Experimental Measles Encephalitis: a Genetic Analysis

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The encephalitogenic potential of nine temperature-sensitive mutants of measles virus was determined in newborn golden Syrian hamsters. The parental virus produced acute encephalitis without any prior adaptation. Six of the mutants were attenuated, two were virulent, and one was associated with hydrocephalus with acute onset. The attenuated mutants, blocked before measles virus antigen and ribonucleic acid synthesis at 39 C, were all members of one complementation group. The virulent temperature-sensitive mutants, defective in hemolysin antigen synthesis at 39 C, were members of a second complementation group. The hydrocephalus-inducing mutant was genetically distinct from the other mutants. The mechanism of attenuation most probably does not involve a temperature-induced inhibition of virus replication, but rather appears to be related to the partial defectiveness of the mutants under permissive conditions.

Measles virus is involved in the pathogenesis of acute encephalitis, subacute sclerosing panencephalitis (SSPE), and possibly multiple sclerosis (10, 19, 24). It has been suggested that a mutant or defective variant of measles virus may be the etiological agent of SSPE (9, 12, 18). Measles virus can produce acute encephalitis in newborn hamsters (2, 21) even without prior adaptation (22). Previous work in this laboratory demonstrated that immunological factors such as maternal antibody can modify the course of acute encephalitis in newborn hamsters (23). The role of virological factors in experimental measles encephalitis, however, is not yet well understood (14).

There is rapidly accumulating evidence that selection for temperature sensitivity results in a co-selection for decreased neurovirulence. Non-neurotropic poliovirus exhibits restricted replication at 40 C (15). More recently, it has been reported that temperature-sensitive (ts) mutants of influenza Ao, reovirus, and vesicular stomatitis virus are highly attenuated (3, 7, 17, 20). Ts mutants of measles virus were therefore isolated as a possible model system for the theorized altered pathogenic properties of virus variants. We present in this report data suggesting that the attenuation of the neurovirulent potential of measles virus ts mutants is at least partially dependent upon the nature of the temperature-sensitive defect.

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MATERIALS AND METHODS

Viruses. Temperature-sensitive mutants of measles virus, induced either by 5-azaacytidine, 5-flourouracil, or proflavine, were isolated and partially characterized (4). The properties of the parental strain of measles virus (CC) were previously reported (5). Nine ts mutants, representative of the three genetic complementation groups, were used in this study. A summary of the physiological properties of the ts mutants is provided in Table 1.

Inoculation of animals. Random-bred golden Syrian hamsters (Lakeview, Newfield, N.J.) were inoculated by the intracranial route within 24 h of birth with 3 × 10⁶ plaque-forming units in 0.05 ml. The mutant inocula contained between <1 and 3 × 10⁶ plaque-forming units measured at 39 C. The titers at the restrictive temperature did not influence the virulence of the mutants. Control animals were inoculated with an extract of uninfected BSC-1 cells. Each experimental group was composed of five to seven litters (45 to 60 animals). Deaths before 4 days were considered related to needle trauma. The period of acute encephalitis extended to 16 days postinoculation.

Virus isolation from hamster brains. A suspension of brain cells was prepared by mincing brain tissue, followed by passage through a 25-gauge hypodermic needle. An alternate means of isolating viable brain cells was to treat coarsely minced brain tissue with 0.25% trypsin for 10 min. Both of these methods successfully isolated viable brain cells, and they were used interchangeably. The brain cell suspension was plated onto confluent monolayer cultures of BSC-1 cells, and the cultures were incubated at 33.5 C, the permissive temperature of the ts mutants. The co-cultivated cells were passaged when confluence was reached. Virus was harvested.
by scraping and sonic oscillation when appropriate levels of cytopathic effect were observed. The cells were harvested 7 to 10 days after co-cultivation if no cytopathic effect was observed. Measles virus was assayed by the plaque method at 33.5 and 39 C as previously described (5). The efficiency of plaquing (EOP) 39/33.5 C was determined for each virus isolate.

