The TO Strains of Theiler's Viruses Cause "Slow Virus–like" Infections in Mice

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Intracerebral inoculation of mice with tissue culture–adapted TO strains of Theiler's mouse encephalomyelitis viruses results in a clinical disease consisting of spastic paralysis due to demyelination after a lengthy incubation period. Thus, in effect, these ordinary picornaviruses are capable of causing a slow infection in their natural host, the mouse. In addition, through the use of tissue culture–adapted virus stocks, virus content in mouse tissues now can be accurately quantified by standard plaque assay.

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Slow viruses are characterized largely by their ability to produce clinical disease months or even years after infection. While the term *slow virus* has come to denote the transmissible group of unconventional viral agents, some conventional animal viruses also behave like slow viruses. These include those responsible for rabies and hepatitis B virus infections and the late-developing forms of panencephalitis due to measles or rubella virus in humans [7, 14, 20]. In addition, certain retroviruses produce transformation leading to neoplasia in a number of animal species [5], encephalomyelitis in Icelandic sheep [6, 16], and paralysis in wild mice [4], all after long delays. Recently it has been reported that mouse hepatitis virus type 3, a coronavirus, causes persistent infection in C3H mice with onset of a wasting syndrome and paralysis as long as three months after inoculation [9]. While there may be other examples, the incubation periods remain to be determined [13]. Nonetheless, the belief persists that conventional animal viruses cause only acute disease that occurs after a short incubation period. This notion certainly still applies to such highly cytolytic viruses as picornaviruses.

Previously, we found that mice develop a biphasic pattern of central nervous system disease after intracerebral inoculation of mouse brain stocks with a murine picornavirus, Theiler's mouse encephalomyelitis virus (TMEV) [10, 12]. During the first three weeks, the virus replicated and produced poliomyelitis-like pathological changes in the CNS gray matter, resulting in flaccid limb paralysis (early disease). Surviving animals generally recovered some function, but they invariably developed chronic CNS infection. In the chronic phase the spinal cord leptomeninges and white matter contained mononuclear cell infiltrates, and there were patchy areas of primary demyelination [1]. By two to three months following the infection, spastic paralysis began to evolve (late disease).

We report here that when tissue culture–adapted stocks are used, these murine picornaviruses regularly produce late neurological disease in mice without antecedent early disease. In this situation, late disease occurs after a lengthy incubation period. In addition, virus content in mouse tissues now can be accurately quantified by a standard plaque assay. This is a great improvement over the previous method of virus titration involving subinoculation of mice [10].

Materials and Methods

At 4 to 8 weeks old, male outbred Swiss and SJL/J mice were inoculated intracerebrally with 10^3 to 10^4 plaque-forming units (PFU) of virus stocks. The Swiss male mice (CD-1) were purchased from Charles River Breeding Laboratories (Portage, MI), and inbred SJL/J male mice from Jackson Laboratories (Bar Harbor, ME).

DA, WW, and TO4, TMEV viruses biologically resembling Theiler's original TO isolates, were adapted to grow in cell culture [11], and virus stocks were prepared in BHK 21 cells. The BHK21 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 0.1 mM L-glutamine, 100 μg of streptomycin and 100 units of penicillin per milliliter, 10% tryptose phosphate broth, and 10% calf serum. The same medium containing 2% calf serum was used for maintenance of these cells.

The virus content of serum and clarified CNS homogenates was determined by standard plaque assay as previously described [18]. In brief, confluent monolayers of

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BHK21 cells in 35 mm plastic FB-6TC multiculture dishes (Linbro Chemical Company, Hamden, CT) were washed once with phosphate-buffered saline (pH 7.4) and inoculated with appropriate virus dilutions (0.1 ml per well). Following adsorption at 37°C for 60 minutes, the inoculum was removed and each well was overlayed with 3 ml of medium consisting of 0.95% bacto-agar (Difco Company, Detroit, MI) in Basal Medium Eagle containing 0.5% bovine plasma albumin, fraction V (Armour Pharmaceutical Company, Chicago, IL), and 0.1 mM L-glutamine with 100 μg of streptomycin and 100 units of penicillin per milliliter. 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 plaques were read 8 to 24 hours later.

Anesthetized mice were sacrificed by total-body perfusion with chilled 3% glutaraldehyde in phosphate buffer (pH 7.4). Brains and spinal cords were fixed, embedded in paraffin or Epon, and stained as previously described [1, 10].

