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NOTES

Relationship Between Host Age and Persistence of Theiler's Virus in the Central Nervous System of Mice

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This study has demonstrated that the ability of BeAn 8386 virus to persist in the central nervous system of mice declines with the increasing age of the host at the time of inoculation. Although persistent infection was established in 1-, 3-, 9-, and 40-week-old mice, there was a significant reduction in both the frequency of virus isolations and the mean virus titers in mice inoculated after 3 weeks of age. The incidence of clinical demyelinating disease (late disease) also decreased in animals infected after 3 weeks of age in parallel with the decline in virus persistence.

The Theiler's murine encephalomyelitis viruses (TMEV) are enteric pathogens of mice and members of the *Picornaviridae* family. Certain strains produce a unique biphasic central nervous system (CNS) disease in their natural host after intracerebral (i.c.) inoculation (7, 8). This is characterized by the occurrence of poliomyelitis during month 1 postinfection (p.i.) (early disease), and surviving mice then develop a chronic, inflammatory demyelinating disorder weeks to months later (late disease). The demyelination is related to a persistent infection in which low levels of infectious virus can be readily recovered from the target organ, the CNS, for many months (8, 14). Persistent infection occurs, although mice develop neutralizing antibodies and cellular immunity to the virus (11).

The susceptibility or resistance to acute virus infections is often age dependent in both humans and experimental animals. On the other hand, there is little known about the effect of age on the persistence of most viruses, including TMEV. A number of viruses persist after a congenital infection in which vertical transmission occurs either by transovarial or by transplacental passage. For one such virus, lymphocytic choriomeningitis virus, mice infected shortly after birth also become lifelong virus carriers as the result of induction of tolerance (5, 6). An epidemiological study of subacute sclerosing panencephalitis has shown that children who develop this form of chronic measles virus infection experience a primary measles virus infection earlier than do matched controls (3). This also suggests that infection earlier in life promotes persistence, but in this case the host develops neutralizing antibodies and cellular immunity to measles virus (1, 4).

In the past few years we have noticed that TMEV has been isolated more often during the persistent phase of infection from mice inoculated as weanlings than as 8- to 12-week-old mice. The present study was conducted to determine the effect of host age on the persistence of TMEV in mice.

Male CD-1 mice (1, 3, 9, and 40 weeks old) obtained from Charles River Breeding Laboratories, Portage, Mich., were inoculated in the right cerebral hemisphere with 1.9×10^6 PFU of virus in 20 μl. The animals were examined one to two times a week for neurological signs as previously described (9).

BHK-21 cells were cultivated in Dulbecco modified Eagle medium (DMEM) as described previously (E. J. Rozhon, J. D. Kratochvil, and H. L. Lipton, Virology, in press). BeAn 8386 virus was isolated from a feral mouse in Belem, Brazil, in 1957 and later was classified as a TMEV by complement fixation serology. This virus was provided as a third-passage mouse brain homogenate by Robert E. Shope, Yale University, New Haven, Conn. A plaque-purified stock was prepared after two passages in BHK-21 cells, one adult mouse brain passage, and three additional passages in BHK-21 cells. The titer of this stock was 9.4×10^8 PFU/ml.

Mice were anesthetized with methoxyflurane at the times indicated below, and the brainstem and spinal cord of individual animals were removed, pooled, and prepared as clarified 10% homogenates in DMEM. The virus concentration in each sample was determined by a standard plaque assay on BHK-21 cell monolayers as described previously (Rozhon et al., in press). Briefly, after adsorption of virus to BHK-21 cells in 60-mm petri dishes at 24°C for 45 min, the monolayers were overlaid with 5 ml of DMEM containing 0.1% (wt/vol) bovine plasma albumin (Reheis Chemical Co., Scottsdale, Ariz.) and 0.9% (wt/vol) Noble agar (Difco Laboratories, Detroit, Mich.). The monolayers were incubated at 33°C for 4 days, and plaques were identified by staining with 0.015% neutral red. The minimum level of sensitivity of the assay is 50 PFU/g of tissue.

To determine the mean 50% dose of virus resulting in persistence (PD_{50}), 3- and 9-week-old CD-1 mice in groups of five were inoculated i.c. with 10-fold dilutions of BeAn 8386 virus. All inoculated animals were sacrificed on p.i. day 40 to assay for virus in their CNS. Calculation of the PD_{50} was by the method of Reed and Muench (12).