**Histological methods.** The calvarium was removed and the brain fixed in situ with 10% neutral-buffered formalin. Paraffin sections were cut at 6 μm and stained with hematoxylin and eosin. Occasionally, sections were stained with Luxol fast blue.

**RESULTS**

**General neurological and histopathological findings.** Neurological symptoms began with evidence of ataxia and hyperactivity. Affected animals exhibited spasms of opisthotonus as well as myoclonic seizures. Later, the injected animals became moribund. Death occurred 2 to 4 days after the onset of the symptoms of neurological impairment.

All measles viruses examined induced diffuse inflammation, whereas the noninfectious control material did not. The infiltrate was predominantly monocyte with few polymorphonuclear leukocytes. Infiltration occurred throughout the parenchyma, including the brain stem and cerebellum. Leptomeningitis was a typical finding. It is interesting to note that there was substantially less infiltration of the neocortex than the lower areas of the brain.

**Parental virus.** Neurological symptoms were first observed 5 days after virus inoculation and appeared in almost all animals during the subsequent 3 days. Between 96 and 100% of the animals had succumbed to a fatal encephalitis within 12 days after virus inoculation (Fig. 1–3). Histological study revealed an acute, highly intense encephalitis. Infectious virus was readily isolated from brains, by both co-cultivation and direct isolation from homogenized brains (Table 2).

**Genetic complementation group I.**

**Highly attenuated mutants.** Neurological symptoms were not observed in animals inoculated with ts mutants A, B, E, or L. Some virus-injected animals died during the 16-day observation period; these mortalities ranged between 2% (tsL) and 17% (tsA) (Fig. 1 and 3). Virus isolation from these infected brains typically required extended co-cultivation and was successful in the case of only 5 of 12 (42%) animals.

All of the isolates were found to have retained the temperature-sensitive defect (Table 2). The body weight of the surviving animals was indistinguishable from that of the control animals. None of the hamsters exhibited any apparent deficits, including demyelination. The only exception was that 2 (17%) out of 12 hamsters autopsied 9.5 months after inoculation with tsL had moderate hydrocephalus. No virus could be isolated from these affected animals. It should be noted that hydrocephalus was not observed in these animals before 8 months after inoculation.

**(ii) Moderately attenuated mutants.** Ts mutants F and S exhibit less phenotypic stability (increased “leakiness”) than the previously described mutants (Haspel and Rapp, unpublished observations). Neurological symptoms were first observed about 10 days after inoculation. Approximately 38% of the hamsters inoculated with tsF (Fig. 2) or tsS (Fig. 3) succumbed to a fatal encephalitis. Virus was successfully isolated from most brains (five of six, 83%). The majority of these isolates (three of five) were still mutant (Table 2). The EOP 39/33.5 C of the 2 "nonmutant" isolates (10−2), although substantially lower than that of the parental strain (10−6), did not meet the criterion for temperature sensitivity (10−4). The animals inoculated with ts mutant F were significantly (P < 0.001) smaller in body weight (mean = 38.6 g) than the control animals (mean = 47.0 g). This running effect by the virus observed at 24 days after

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**Table 1. Biological properties of complementation groups of temperature-sensitive mutants of measles virus**

<table>
<thead>
<tr>
<th>Property at 39 C</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Parental</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicationb</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Cell penetrationc</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Virion RNA synthesisd</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Nucleocapsid antigen</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hemagglutininf</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Hemolysin antigeng</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

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* From reference 4.
* Yields of the mutants at 39 C in BSC-1 cells were 1,000- to 10,000-fold lower than at 33.5 C.
* Defined as the ability to become refractory to measles virus-neutralizing antibody after incubation at 39 C.
* RNA, Ribonucleic acid. Synthesis defined as the resistance to 5-azacytidine present in the cell culture medium after the shutdown of the mutant-infected cells from 39 C to the permissive temperature. The resistance was measured by the production of infectious progeny under these conditions.
* Measured by indirect immunofluorescence using antisera prepared against purified virion components.
* Hemadsorption by infected cells with African green monkey erythrocytes.
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inoculation was temporary, since the adult animals were indistinguishable from the controls. None of the tsF-inoculated animals examined up to 8 months after inoculation exhibited any apparent neurological dysfunction. However, 45% (5 of 11) of the animals autopsied 9.5 months after inoculation with ts mutant F had a moderate degree of hydrocephalus. Virus could not at that time be isolated from the brains of tsF-inoculated animals.

