On the Mechanism of Neurotropism of Vesicular Stomatitis Virus in Newborn Hamsters. Studies with Temperature-sensitive Mutants

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SUMMARY

The virulence of temperature-sensitive mutants of vesicular stomatitis virus (VSV) injected subcutaneously into newborn hamsters was positively correlated with their tendency to generate revertants and with their leakiness in cultured hamster embryo fibroblasts maintained at 37 °C, the measured body temperature of the animals under our experimental conditions. The complementation group of the mutants seemed important only in that it tended to determine reversion frequency and leakiness. One non-reverting group I mutant (T1026), however, was much less virulent than would be expected from its extreme leakiness at body temperature.

The disease produced by the less virulent mutants was characterized by neurological symptoms and led to delayed death, unlike the rapid death produced by virulent mutants. Infectious virus could be found in higher titres in the brains than in peripheral organs of such animals (with ratios as high as 10⁸). This neurotropism was not correlated with the complementation group of the mutant but was shown to be the consequence of survival for more than 3 days after injection. Age was not responsible for the effect. Animals injected at birth with T1026 were completely resistant to subcutaneous superinfection with the highly virulent wild-type virus HR at 3 to 4 days, though non-T1026-protected animals were completely sensitive. When HR was injected intracerebrally at 3 to 4 days, the T1026-protected animals allowed replication to high titres in the brain but not in peripheral organs, whereas non-T1026-protected animals allowed replication to high titres in both brain and in peripheral organs.

We suggest from these results that the observed neurotropism is produced by a resistance mechanism operative in peripheral organs but not in the brain; this resistance develops rapidly in newborn animals on exposure to virus and clears virus from the peripheral organs leaving it in the brain. It is possible that our effect represents a controlled and accelerated induction of the classical peripheral resistance of animals to various viruses which normally develops with age.

INTRODUCTION

Temperature-sensitive (ts) mutants of animal viruses have proven useful in the delineation of genetic and biochemical events involved in virus development in vitro. The use of such mutants to look for correlations between viral molecular development and disease in vivo...
has obvious appeal, and several groups have recently used this approach (Clark & Koprowski, 1971; Fields, 1972; Stanners, Farmilo & Goldberg, 1975). Though no easily interpretable correlations have emerged from such studies, in some cases the diseases produced by the mutants were different from those produced by the wild type virus, thus creating possible model systems for human diseases of unknown aetiology.

Our previous work has concerned a ts mutant of VSV known as T1026. Wild type VSV is normally highly cytocidal. The mutant, however, under semi-permissive conditions (38.5 °C), allows cellular DNA synthesis and division in cells which synthesize viral RNA and which display the viral glycoprotein on their surface (Farmilo & Stanners, 1972; Stanners et al. 1975). When injected subcutaneously into newborn hamsters, the parental VSV caused rapid death with virus disseminated throughout the body. T1026, on the other hand, caused a delayed death preceded by distinct neurological symptoms, and virus could be recovered only from the brain (Stanners et al. 1975). The mechanism of this neurotropism is the subject of this report.

We have explored the role of the viral genotype in virulence and neurotropism by the use of a number of VSV mutants having ts lesions in each of the five known complementation groups (Pringle, 1970; Wong, Holloway & Cormack, 1972; Flamand, 1970). The effects of age and of body temperature have also been studied. The findings point to the development of an unusual resistance mechanism, operative in all organs of the body except the brain, which depends upon exposure to the virus. The relationship of these findings to the classical observation of increasing resistance of animals with increasing age to peripheral infection with viruses, with retention of sensitivity to intracerebral infection (Sabin & Olitsky, 1938; Johnson, 1964; Falke & Rowe, 1965), is discussed.

METHODS

Animal procedures

Source. Outbred Syrian hamsters designated HOR-F1 were obtained from High Oak Ranch Ltd, RR no. 1, Goodwood, Ontario, as pregnant females, 2 or 9 days before term. The animals were maintained at 21 ± 1 °C in plastic cages, with sawdust and absorbent cotton for nesting.

Virus infection. To minimize cannibalism, newborn animals were handled only with rubber gloves. Subcutaneous injections high on the back and intracerebral injections through the top of the skull were carried out using a Hamilton Repeating Dispenser, model PB600-1 modified to accept a standard disposable 1 ml tuberculin plastic syringe with a 27 gauge needle. The syringe delivered 0.020 ml/shot. The mortality due to the procedures alone was tested using virus-free medium and was found to be negligible. After injection with virus, animals were observed and handled in a vertical laminar flow tissue culture hood. Cage bedding was heated to 100 °C before disposal.

Organ preparation. At various times after injection, animals were removed from their litters, and killed by decapitation. Various organs were removed using freshly sterilized instruments for each organ, and were frozen at −70 °C. In preparation for virus assay, the organs were thawed and homogenized in 2·0 ml z-MEM, by pipetting (brain) or by the use of a loose fitting Dounce glass homogenizer (other organs).