Results

The animals were examined daily for the first month after inoculation and weekly thereafter for signs of disease, particularly gait abnormality. As shown in Table 1, all the TMEV strains produced clinical neurological illness in Swiss mice. Depending on the virus strain, 58 to 90% of the mice developed disease between 11 and 22 weeks after inoculation. The CNS involvement in these animals was primarily upper motor neuron in character with prominent spasticity, resulting in an easily recognizable hesitant, waddling gait. This clinical picture is identical to that in late disease following infection with mouse brain stocks of the DA virus [10, 12]. In the present experiments, however, the mice remained well throughout the first month of infection. TO4 virus also produced late disease in SJL/J mice, but the onset of spastic paralysis occurred somewhat earlier than in the Swiss mice (see Table 1). In this connection, SJL/J mice have been shown to be more susceptible to chronic TMEV infection than other inbred strains of mice and Swiss mice [10, 12]. The animals have been followed for as long as eight months, and late disease in Swiss and SJL/J mice has been progressive without clinical evidence of remission.

Previously, the level of virus in tissues of mice chronically infected with TMEV had to be determined by titration back into mice. We have now found that by using tissue culture–adapted TO virus strains, virus content can be measured by standard plaque assay in BHK21 cells. Therefore, in additional experiments, Swiss mice inoculated intracerebrally with 10^3 to 10^4 PFU of TO4 virus were sacrificed for virus assay. Virus titers in blood and CNS of individual animals at several representative times after infection are shown in Table 2. While virus was not detectable in serum, it was present in the brains or cords of all mice examined. It should be noted that after the onset of late disease, virus was found only in the spinal cord, the site of pathological involvement. Furthermore, the virus obtained from the CNS of infected mice always produced minute plaques which were identical to those in the TO4 virus inoculum.

Table 1. Theiler Virus–induced CNS Demyelinating Disease Occurring after a Prolonged Incubation Period

Virus	Mouse Strain	Incubation Period (wk)	Clinical CNS Disease[a]
DA	Swiss	11–14	7/12
WW	Swiss	11–22	10/12
TO4	Swiss	11–15	10/11
TO4	SJL/J	5–7	20/20

[a]Number of animals showing spastic paralysis/number inoculated.

Table 2. Titers of Virus in CNS and Blood of Individual Adult Mice Inoculated Intracerebrally with TO4 Virus

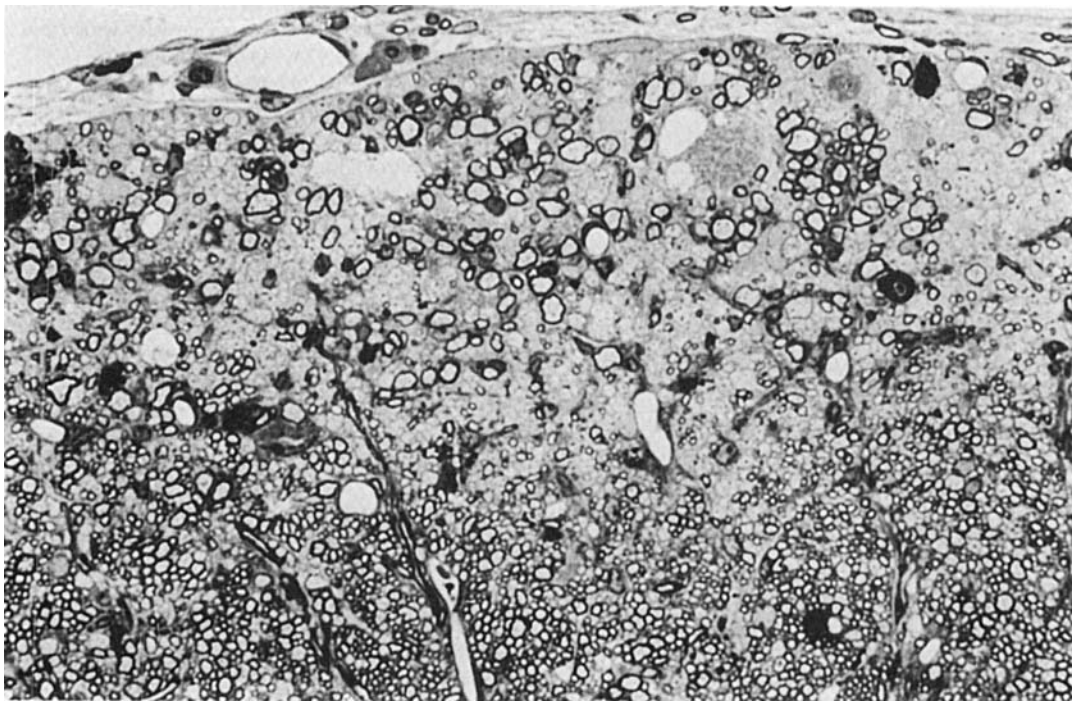
Weeks after Infection	Log_{10} PFU per Ml^{-1} or Gram[a]		
	Serum	Brain	Spinal Cord
1	ND	$10^{5.1}$	$10^{4.2}$
	ND	$10^{4.2}$	$<10^2$
	ND	$10^{4.2}$	$10^{4.2}$
4	$<10^2$	$<10^2$	$10^{1.6}$
	$<10^2$	$10^{2.6}$	$10^{3.3}$
	$<10^2$	$10^{3.2}$	$10^{5.1}$
11[b]	$<10^2$	$<10^2$	$10^{3.2}$
	$<10^2$	$<10^2$	$10^{2.8}$