Genetic complementation group II. Neuro-

### Fig. 1. Mortality of hamsters after intracranial inoculation of parental measles virus, control material, or temperature-sensitive (ts) mutants A or B. Newborn hamsters were inoculated with $3 \times 10^4$ plaque-forming units of measles virus in 0.05 ml. Control hamsters were inoculated with a homogenate of noninfected cells.

### Fig. 2. Mortality of hamsters after intracranial inoculation of parental measles virus, control material, or temperature-sensitive (ts) mutants E, F, or G. Newborn hamsters were inoculated with $3 \times 10^4$ plaque-forming units of measles virus in 0.05 ml. Control hamsters were inoculated with a homogenate of noninfected cells.
plaque-forming units of parental measles inoculation of noninfected control hamsters, respectively. The survivors appeared (Fig. 3).

Virus isolations attempted in encephalitis followed somewhat more rapid course in tsN-inoculated animals; however, by day 13 the differences between tsC and tsN had disappeared (Fig. 3). Both mutants had caused fatal encephalitis in 98% of the infected animals. All attempted virus isolations were successful and all 11 isolates were mutant (Table 2).

Genetic complementation group III. Animals first exhibited neurological symptoms 8 days after inoculation with ts mutant G. During the 16-day experimental period, 63% of the animals died (Fig. 2). The survivors at 24 days after inoculation were significantly (P < 0.001) smaller (mean = 34.0 g) than the control animals (mean = 47.0 g). As was the case with tsF-inoculated animals, the running was not permanent. Infectious virus was successfully isolated from tsG-infected brains by co-cultivation (Table 2). Five of the six isolates were still mutant. Although the nonmutant isolate did not meet the criterion for temperature sensitivity, its EOP was less than that of the parental virus. Unlike the other mutants, ts mutant G induced hydrocephalus rapidly after inoculation. We reported that 76% (42 of 55) of tsG-injected animals autopsied between 14 and 33 days after inoculation were hydrocephalic (6). In a subsequent experiment, 92% (11 of 12) of hamsters autopsied between 12 and 16 days after inoculation with ts mutant G had moderate to severe hydrocephalus. Severely hydrocephalic animals were, however, able to survive even as long as 9.5 months after inoculation.

DISCUSSION

In agreement with our previous findings (22), the parental measles virus was able, without prior adaptation, to induce a fatal encephalitis in all inoculated hamsters. All of the members of complementation group I were attenuated when compared with the parental virus. This attenuation was manifested by a decrease in mortality rather than an increase in survival time. Mutants in group II showed an increase in survival time, although mortality was the same as that of wild-type virus. Thus, group II mutants also appear to be slightly less virulent than wild type. The nature of the genetic defect thus appears to determine the virulence of the mutant. This relationship between the genetic

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of successful isolations*</th>
<th>No. of mutant isolates*</th>
<th>EOP 39/33.5 C°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>5/5</td>
<td>0/2</td>
<td>10^-3.2</td>
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<tr>
<td>Complementation group I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tsA</td>
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<td>1/1</td>
<td>10^-4.7</td>
</tr>
<tr>
<td>tsB</td>
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<td>3/3</td>
<td>10^-3.2</td>
</tr>
<tr>
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<td>tsF</td>
<td>4/4</td>
<td>2/4</td>
<td>10^-3.6</td>
</tr>
<tr>
<td>tsL</td>
<td>0/2</td>
<td>0/0</td>
<td>10^-3.8</td>
</tr>
<tr>
<td>tsS</td>
<td>1/2</td>
<td>1/1</td>
<td>10^-3.0</td>
</tr>
<tr>
<td>Complementation group II</td>
<td></td>
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<td></td>
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<tr>
<td>tsC</td>
<td>6/6</td>
<td>6/6</td>
<td>10^-1.0</td>
</tr>
<tr>
<td>tsN</td>
<td>5/5</td>
<td>5/5</td>
<td>10^-4.4</td>
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<tr>
<td>Complementation group III</td>
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<td></td>
</tr>
<tr>
<td>tsG</td>
<td>6/6</td>
<td>5/6</td>
<td>10^-4.6</td>
</tr>
</tbody>
</table>

* Virus isolation by co-cultivation of brain cells with BSC-1 cells at 33.5 C as described in Materials and Methods. Virus was assayed by the plaque method at 33.5 and 39 C (5).