Measurement of temperature. The body temperature of young hamsters was measured using a tiny (1 mm diam.) thermistor bead (51A2-2W6, Victory Engineering Corp., Springfield, N.J.) with a resistance of about 100,000 ohms at 25 °C connected to a standard digital multimeter (Systron-Donner, Model 7050) adjusted to read resistance. The precise relation-
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ship between resistance and temperature for the thermistor was determined by immersion in water at temperatures defined by a calibrated mercury thermometer. Oral temperatures were measured by insertion of the thermistor bead into the side of the mouth towards the cheek pouch. The measurement was completed within 5 to 15 s after removal of the hamster from the litter. Rectal and intraperitoneal (measured by insertion of the thermistor through a small incision in the abdomen) temperatures were usually the same as or lower than the oral temperature.

Tissue culture procedures. Hamster embryo fibroblasts (HA) obtained from 13-day-old embryos were grown as monolayer cultures in glass bottles (Brockway Glass Co.) at 37 °C and were used in the 3rd to 5th subculture after explantation. The growth medium consisted of α-MEM (Stanners, Eliceiri & Green, 1971) containing asparagine at 50 μg/ml and supplemented with 10% foetal calf serum (FCS) (Flow Laboratories, Rockville, Md).

Virus procedures

Source. All virus strains and derived temperature-sensitive (ts) mutants originated from the Indiana strain of VSV. The ts mutants were obtained from four sources: those prefixed with ‘G’ for Glasgow were provided by Dr C. R. Pringle (Pringle, 1970), those prefixed with ‘W’ for Winnipeg were provided by Dr D. Cormack and Dr A. F. Holloway (Wong et al., 1972), those prefixed with ‘O’ for Orsay were provided by Dr N. Genty (Flamand, 1970), and those prefixed with ‘T’ for Toronto were provided by Dr T. Nakai and Dr A. F. Howatson. \( \text{ts} \) was the parental wild-type (WT) strain of the G mutants, while HR is a heat-resistant variant of VSV (Schincariol & Howatson, 1970) and was the parental strain of the T mutants.

Lysates. A lysate of each strain or mutant was prepared by infection of exponentially growing HA cultures of 2 to 3 × 10⁶ cells with 10⁴ to 10⁵ p.f.u. of virus picked from a single plaque. The infected cultures were incubated at 29 °C for 1 to 2 days using α-MEM plus 2% FCS. The supernatant medium was then removed and subjected to light centrifuging to remove cell debris. Samples of the supernatant were frozen at −70 °C. A sample of each lysate was then thawed to determine the plaque titre at 29 °C for ts virus and at 38.5 °C for ts revertant and WT virus. The frequency of revertants (p.f.u. 38.5 °C/p.f.u. 29 °C) in lysates of the ts mutants varied from <10⁻⁸ to about 10⁻². In experiments where ts virus was injected into hamsters or tested for leakiness in vitro (Fig. 1), the inoculum was sufficiently small to exclude the presence of revertants.

This procedure for preparation of lysates was designed to minimize the proportion of defective T particles to infectious B particles produced. By sucrose density gradient analysis of [¹⁴C]-valine labelled lysates, the ratio of T particles to B particles was less than 0.05 for HR and less than 0.01 for T1026 (the limit of sensitivity of the assay). Lysates of the other mutants were not checked for T particles.

Plaque assay. Infectious virus was measured by plaque assay using HA cells and a methyl cellulose-containing overlay as described previously (Farmilo & Stanners, 1972). In some experiments the procedure was miniaturized by the use of 24-well 16 mm Linbro plastic tissue culture trays in place of 60 mm Petri dishes.

The type of virus in various organ or culture preparations was assessed from the titre and size of plaques obtained after incubation of the dishes at 38.5 °C, in comparison with replicate dishes incubated at 29 °C. The virus type is denoted ‘ts’ for temperature sensitive, ‘r’ for revertants producing plaques smaller than WT, and ‘R’ for full-sized revertants. In Fig. 1 and Table 3, where more than one type of virus was produced, the majority type is listed first. Only virus types present at a level of >1% of the majority type are listed.
Complementation assay. Exponential cultures of about $2.5 \times 10^6$ hamster embryo fibroblasts in 4 oz bottles were infected at 39 °C at an input multiplicity of 2 p.f.u./cell with ts mutants singly and in pairs. Temperature was controlled by total immersion of culture bottles in a water bath at 39 °C kept in a warm room at 38.5 °C. After 45 min for virus adsorption, the cultures were washed twice, 10 ml of α-MEM + 2% FCS was added to the cultures and incubation was continued for a further 7 h. At this time the cultures were cooled rapidly to −20 °C, thawed, and the medium was assayed for infectious virus at 29 °C and at 38.5 °C by the plaque assay. Complementation indices were calculated by the method of Pringle (1970), and represent the yield of virus from double infection assayed at 29 °C minus the yield assayed at 38.5 °C (revertants) divided by the sum of yields from single infection assayed at 29 °C.

RESULTS

Mutant characterization

To interpret the results obtained on infection of animals with the various ts mutants of VSV, it was first necessary to characterize the mutants for their complementation group and for their tendency to leak or produce revertants at various temperatures.