For the analysis for significance, the X^2 and the two-sample *t* tests were used.

Although the main focus of this study was virus persistence, the clinical data from the initial experiment provided a correlation between persistence and disease occurrence. In contrast to the results of an earlier study in which a brain-derived stock of TMEV was used (2), the development of flaccid paralysis due to poliomyelitis (early disease) was found to be age dependent in the mice receiving this tissue

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TABLE 1. Early and late disease in mice of various ages inoculated i.c. with BeAn 8386 virus

Age (wk)	No.	Early disease[a] (no. paralyzed [no. dead])	Late disease[b] (no. affected [no. dead])
1	27	17 (10)	4 (1)
3	21	1 (0)	7 (2)
9	20	1 (1)	2 (1)
40	17	1 (1)	2 (1)

[a] Flaccid paralysis.
[b] Spastic paralysis and extensor spasms occurring after 1 month p.i. Note that seven mice in each group were sacrificed for virus assay at 1 month p.i.; thus, both the early deaths and sacrificed animals should be subtracted to give the total at risk for late disease.

culture-adapted stock of BeAn 8386 virus (Table 1). Two-thirds of the 1-week-old animals injected with virus developed flaccid paralysis, whereas only 5% of the older groups of mice were affected. In addition, 10 of 27 mice inoculated at 1 week of age died, a substantially greater mortality than observed in the older groups of mice. Although few of the 3-, 9-, and 40-week-old animals developed early disease, a somewhat higher incidence of late disease was seen in these same age groups (Table 1).

Since the early phase of logarithmic virus growth is over by p.i. day 21 (8), the persistent phase of infection can be defined as beginning after this time. To determine whether there was virus persistence, 7 to 11 mice in each age group were sacrified for virus assay at ca. 1 and 3 months p.i. These times were believed to be representative of the initial part of the persistent infection, which can last for longer than a year (14). The CNS of most animals had detectable infectious virus, but a decrease in the frequency of virus isolations was observed between the animals sacrificed at 1 and 3 months p.i. for each age group (Table 2). When the results for both times were combined, animals 3 weeks of age and younger had a significantly higher frequency of persistent CNS infections than did mice inoculated at an older age (see Table 2 for P values).

The virus titers for animals from all age groups are shown in Fig. 1. A decline in the mean virus titer between 1 and 3 months p.i. was found for each age group. The difference in the mean virus titer between the age groups correlated closely with the difference in the frequency of virus isolations presented in Table 2. At 1 month p.i, the mean titers were significantly higher in 1-week-old mice compared with all older ages ($P < 0.001$) and in 3-week-old mice compared with the 9-week-old mice ($P < 0.01$). At 3 months p.i., the mean titers in the 1- and 3-week-old mice were significantly higher than in the 9- and 40-week-old animals ($P < 0.001$).

Since there was a difference in persistence between 3- and 9-week-old mice, we determined whether the 50% mean virus dose required to produce a persistent CNS infection in both age groups also would be different. The amount of virus required to establish a persistent CNS infection in 9-week-old mice (3.8×10^4 PFU) was more than that required for 3-week-old mice (1.2×10^4 PFU) (Table 3).

TABLE 2. Frequency of virus isolations in the persistent phase of infection in mice inoculated i.c. with BeAn 8386 virus

Age (wk)	No. positive/total[a] (%)		
	1 mo. p.i.	3 mo. p.i.	Total
1	7/7 (100)	5/7 (71)	12/14[b] (86)
3	7/7 (100)	9/11[c] (82)	16/18[c] (89)
9	6/7 (86)	3/11 (27)	9/18[d] (50)
40	3/7 (43)	1/7 (14)	4/14 (29)
Total	23/28 (82)	18/36 (50)	41/64 (64)

[a] The numerator is the number of animals from which virus was isolated, and the denominator is the total number of animals assayed.
[b] Statistically significant compared with 40-week-old mice ($P < 0.01$ by X^2).
[c] Statistically significant compared with 9 ($P < 0.05$)- and 40 ($P < 0.01$)-week-old mice (X^2).
[d] Not significantly different compared with 40-week-old mice ($P > 0.05$ by X^2).

