Evidence for a Mouse Pathogenicity Locus in Certain Temperature-Sensitive Mutants of Foot-and-Mouth Disease Virus

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Serial tissue culture passaging of three foot-and-mouth disease temperature-sensitive mutants demonstrated the stability of their temperature sensitivity and mouse avirulence characteristics. Recovery of mouse-virulent temperature-sensitive viruses after passage of the mutants in mice suggested that these were not covariant expressions of the same locus, but were under the control of different genes.

Various mutational events can occur in the viral genome resulting in a temperature-sensitive (ts) phenotype. Most viruses carrying a ts lesion also exhibit reduced virulence for susceptible hosts (11, 12, 15-17, 19, 26). This close association between the ts marker and virulence has led to the suggestion (10, 14, 16, 21) that ts mutants might be selected as live-virus vaccines. However, some ts mutants retain essentially parental levels of virulence (12, 17), suggesting that additional loci may be involved; virulence for intact animals may be affected by mutations in many different genes (9).

Three type A44 foot-and-mouth disease (FMD) virus ts mutants isolated in our laboratory were unable to synthesize viral ribonucleic acid (RNA) at 38.5°C (nonpermissive temperature). Defining complementation groups for ts mutants of FMD viruses (13, 22) had been difficult. Both virus recovery and isotope incorporation procedures were used to establish that these three mutants belonged to the same complementation group (24). Two of these mutants exhibited reduced virulence for infant mice; they were immunogenic and induced resistance to subsequent infection by the parental virus, but not to a heterologous type of FMD virus (23). These mutants produced reduced clinical manifestations of FMD in steers, and the viruses recovered from blood were ts+. One of the ts+ revertants, however, remained avirulent for mice; thus, two cistrons may have been involved. In the present study, the hypothesis that the loci controlling the ts characteristic and mouse pathogenicity may be different in these mutant viruses was examined further.

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MATERIALS AND METHODS

Tissue culture and virus assay. Primary bovine kidney (BK) cell cultures were prepared by the Research Services section, Plum Island Animal Disease Center. The cells, seeded in stopped suppression bottles, were propagated in Hanks balanced salt solution containing 0.5% lactalbumin hydrolysate (HLH medium) with 6% bovine serum. After 4 days the medium was discarded and replaced with HLH containing 2% bovine serum. The cultures were washed with serum-free HLH before virus infection. Drained cultures were inoculated with 0.1 ml of virus and incubated for 1.5 h at 37°C. After appropriate overlay was added, the cultures were incubated in air incubators at 33 and 37°C, or at 37.5, 38.0, and 38.5°C in water baths.

Three types of overlay media were used. Infected cultures were overlaid with 10 ml of HLH for virus production. Virus was routinely assayed at the permissive (39°C) temperature by overlaying the infected cultures with gum tragacanth medium (20). For comparing plaque development at various temperatures, agar medium was added (3).

Mice. PIADC:M-1(S)CV mice were used in these experiments. These Rockefeller-H strain mice have been reared in a closed colony at this center for over 20 years, and the young are highly susceptible to infection by FMD virus (6). Five-day-old mice, caged 10 per nursing mother, were inoculated (0.03 ml) intraperitoneally with virus dilutions and observed for 7 days. Hind leg skeletal muscle (5) was homogenized in 10 ml of 0.16 M tris(hydroxymethyl)amino methane buffer, pH 7.4, and the supernatant fluids were frozen (−56°C) for subsequent virus assays.

Stock virus. FMD wild-type (wt) virus, type A44, and three ts mutants previously isolated and partly characterized (21, 22) were used in these experiments. The designation mou denotes the hypothesized locus controlling mouse pathogenicity. Mu-
RESULTS

Stability of viruses during tissue culture passage. The wt and ts mutant viruses were grown in BK cells under liquid overlay at 33°C; the progeny were assayed under agar overlay at both 33 and 38.5°C and in infant mice. The results obtained after six serial passages are shown in Fig. 1 and indicate that the general characteristics for each virus were retained. After three BK cell passages at 33°C, the ability of recovered ts-22 (mou) virus to form plaques at 38.5°C was reduced.

Recovery of virus from infected mice. Mice were injected with 10⁶ plaque-forming units (PFU) (determined by 33°C titrations) of ts-22 (mou); after 4 days all mice were alive, but could be divided into two groups. “Affected” mice exhibited hind leg muscle paralysis, lethargy, hyperventilation, or other signs of being in extremis. The “normal” mice were indistinguishable from uninoculated control mice. The affected mice were separated from the normals, and all mice were recaged, 10 infants per nursing mother, and observed 3 days later for survivors. Of the normal mice, 110/120 (91.6%) survived, whereas only 56/130 (43.1%) of the affected mice survived. The amount of virus recovered 4 days postinoculation from homogenized muscle tissue of both normal and affected living mice indicated that mutant virus replicated in both groups to a similar extent. Virus that was recovered 4 days postinoculation from affected mice was injected undiluted into additional mice for subsequent passaging. Recovered virus was assayed at 33 and 38.5°C in BK cultures and in mice after each of six serial passages (Fig. 2). After two passages of ts-22 (mou) or ts-24 (mou) in mice, the recovered virus was pathogenic for mice (mou+), but unable to produce plaques at 38.5°C (ts). Relative virus titers did not change in the wt or ts-42 (mou+) populations after each passage.