With regard to genotype, most of the mutants had already been placed in one of five complementation groups of VSV by other workers. We considered it necessary to determine the complementation group of T1025 and T1026, as the latter mutant precipitated this study, and to confirm the complementation groups of the other mutants using our cell system. To this end, cultures were infected with the mutants, two at a time, and incubated at the non-permissive temperature of 39 °C. Complementation indices, which represent the yield of infectious particles for double infection divided by the sum of the yields for single infection (with corrections for revertants), are shown in Tables 1 and 2. The results confirm in our cell system the previous assignments of the mutants, and show clearly that T1025 and T1026 belong in complementation group I. T1026 showed complementation indices of 85 to 1800 with mutants of other complementation groups, and indices of 0.25 up to 6.9 with 8 other group I mutants. We feel that, in view of the very low yield obtained, the index of 6.9 obtained between T1026 and G12 is not a significant indication of complementation. From work done on the molecular characterization of other group I mutants (Szilágyi & Pringle, 1972), it is thus likely that T1026 has a temperature sensitive virion RNA-dependent RNA polymerase. The significance of this finding will be considered later.

To measure the leakiness and reversion of the mutants versus temperature, HA cultures were infected with each of the mutants at low multiplicities, then incubated for 48 h at temperatures ranging from 29 to 39 °C. The titre and type (ts, R: revertant, or r: partial revertant) of the virus produced was then determined by the plaque assay. The results, presented as the ratio of total infectious virus produced at any temperature to that produced at 29 °C (with the virus type in brackets), are shown in Fig. 1. The outcome of an infection at any given temperature is evidently a balance between the number of infectious particles produced due to leakiness of the mutation, the probability of reversion per infectious particle produced, and the selective advantage of any revertants generated over the original ts virus. It can be seen from Fig. 1 that mutants in groups II, III, IV and V all tend to generate revertants, whereas five out of the eight mutants in group I do not. This may be partially due to the specific failure of group I mutants to synthesize viral RNA with increasing temperature (Szilágyi & Pringle, 1972), thus reducing the pool of potentially revertable RNA molecules. T1026 appears to be unique in being a leaky, non-reverting group I mutant with a very high cut off temperature (39 °C).
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Table 1. Complementation indices for pairs of ts mutants*

<table>
<thead>
<tr>
<th>Mutant (group)</th>
<th>T1025 (I)</th>
<th>W4 (I)</th>
<th>G1 (I)</th>
<th>G22 (II)</th>
<th>G31 (III)</th>
<th>G41 (IV)</th>
<th>O45 (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1026 (I)</td>
<td>1.7</td>
<td>0.35</td>
<td>1.1</td>
<td>1800</td>
<td>900</td>
<td>85</td>
<td>1200</td>
</tr>
<tr>
<td></td>
<td>(8 x 10⁴)</td>
<td>(5 x 10⁴)</td>
<td>(3 x 10⁴)</td>
<td>(5 x 10⁶)</td>
<td>(3 x 10⁴)</td>
<td>(8 x 10⁴)</td>
<td></td>
</tr>
<tr>
<td>T1025 (I)</td>
<td>3.4</td>
<td>0.35</td>
<td>3.4</td>
<td>3900</td>
<td>1100</td>
<td>390</td>
<td>2300</td>
</tr>
<tr>
<td></td>
<td>(6 x 10⁵)</td>
<td>(1 x 10⁵)</td>
<td>(6 x 10⁵)</td>
<td>(6 x 10⁶)</td>
<td>(7 x 10⁵)</td>
<td>(8 x 10⁶)</td>
<td></td>
</tr>
<tr>
<td>W4 (I)</td>
<td>0.29</td>
<td>0.29</td>
<td>2.500</td>
<td>1400</td>
<td>1000</td>
<td>4300</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3 x 10⁵)</td>
<td>(5 x 10⁶)</td>
<td>(3 x 10⁶)</td>
<td>(1 x 10⁶)</td>
<td>(1 x 10⁶)</td>
<td>(1 x 10⁶)</td>
<td></td>
</tr>
<tr>
<td>G11 (I)</td>
<td>0.35</td>
<td>0.35</td>
<td>2.000</td>
<td>2000</td>
<td>2500</td>
<td>310</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3 x 10⁵)</td>
<td>(5 x 10⁶)</td>
<td>(3 x 10⁶)</td>
<td>(1 x 10⁶)</td>
<td>(1 x 10⁶)</td>
<td>(1 x 10⁶)</td>
<td></td>
</tr>
<tr>
<td>G22 (II)</td>
<td>0.64</td>
<td>0.64</td>
<td>0.56</td>
<td>0.56</td>
<td>0.64</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4 x 10⁴)</td>
<td>(1 x 10⁵)</td>
<td>(1 x 10⁵)</td>
<td>(1 x 10⁵)</td>
<td>(1 x 10⁵)</td>
<td>(1 x 10⁵)</td>
<td></td>
</tr>
</tbody>
</table>

* Complementation between pairs of ts mutants was measured as described in Methods. The numbers in brackets represent the yield of virus from double infection assayed at 29 °C in p.f.u./ml. Cultures infected with HR at 4 p.f.u./cell (the combined input multiplicity of the two ts mutants) under these conditions yielded 2 x 10⁷ p.f.u./ml.

† An input multiplicity of 5 p.f.u./cell was used for this pair.