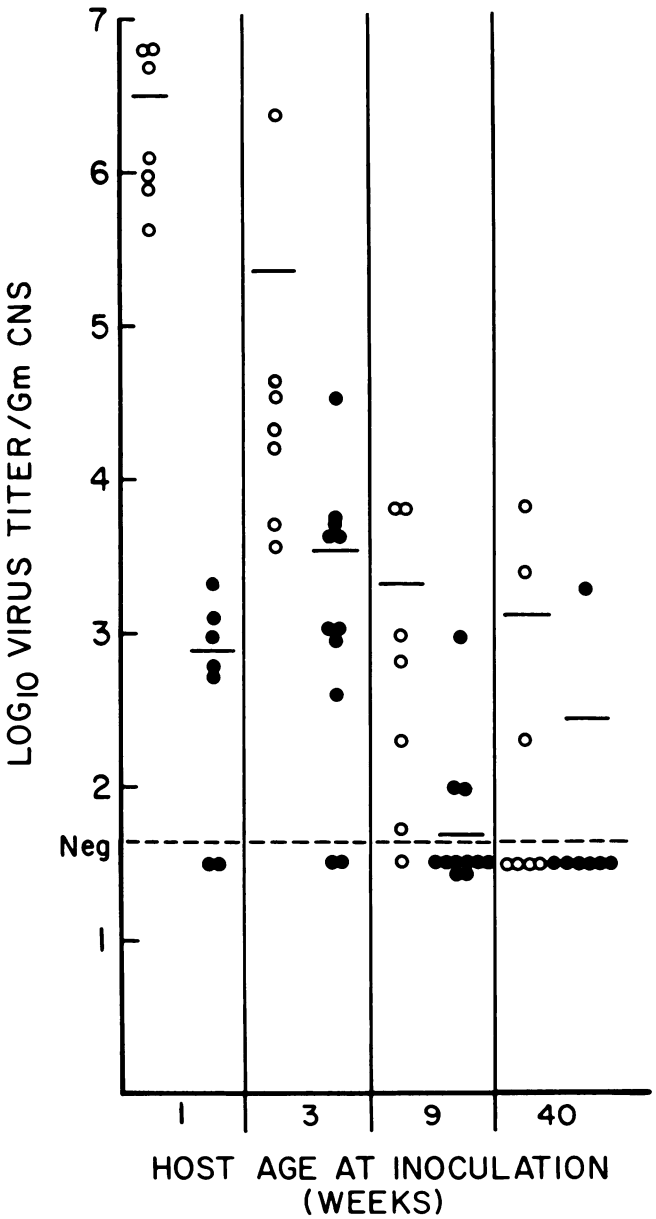

FIG. 1. Central nervous system virus titers in individual mice inoculated i.c. with 1.9×10^6 PFU of BeAn 8386 virus at various ages. Symbols: ○, 1 month p.i.; ●, 3 months p.i. The mean titers are indicated by horizontal bars, and the level of sensitivity of the virus assay is indicated by the dashed line.

TABLE 3. PD_{50} in 3- and 9-week-old mice inoculated i.c. with BeAn 8386 virus

Age (wk)	Log_{10} PD_{50}[a]	PFU/PD_{50}
3	3.25	1.06×10^4
9	2.65	4.21×10^4

[a] Determined by virus assay of the CNS from mice inoculated i.c.with serial 10-fold dilutions of virus. There were five mice per dilution.

The present study has demonstrated that the ability of TMEV to persist in the CNS of mice declines with the increasing age of the host at the time of infection. On the basis of both the frequency of virus isolation and the mean virus titers for the four age groups studied (Table 2 and Fig. 1), this decline was particularly evident in mice older than 3 weeks of age. In addition, the virus dose required to establish a persistent infection in 9-week-old mice was approximately four times the dose required for 3-week-old mice (Table 3). Since mice are immunologically mature by 3 weeks of age, it would seem that the decline in persistence after this age does not relate to maturation of the immune system. The exact mechanism responsible for the age dependency of susceptibility to TMEV persistence remains to be determined.

The incidence of demyelinating disease (late disease) tended to be less in mice inoculated after 3 weeks of age and essentially paralleled the decline in virus persistence. Although the inflammatory demyelinating lesion that is responsible for late disease appears to be immune mediated (10, 13), it would also appear from the above results to be related to the level of virus replication. Conceiveably, higher virus antigen loads may amplify immune responses leading to demyelination.

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