* Isolates with an EOP 39/33.5 C less than 10^-2 were considered to have retained the temperature-sensitive defect.

* From reference 4.
defect and the pathogenic potential of measles virus was further supported by the finding that tsG, unique among the mutants in its ability to rapidly induce hydrocephalus, is the sole member of its complementation group. The development of a moderate degree of hydrocephalus among a minority of tsE- and tsF-inoculated animals is suggestive of a slow developmental process. A minor degree of damage to the aqueduct of Sylvius, occurring early in infection, would result in a gradual build-up of cerebrospinal fluid. Thus, hydrocephalus was not observed in autopsied animals before 9.5 months after inoculation. On the other hand, severe hydrocephalus was observed as early as 12 days after inoculation with ts mutant G. The few animals inoculated with the parental virus and surviving up to 12 days did not exhibit hydrocephalus. Other mutants in group III are being sought, but these preliminary results already suggest that G may be a rare mutant.

The in vivo replication of ts mutants can be inhibited when the host body temperature is the same as the nonpermissive temperature (7, 11, 25). Reovirus ts mutants possess altered pathogenicity even under conditions where the host body temperature is lower than the nonpermissive temperature (3). Virus, when isolated from infected brains, was found to have retained the ts defect (13). The basal temperature of the hamster is approximately 37 C (16). A virulent measles ts mutant (tsC) exhibited only semipermissive in vitro replication at 37 C, whereas highly attenuated mutants such as tsA replicated as well at 37 as at 33.5 C. Inflammation within the central nervous system could conceivably induce a fever approaching or exceeding the nonpermissive temperature. The in vitro incubation of mutant-infected cells at the nonpermissive temperature results in a selection for revertant virus (Haspel and Rapp, unpublished observations). Yet, the vast majority (24 of 27, 89%) of the isolates from mutant-infected brains were still mutant. Thus it appears unlikely that the host body temperature plays a major role in the attenuation of the mutants.

There was variation in the degree of attenuation among the group I mutants. This variation could not be attributed to the genetic stability of the mutants, as tsE, despite a high reversion frequency (EOP = 10\(^{-2}\)), was more attenuated than tsF (EOP = 10\(^{-3}\)-9). The genetic instability (EOP 39/33.5 C) was manifested by the production of large wild-type plaques at 39 C. The progeny of these plaques were indistinguishable from the parental virus. Furthermore, all viruses were passaged by diluted inocula an equivalent number of times through BSC-1 cells; thus it is doubtful that the differences in neurovirulent potential were due to differences in virus passage history (1, 8). The phenotypic stability of the mutants (the production of small non-wild-type plaques at 39 C) appears to be the most likely factor. The leaky ts mutants were the least attenuated, whereas the most phenotypically stable mutants were the most attenuated.

The in vitro replication of the ts mutants at the permissive temperature is less efficient than that of the parental strain; the mutants are thus semidefective under permissive conditions. It is conceivable that this semidfectiveness under permissive conditions rather than an inhibition of the mutant by the host body temperature may be responsible for the attenuation. A mutant that expresses partial activity (leakiness) at the nonpermissive temperature would be less defective under permissive conditions and consequently would be less attenuated. The data suggest that a partial defect early in the replicative cycle results in attenuation whereas a mutant with a late defect remains virulent. Studies are presently in progress to isolate other types of mutants to determine whether partial defectiveness of an early replicative event rather than temperature sensitivity per se is responsible for the observed attenuation of neurovirulence.

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