Table 2. Complementation indices between T1026 and other group I mutants*

<table>
<thead>
<tr>
<th>Mutant (group)</th>
<th>O5 (I)</th>
<th>W11 (I)</th>
<th>G12 (I)</th>
<th>G114 (I)</th>
<th>G13 (I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1026 (I)</td>
<td>1.1</td>
<td>2.9</td>
<td>6.9</td>
<td>0.56</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>(4 x 10²)</td>
<td>(1 x 10⁵)</td>
<td>(1 x 10⁵)</td>
<td>(1 x 10⁵)</td>
<td>(1 x 10⁵)</td>
</tr>
</tbody>
</table>

* For explanation of entries and description of experimental procedures, see Methods and the legend of Table 1.

It will be shown later that the mean body temperature of hamsters from birth to age 4 days is 37 °C. This value is shown in Fig. 1 as vertical dotted lines. The effect of a mutant on newborn hamsters can be predicted from the results of Fig. 1 at 37 °C (see below).

Effect of mutants on survival

To determine the effect of the mutants in vivo, hamsters were injected subcutaneously within 24 h of birth with each mutant using low doses so as to minimize the probability of injection of revertants. The time of death or imminent death (animals were often sacrificed when their condition indicated death within a few hours) was recorded and the titre and type of infectious virus recovered from various organs was determined by the plaque assay. Survival curves for a WT strain, Its⁺, and three group I mutants, T1025, T1026 and G114 are presented in Fig. 2. It can be seen that, whereas the WT strain was extremely virulent, killing all of the hamsters within 2 days after injection, the three mutants varied widely in their virulence. T1025 killed 50% of the animals in 1.5 days, T1026 had little effect until about 5 days after injection, then killed 80% of the animals between 5 and 10 days, and G114 had little or no effect. These results can be predicted from Fig. 1. T1025 was virulent because it tends to generate full revertants. T1026 was virulent but much less so because it is extremely leaky at 37 °C, but does not generate revertants. The fact that the relative yield of
T1026 at 37 °C was the same as that of its WT parent, HR, yet its virulence was much less is somewhat anomalous, though consistent with recent observations that even at 37 °C the cytopathogenicity of T1026 is low (A. Kabal, M. Francoeur & C. P. Stanners, unpublished observations). G114 had very little effect because it cannot initiate productive infection at 37 °C and does not generate revertants. In the data of Fig. 2, a range of virus doses was used to eliminate differences between mutants which might arise at one particular dose. In point of fact dose had little effect, as in separate experiments where groups of animals were infected with single doses, survival was only slightly dependent on dose (see, for example, results for T1026 in Fig. 2).

A summary of such results for all of the mutants, including the in vitro results at 37 °C taken from Fig. 1, is given in Table 3. The mutants are listed there in order of their decreasing virulence. The correlations between virulence in vivo and revertant production and leakiness in vitro noted for the three group I mutants above hold well for this larger group of mutants, though there are some exceptions (e.g. T1025 vs O5). It is also clear that revertant production correlates more strongly with virulence than does leakiness. Comparing the type of virus recovered from infected animals with that recovered from infected cultures at 37 °C, it is also apparent that the animals tend to select more strongly for revertants.

These results indicate that, as far as virulence is concerned, the genotype of ts VSV
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Fig. 2. Survival of newborn hamsters injected subcutaneously with WT and three ts VSV mutants. For Its+, 30 animals were injected with $10^7$ to $10^8$ p.f.u./animal; for T1025, 30 animals with $10^6$ to $10^7$ p.f.u./animal; and for G114, 30 animals with $10^7$ to $10^8$ p.f.u./animal. For T1026, the results of four independent experiments three of which employed single but different doses ranging from $10^2$ to $10^6$ p.f.u./animal and involving approx. 20 animals each, are shown.

Table 3. *Virulence in vivo and in vitro of ts VSV mutants*

<table>
<thead>
<tr>
<th>Mutant group</th>
<th>Time to death of 50% of animals (days)</th>
<th>Type of virus recovered</th>
<th>Relative yield p.f.u. 37°C/p.f.u. 29°C</th>
<th>Type of virus recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (WT)</td>
<td>1</td>
<td>WT</td>
<td>$6 \times 10^8$</td>
<td>WT</td>
</tr>
<tr>
<td>Its+ (WT)</td>
<td>1</td>
<td>WT</td>
<td>$2 \times 10^{-1}$</td>
<td>WT</td>
</tr>
<tr>
<td>G22 (II)</td>
<td>1.5</td>
<td>R, r</td>
<td>$4 \times 10^{-1}$</td>
<td>R</td>
</tr>
<tr>
<td>O45 (V)</td>
<td>1.5</td>
<td>R, r</td>
<td>$10^{-3}$</td>
<td>r, R</td>
</tr>
<tr>
<td>T1025 (I)</td>
<td>1.5</td>
<td>R</td>
<td>$8 \times 10^{-4}$</td>
<td>ts, R</td>
</tr>
<tr>
<td>G41 (IV)</td>
<td>2</td>
<td>r</td>
<td>$&lt; 10^{-8}$ to $8 \times 10^{-1}$</td>
<td>r</td>
</tr>
<tr>
<td>G31 (III)</td>
<td>2.5</td>
<td>R, r, ts</td>
<td>$10^{-3}$</td>
<td>r</td>
</tr>
<tr>
<td>W4 (I)</td>
<td>2.5</td>
<td>R</td>
<td>$3 \times 10^{-1}$</td>
<td>ts, R, r</td>
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<tr>
<td>W11 (I)</td>
<td>1 to 4</td>
<td>R</td>
<td>$2 \times 10^{-5}$</td>
<td>ts</td>
</tr>
<tr>
<td>O5 (I)</td>
<td>4.5</td>
<td>r, R</td>
<td>$3 \times 10^{-2}$</td>
<td>ts, R</td>
</tr>
<tr>
<td>T1026 (I)</td>
<td>7.5</td>
<td>ts</td>
<td>$7 \times 10^{-2}$</td>
<td>ts</td>
</tr>
<tr>
<td>G12 (I)</td>
<td>5 to &gt;400</td>
<td>ts</td>
<td>$7 \times 10^{-6}$ to $10^{-4}$</td>
<td>ts</td>
</tr>
<tr>
<td>G11 (I)</td>
<td>&gt;400</td>
<td>--</td>
<td>$5 \times 10^{-6}$ to $10^{-6}$</td>
<td>ts</td>
</tr>
<tr>
<td>G13 (I)</td>
<td>&gt;400</td>
<td>--</td>
<td>$&lt; 10^{-8}$ to $10^{-6}$</td>
<td>ts</td>
</tr>
<tr>
<td>G114 (I)</td>
<td>&gt;400</td>
<td>--</td>
<td>$&lt; 10^{-8}$ to $10^{-8}$</td>
<td>--</td>
</tr>
</tbody>
</table>

