from experimental animal studies on mechanisms of host resistance to ic challenge with neurotropic viruses. In the 1940s it was shown that animals survived second infections with neurotropic viruses only if they had high titers of antibody in serum or if antibodies were present in the CNS (in CSF or CNS homogenates) [5, 6]. In this circumstance the serum-CNS antibody ratio was usually reduced, a finding which suggested that antibody was formed in the CNS. Subsequently, Reid and coworkers [8] showed more convincing evidence of local production of antibody in the CNS of sheep infected with louping ill virus. Not only were reduced serum-CSF ratios of antiviral antibody present in infected sheep, but also normal ratios of antibody to a heterologous antigen (ovalbumin) were found. Recently, in a human demyelinating disease suspected of having a viral etiology, mononuclear cells from the CSF were shown to synthesize immunoglobulins in vitro [14, 15], and in an experimental parainfluenza virus infection of mice, evidence was presented that virus-specific memory, bone marrow-derived (B) cells in fact persist in the CNS [16].

On the other hand, the events leading to the appearance of antibodies in the CNS are still not precisely known. Since the CNS does not possess organized lymphoid tissue or thymus-derived (T) or B lymphocyte precursors, lymphocytes participating in local CNS inflammatory reactions and the accompanying antibody response probably originate from peripheral lymphoid tissues. Lymphocytes can enter the CNS because ultrastructural studies have shown mononuclear cells penetrating the blood-brain barrier by migrating through the intact endothelium of small blood vessels [17]. Once inside the blood-brain barrier, B lymphocytes differentiate into antibody-forming cells upon interaction with antigen. Furthermore, there is some indirect evidence that clones of sensitized lymphocytes may remain discretely localized within the CNS. In investigations of the immunity of rhesus monkeys that recovered from poliovirus infections of the CNS, resistance to reinfection by ic challenge was found to be limited to those animals given inoculations in regions of the CNS that actually were invaded by virus during the initial infection [18]. Monkeys that had recovered were susceptible when challenged with virus in regions of the CNS not previously invaded by virus. Recently Ogra and coworkers [7] found substantial titers of antiviral antibody in the thalamus, cerebellum, and spinal cord of monkeys infected with type 1 poliovirus but not in the occipital cortex. Pathologic change and, probably, replication of poliovirus do not occur in the occipital lobes but do occur in these other areas of the CNS.

Since we have now demonstrated that the CNS of TMEV-infected mice contains neutralizing antibody, it seems paradoxical that infectious virus can be consistently isolated from the CNS [1]. Why virus is not neutralized by antibody and why chronic infection is not aborted are unclear.

These are particularly intriguing questions since TMEV is a nonbudding, highly cytolytic virus and is able to spread to other susceptible cells only by extracellular extension. In this circumstance antibody should be a first line of host defense, and, indeed, in protection against other picornaviruses, humoral immunity is considered to be more important than cellular immunity [19]. Of a number of possible explanations for this failure of viral clearance, formation of complexes of infectious virus and antibody and production of antibodies with low affinity for TMEV deserve consideration. Circulating complexes of infectious virus and antibody have been demonstrated in a number of chronic viral infections [20]. In contrast to these infections, in TMEV infection there is only an evanescent viremia and no evidence of immune complex deposition in renal glomeruli by histology or fluorescent antibody staining (authors' unpublished observations). However, it seems plausible for complexes of infectious virus and antibody to form in the CNS, remain trapped inside the blood-brain barrier, and not reach the circulation. This possibility as well as that of the production of antibodies with low affinity for TMEV are currently under investigation.

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