Mice injected with stock wt or ts-42 (mou+) virus generally died after 3 to 7 days. The viruses appeared to kill mice faster after passages through mice. In efforts to examine this observation further, mice were injected with stock viruses and viruses recovered after three mouse passages (10⁶ PFU of mou virus or 100 mean lethal doses [LD₅₀] of wt or mou+ virus). Mouse deaths recorded 48 h later (Table 1) indicated that the killing time had changed. The killing rate at 48 h increased dramatically after a single passage of either wt or ts-42 virus, after two mouse passages of ts-24 (mou+) virus, or three mouse passages of ts-22 (mou+) virus.

Cutoff temperature determinations. The stock viruses and viruses recovered after mouse passage 6 were grown under agar overlay at various temperatures. The resulting titers (Table 2) indicated that the mutant viruses selected after mouse passage exhibited an increase of 0.5°C in cutoff temperature. Mutant

![Fig. 1. Titers of FMDV wt and ts mutants obtained after passage in BK cell cultures at 33°C. Symbols: A, PFU per milliliter, 33°C BK agar overlay assay; ■, PFU per milliliter, 38.5°C agar overlay assay; O, LD₅₀ per milliliter, mouse assay.](http://iai.asm.org/)

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The multifunctional RNA polymerase factors identified in picornavirus replication (1, 2, 8), and the role of progeny RNA as messenger RNA in the same growth cycle (7) invoke alternative means for interpreting these data.

MacKenzie (17) suggested that most reversions in his FMD ts mutants were the result of a second spontaneous mutation within the cistron rather than true "back mutations," since ts+ revertants did not uniformly exhibit restoration of virulence. Should a single base substitution alter a multifunctional gene product involved with both viral replication at elevated temperatures and mouse lethality, then a new mutational event within the cistron could result in partial restoration of the wt function.

Alternatively, base substitutions or deletions in one cistron may affect the translation of the adjacent polycistronic information (polarity effect), resulting in more than one aberrant post-translational cleavage product. A second mutational event (e.g., frame-shift) might restore the normal translation of the cistron(s) governing mouse virulence without affecting the ts expression.

If independent loci are responsible for these two functions, then the genome could also revert to mou+ while retaining the ts characteristic. It has been shown that ts+ mou revertants were recovered from steers inoculated with ts-24 (mou) mutant virus (23), indicating that temperature sensitivity varies independently from mouse virulence.

Virulence, or the varying degrees of reduced virulence associated with mutant viruses, must be interpreted as a consequence of the complex interactions between the host genes and the altered physiological characteristics of these conditional-lethal mutants. The influence of the genetic "background" of the host animal must be considered in the evaluation of virulence since susceptibility to picornavirus infection is controlled by the host cell (18). Campbell (6) showed that the mouse strain used in these experiments may carry genes regulating the susceptibility and resistance to infection by FMD viruses. But viral genes are also involved. Wittmann and Ahl (25) identified host-specific virulence markers of attenuated FMD viruses and showed that, during the course of attenu-
tion, virulence was sequentially lost for cattle, swine, and then mice.

As was also shown previously (17), FMD virus ts mutants that are attenuated for infant mice retain the ts marker and multiply successfully in this host without causing clinical manifestations. No correlation has been observed between cutoff temperature and mouse virulence in a large sample of FMD ts mutants (17), in contrast to the results generally observed with such mutants. Virus recovery from affected mice favored the various selective pressures encouraging growth of viruses virulent for suckling mice. The changes in killing rate (Table 1) probably reflect both "enrichment" of viruses containing mouse pathogenicity loci and selection of variants able to multiply successfully at slightly elevated temperatures (Table 2).

The data presented here neither delineate the mechanism involved in the expression of these mutations or the production of revertants nor determine whether more than one mechanism can occur. What these data show is that: (i) temperature sensitivity and mouse avirulence do not covary during tissue culture passage; (ii) these markers do covary under specific selective pressures; and (iii) these new (revertant) populations remain stable. The possibility that pathogenicity for a particular host and temperature sensitivity are under the control of separate loci is important in attempting to isolate attenuated viruses suitable for live-virus vaccines.

ACKNOWLEDGMENTS

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LITERATURE CITED


Table 2. Growth of wt and ts mutant viruses under agar overlay at various temperatures

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Mouse passage 6

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NONCOVARIANCE OF $ts$ AND VIRULENCE MARKERS