* $10^9$ to $10^8$ p.f.u. of each of the mutants listed was injected subcutaneously into approx. 30 hamsters within 24 h after birth. The type of virus recovered from brain homogenates from dead or dying animals was determined by the plaque assay, as described in Methods. The *in vitro* data was obtained from Fig. 1.
Table 4. Neurotropism with T1026*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Age at injection (days)</th>
<th>Age at death (days)</th>
<th>p.f.u. in brain</th>
<th>p.f.u. in kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Its+</td>
<td>&lt;1</td>
<td>1</td>
<td>$2 \times 10^8$</td>
<td>$4 \times 10^8$</td>
</tr>
<tr>
<td>HR</td>
<td>3</td>
<td>4</td>
<td>$4 \times 10^7$</td>
<td>$&gt;2 \times 10^8$</td>
</tr>
<tr>
<td>HR</td>
<td>6</td>
<td>7</td>
<td>$4 \times 10^7$</td>
<td>$&gt;2 \times 10^8$</td>
</tr>
<tr>
<td>HR</td>
<td>6</td>
<td>8</td>
<td>$2 \times 10^7$</td>
<td>$4 \times 10^7$</td>
</tr>
<tr>
<td>T1026</td>
<td>&lt;1</td>
<td>7</td>
<td>$1 \times 10^9$</td>
<td>$&lt;50$</td>
</tr>
<tr>
<td>T1026</td>
<td>&lt;1</td>
<td>8</td>
<td>$4 \times 10^7$</td>
<td>$&lt;50$</td>
</tr>
<tr>
<td>T1026</td>
<td>&lt;1</td>
<td>8</td>
<td>$6 \times 10^7$</td>
<td>40</td>
</tr>
<tr>
<td>T1026</td>
<td>3</td>
<td>6</td>
<td>$2 \times 10^7$</td>
<td>$&lt;5$</td>
</tr>
</tbody>
</table>

* Hamsters were injected subcutaneously with $10$ to $10^3$ p.f.u. Its+ or HR, and $10^3$ to $10^6$ p.f.u. T1026. Shortly before or shortly after death, animals were removed and the total amount of infectious virus in brain and kidney was determined as described in Methods. The results shown are for individual animals representative of much larger groups and were relatively independent of the precise dose of virus injected.

mutants is important only to the extent to which it determines reversion and leakiness. T1026, however, seems anomalous in being less virulent than would be predicted from its extremely high leakiness at 37 °C.

Neurotropism with T1026

Though the precise cause of death induced by the various strains and mutants in the above studies was not ascertained, the symptoms observed with WT strains and mutant T1026 were clearly different. WT strains produced symptoms which were evident only just before death and were characterized by extreme lassitude and gasping. T1026 on the other hand produced symptoms, lasting for days and occasionally weeks, which were obviously neurological. They included hemiplegia, tremor, and selective paralysis of various limbs. A detailed histological study of such infected animals is in progress.

Various organs of dying infected animals were tested for the presence of virus by the plaque assay. The results for brain and kidney are presented in Table 4. Whereas WT strains of VSV were found in similar or higher titres in the kidney than in the brain, T1026 was found only in the brain, with little or no virus in the kidney. The extent of this rather striking neurotropism was greater than $10^7$ (p.f.u. brain/p.f.u. kidney) in some cases. (We do not imply by the use of the term 'neurotropism' that the virus concerned has a tendency to 'home' for the brain. The term refers to the situation where virus is found in higher titre in the brain than in other peripheral organs. As will be shown, this situation is the result of a host–virus interaction.) This observation is not simply a reflection of the known effect of age on inhibiting peripheral versus cerebral development of viruses (Sabin & Olitsky, 1938; Johnson, 1964), as 6-day-old animals injected with WT virus also showed similar or higher titres of virus in kidney than in brain (Table 4).