[a]Determined by standard plaque assay in BHK21 cells. Plaque assay is unable to detect less than 2 log_{10} of virus.
[b]These 2 mice had clinical evidence of late disease.

ND = not done.

All three viruses produced similar late pathological changes in the spinal cord, consisting of numerous demyelinated axons; some remyelinated axons, which could be identified by their abnormally thin myelin sheaths; and a low-grade mononuclear cell inflammatory response (Figure). Inflammatory cells were mainly lymphocytes and macrophages. In addition, there was a mild astroglial response. The demyelinated areas in spinal cord were always rather sharply demarcated from the surrounding normal white matter. In general, these findings resemble the chronic pathological picture of TMEV infection following intracerebral inoculation of DA virus grown in mouse brain [1].

To determine if these tissue culture–adapted viruses fail to produce the pathological changes of early



Coronal section of spinal cord from an outbred Swiss mouse killed three months after intracerebral inoculation with WW virus. Beneath the leptomeninges is a demarcated area of demyelination showing numerous naked axons, some remyelinated axons, and an occasional fragment of myelin debris. (Toluidine blue; ×640.)

poliomyelitis, 4 mice inoculated with the WW strain were killed during the third and fourth weeks after infection. None of the Epon-embedded material from these animals showed any abnormality. While this finding does not rule out the occurrence of early virus replication in spinal cord motor neurons, it does support the notion that the adaptation process has resulted in attenuation of this viral property.

Discussion

The present study demonstrates that TO strains of TMEV—ordinary picornaviruses—are capable of causing a slow infection in their natural host, the mouse. In this circumstance, TMEV-induced disease satisfies the major criteria for slow infections set forth by Sigurdsson in 1954 [19]. He defined a slow infection as one in which a protracted course of illness with predominant involvement of a single organ system, usually resulting in death of the host, occurs after a long latency. While our infected animals have not as yet died, their disease has been progressive and closely resembles late disease induced with brain-derived DA virus, which is fatal.

We are not certain why adaptation of the TMEV strains to cell culture eliminated the earlier clinical phase of poliomyelitis. While it probably merely represents attenuation of the original virus, this result has uncovered a unique virus-host interaction for a picornavirus: a slow infection. It is indeed remarkable that the incubation period, which ranged from one to five months, represents an appreciable portion of the life span of the mouse. Although the minimum infectious dose for induction of chronic CNS infection has not been determined, there is reason to believe that lower doses of virus may result in even longer incubation times [8].

Since infectious virus can be recovered from the CNS of these animals throughout the incubation period and after onset of disease (see Table 2), it appears that persistent infection is maintained through continuous production of infectious virus. In contrast to other viruses, there is little experimental evidence to suggest that picornaviruses can remain latent, either integrated into the cell genome or existing in the cytoplasm in the form of defective particles. From investigations of carrier culture systems it is known that picornaviruses establish steady-state infections with virus replicating in only a fraction of the cell population at any one time [21]. In this situation it is believed that when a cell becomes infected, virus is probably allowed to complete its normal replicative cycle, and the cell is destroyed. This notion is strongly supported by the fact that picornaviruses are highly cytolytic agents, probably due to their ability to rapidly suppress host cell protein synthesis [15] so that it is unlikely a cell can survive once it is infected. Further, overwhelming infection is probably pre-

vented by innate resistance of the majority of the cell population at risk or by the host's immune response. Obviously, the precise mechanisms of picornavirus persistence remain to be elucidated.

Finally, these observations may have some relevance to human disease since TMEV infection in mice is one of the few available experimental animal models of virus-induced demyelination [2]. Epidemiological evidence suggests that multiple sclerosis, a human demyelinating disease strongly suspected of having a viral origin, may have an incubation period lasting many years [3, 17]. The prolonged incubation period of TMEV infection in mice furnishes a possible additional parallel with multiple sclerosis.

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