Other organs, including liver, heart, lung and testis did not harbour infectious virus in ‘neurotropic’ animals injected with T1026, though these organs did contain infectious virus in ‘non-neurotropic’ animals infected with WT virus (data not shown).

Neurotropism vs mutant

To look for correlations between neurotropism and mutant complementation group, leakiness or reversion, each of the mutants was injected subcutaneously into newborn hamsters. Brains and kidneys of dead or dying animals were then tested for infectious virus by the plaque assay. A summary of these results is shown in Fig. 3 where 'neurotropism',
Fig. 3. Neurotropism in newborn hamsters versus VSV mutant and time of death. Hamsters were injected subcutaneously with doses of different mutants of VSV ranging from $10^3$ to $10^6$ p.f.u./animal within 24 h after birth (a few animals were injected 3 days after birth). The ratio of p.f.u. in the brain to p.f.u. in the kidney is presented for individual dead or dying animals. The dashed line represents the approximate logarithmic average.

expressed as the ratio of p.f.u. in brain to p.f.u. in kidney, is plotted against time between injection and sacrifice. Each point represents one animal injected with the indicated mutant. Where no virus could be recovered from the kidney the point is surrounded with a box and represents the actual brain titre. These results are for selected animals which were dead or dying at the indicated times and are not necessarily representative of all animals still alive at those times. Thus we cannot use the data of Fig. 3 to predict the development of virus in animals from injection to death.

The results indicate that any mutant, regardless of the genotype, leakiness or tendency to revert can show neurotropism if it allows survival of the animal for more than approx. 3 days after injection. Thus WT strains (HR and It+) rarely show neurotropism because they kill all animals before 2 days after injection while T1026 nearly always shows neurotropism because it allows almost full survival until 4 to 5 days after injection. It should be noted that the occasional T1026 injected animal which died at 1 or 2 days after injection showed approx. the same titre of virus in kidney as in brain.
Fig. 4. Decline in oral temperature (T) of newborn and 3-day-old hamsters under three different conditions. 'Dead alone' refers to a newborn animal removed from the litter and killed by severing the spinal cord. 'Alive alone' refers to animals removed from the litter, and 'alive in litter' refers to animals left in a litter and selected randomly for measurement after removal of the mother. Time zero was the time of the initial temperature (T0) measurement. The final temperature (Tf) was the average ambient air temperature and was usually about 21 °C.

The role of body temperature in neurotropism

It was conceivable that, if the mean body temperature of hamsters increased during the first days of life as has been reported for mice (Teisner & Haake, 1974), the effect on replication of ts virus could be greater in visceral organs than in the brain, thus giving the observed delayed neurotropism. Such a situation might arise if, for example, a possibly higher protein synthesizing capacity of neurons allowed replication of viral ts mutants at higher temperatures. This hypothesis was tested by measuring the body temperature of newborn and 3- to 4-day-old hamsters.

If newborn hamsters are removed from their nest, their body temperature immediately declines. Similarly, removal of the mother from the nest results in a decline, though less rapid, in body temperature of all of the young hamsters in the nest. To estimate the body temperature under normal nesting conditions, the body temperature of littermates removed at random was measured as a function of time after removal of the mother. Plots of temperature versus time were then constructed and extrapolated to zero time to give the average body temperature of the litter at the time of removal of the mother. The data (not shown) indicated an average daytime litter temperature of 37 °C for newborn and 36.8 °C for 3-day-old hamsters with a range of ±1 °C in both cases. Thus there is no increase in the body temperature of hamsters from birth to age 3 days.
Neurotropism with VSV

If 3- to 4-day-old hamsters are less poikilothermic than newborns, one might expect that their temperature would be better maintained during brief normal departures of the mother from the nest. The rate of decline in body temperature of animals killed immediately after removal from the nest, of alive animals removed from the nest, and of alive animals left in the litter after removal of the mother is shown in Fig. 4. The data is presented so as to eliminate the effects of initial litter temperature and room temperature; a warm body which cools according to Newton’s law of cooling should give a straight line when analysed in this way. It can be seen that dead animals do in fact give a straight line, and that alive animals deviate from this behaviour, cooling less rapidly, while alive animals in the litter cool even less rapidly. The essential result, however, is that the data for newborn and 3- to 4-day-old animals, whether in the nest or not, is superimposable.

It is, therefore, apparent that changes in body temperature with age cannot account for the observed delayed neurotropism. It is also apparent that the body temperature of newborn hamsters can drop very quickly (in about 5 min) into the permissive range of the mutants if the mother leaves the nest. This could account for some litter-specific variation in the virulence of certain $ts$ mutants which has been observed in our virulence studies.

A resistance mechanism dependent on prior infection

As an alternative hypothesis to the above, we suggest that, after subcutaneous injection, the virus replicates first at the site of injection, then spreads to other body organs including kidney and brain; within 3 days after infection, a resistance mechanism is stimulated which clears the virus from the peripheral organs leaving it in the brain. The postulated resistance mechanism would depend on prior exposure to virus and would not operate in the brain.

To test this hypothesis, animals were injected subcutaneously with T1026 at birth, then superinfected subcutaneously with WT strain HR, 3 or 4 days later. Their survival was recorded, and virus production in brain and kidney in dead or dying animals was determined by the plaque assay. T1026 and HR production could be distinguished easily using the plaque assay at 38.5 °C since T1026 does not produce revertants in vivo (see Fig. 1 and Table 3). The results are shown in Fig. 5 and in Table 5. It can be seen from Fig. 5 that pre-infection with T1026 afforded essentially complete protection from the rapid death produced by subcutaneous injection of WT strain HR at 3 days of age. The HR superinfected animals died at the same time as those infected with T1026 alone. The virus recovered (Table 5) was largely due to T1026 and was found in the brain only, though the occasional animal showed small amounts of HR virus in the brain along with large amounts of T1026. If the superinfecting dose of HR was increased by a factor of 100 to $5 \times 10^5$ p.f.u./animal, HR virus could be recovered in both brain and kidney at high titres in approx. half of the animals (Table 5). These results are consistent with the resistance hypothesis but require that the resistance mechanism can be ‘swamped’ by a large amount of superinfecting virus.

More convincing evidence for the hypothesis would be a demonstration that superinfecting WT virus can replicate in brain in pre-infected animals which are resistant to replication in peripheral organs. To this end newborn animals injected or not injected at birth with T1026 were superinfected at 3 or 4 days of age with WT strain HR by the intracerebral route. The results are shown in Table 5 and Fig. 5 and 6. Regarding survival, all animals died within 24 h when injected intracerebrally with HR alone (Fig. 5). Prior injection with T1026 at birth afforded some protection, but less than was observed for subcutaneous superinfection with HR. Animals receiving intracerebral injection of HR alone showed replication of virus to extremely high titres in both brain and kidney, indicating that virus derived from intracerebral injection could easily enter the kidney (Table 5). Animals
Fig. 5. Survival of hamsters injected or not injected subcutaneously at birth with T1026 followed by (a) subcutaneous or (b) intracerebral infection with HR at 3 days or 4 days of age, respectively. For the subcutaneous experiment, animals were injected with $2 \times 10^4$ p.f.u./animal for both T1026 and HR. For the intracerebral experiment, animals were injected with $3 \times 10^8$ p.f.u. T1026 and $5 \times 10^8$ p.f.u. HR. Approx. 30 animals were used for each category. $\times--\times$, T1026 alone; $\bigcirc--\bigcirc$, T1026 plus HR; $+--+$, HR alone.

Table 5. Virus development in doubly infected animals*

<table>
<thead>
<tr>
<th>T1026 injected p.f.u./animal</th>
<th>HR injected p.f.u./animal</th>
<th>p.f.u. recovered brain</th>
<th>p.f.u. recovered kidney</th>
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<td></td>
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<td>ts</td>
</tr>
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<td>$&lt;5$</td>
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<td>$&lt;50$</td>
</tr>
</tbody>
</table>

* Hamsters were injected or were not injected subcutaneously with the indicated doses of T1026 within 24 h of birth. Three to four days later the animals were injected subcutaneously (SC) or intracerebrally (IC) with the indicated doses of WT strain HR. The total amount and type of virus recovered from organs of dead or dying animals is shown. Animals injected with HR were taken 1 and 2 days after injection; those injected with T1026 and HR were taken 1, 2, 3 and 4 days after injection of HR. The data shown are for individual animals representative of a much larger number involved in several independent experiments, and indicate the range of results obtained.

† Not detectable, due to the presence of large numbers of WT plaques.
Fig. 6. The distribution of neurotropism among animals injected intracerebrally with WT strain HR at 3 to 4 days of age, or subcutaneously with Tio26 at birth and intracerebrally with HR at 3 to 4 days of age. ‘Neurotropism’ is here defined as the ratio of WT p.f.u. in the brain to WT p.f.u. in the kidney. Tio26 was injected at $3 \times 10^3$, $5 \times 10^5$ and $2 \times 10^6$ p.f.u./animal, and HR at $5 \times 10^3$ and $2 \times 10^4$ p.f.u./animal. Results at the different doses were similar. In some cases, no virus could be detected in the kidney. In these cases, the minimum ratio, using the sensitivity of the assay as the maximum possible kidney titre, is plotted. This figure represents a summary of data from three separate experiments.

injected with Tio26 at birth followed by intracerebral superinfection with HR, however, showed high titre replication of HR in the brain and little or no replication of HR in the kidney (Table 5). The magnitude of this selectivity is shown in Fig. 6 where it can be seen that, in three separate experiments, the ratio of p.f.u. recovered from the brain to p.f.u. recovered from the kidney varied from $10^3$ to $10^8$ in 17 out of 18 animals tested.

**DISCUSSION**

We have shown that the virulence of *ts* VSV mutants for newborn hamsters correlates well with their tendency to generate revertants and to a lesser extent with their leakiness both *in vitro* and *in vivo*. The complementation group of the mutants seems relevant only in that it can determine reversion and leakiness except, perhaps, for the group I mutants (see below). A relatively high proportion of the group I mutants studied did not generate revertants, unlike various representatives of all other complementation groups. This could be due to the fact that the temperature-sensitive block occurs very early in the growth cycle of these viruses thus preventing a build-up of potentially reversible RNA molecules.

Tio26, the mutant which precipitated this study, was shown to be a non-reverting group I mutant with a very high cut-off temperature. At 37 °C, the average body temperature of newborn hamsters, Tio26 was shown to be the most leaky and also the most virulent of the non-reverting group I mutants. The yield of Tio26 at 37 °C *in vitro*, however, was actually as high as that of its WT parental strain, HR, though it was much less virulent. It is, therefore, possible that the defect of group I mutants, presumably the virion RNA-dependent
RNA polymerase (Szilágyi & Pringle, 1972), results in a cell-virus interaction which is less
cytocidal even under virtually permissive conditions. This would be consistent with our
previous finding that T1o26 at the semi-permissive temperature of 38.5 °C is much less
cytocidal than HR (Farmilo & Stunners, 1972), and only partially consistent with the sug-
gestion of Marcus & Sekellick (1975) that the virion polymerase, viral transcription and an
event dependent on transcription are all involved in cell killing. Our suggestion obviously
requires confirmation with other non-reverting, highly leaky group I mutants, however.

The preliminary finding that T1o26, unlike WT-HR, was highly neurotropic in newborn
hamsters (Stunners et al. 1975) was confirmed in this work. This observation is consistent
with the fact that T1o26 causes a delayed death characterized by neurological symptoms.
Neurotropism, however, was shown not to be a peculiar feature of T1o26 or of group I
mutants, but to be the inevitable consequence of prolonged survival after injection. Thus,
any mutant which in any particular animal allowed survival for 3 days or more after injec-
tion was recovered in much higher titre in the brain than in other body organs. The ratio of
p.f.u. recovered from the brain to that recovered from the kidney could be as high as 10^8.
The key attributes of a mutant in establishing a neurotropic state are thus reduced virulence
coupled with continued viral replication. T1o26 possesses these attributes to a greater extent
than any of the other mutants, and was more consistently neurotropic (see Fig. 3). It is
possible that the presence of defective interfering T particles in a viral inoculum could also
enhance neurotropism by reducing virulence. Doyle & Holland (1973) observed reduced
virulence of mixtures of infectious and defective T particles in intracerebral injection of mice.
It is unlikely that T particles represent an important factor in our experiments, however, as
our method of preparing viral lysates minimizes their production, and T1o26 in particular
does not appear to produce T particles, at least as detected by electron microscopy or by
velocity sedimentation analysis of labelled virus preparations (A. J. Farmilo & C. P.
Stunners, unpublished observations).

The observed neurotropism was not simply a consequence of age, as WT strains were not
neurotropic even when injected into animals up to 6 days of age. We suggest that a resistance
mechanism, dependent upon prior exposure to the virus, develops rapidly within the animals
and clears the virus out of all peripheral organs leaving it to develop in the brain. The
postulated resistance mechanism would, of course, not operate in the brain. Consistent with
this hypothesis was our observation that prior infection with T1o26 rendered hamsters
completely resistant to subcutaneous superinfection with HR 3 to 4 days later while non-
T1o26-infected animals were fully sensitive. Also when such T1o26 pre-infected animals
were superinfected with HR by the intracerebral route, the latter virus replicated to very
high titres in the brain but was almost totally excluded from the kidney, though intra-
cerebral infection of non-pre-infected animals with HR led to equally high titres in both
brain and kidney.

What is the nature of this resistance mechanism? Preliminary experiments exclude
interferon which in T1o26 injected 'neurotropic' animals, was detectable in low titre only
in the brain, the opposite of what would be expected. A normal antibody response is also
unlikely, since the resistance develops in newborns and develops within 3 days. In pre-
liminary tests, no soluble factors in brain or kidney were found which could inactivate the
infectivity of VSV. Also, preliminary experiments indicate that resistance produced by
injection of T1o26 (Indiana serotype) can also render the animals resistant to subcutaneous
infection with a serologically unrelated strain of VSV (New Jersey serotype). Experiments
are in progress to confirm these observations and to determine the extent of cross resis-
tance to other viruses. The relationship of our findings to the recent report (Wagner, 1974) of
successful vaccination of 3-week-old mice with ts mutants of VSV is unknown at present. In that case resistance also appeared extremely soon after injection.

The classical observation that with increasing age animals become resistant to significant viral replication from peripheral injection of various viruses while retaining sensitivity to intracerebral injection (Sabin & Olitsky, 1938; Johnson, 1964; Falke & Rowe, 1965) may be relevant to these observations and may provide some clues. It is possible that we are observing here a controlled and accelerated induction of the same resistance mechanism which normally develops with age. Sabin & Olitzky (1938) originally proposed the development of physical barriers for VSV between peripheral nerves and the brain. Johnson (1964), working with herpes virus, showed that no such anatomical barriers existed but that infected peritoneal macrophages in older animals, unlike those in young animals, did not spread the virus to other cells. Hirsch, Zisman & Allison (1970) have also demonstrated a more effective clearance of herpes virus by peritoneal macrophages from older animals. This model would require that such macrophages be absent in significant numbers from the brain. If our suggested peripheral resistance mechanism is similar to that involved in the age-related resistance, then our evidence also excludes physical barriers and indicates that the mechanism can be primed by prior exposure to virus. We are now studying the properties of peritoneal macrophages from primed and pristine animals.

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