Pathogenicity and Immunogenicity for Mice of Temperature-Sensitive Mutants of Vesicular Stomatitis Virus

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Received for publication 11 March 1974

Temperature-sensitive (ts) mutants of vesicular stomatitis (VS) virus were tested for their pathogenicity and immunogenicity in weanling mice. Compared with the wild-type virus (ts+), ts mutants representing genetic complementation groups I, II, and IV were considerably less pathogenic for mice infected by the intracerebral route and caused few deaths after intranasal inoculation. Mice were completely resistant to ts+ and ts mutants by the intraperitoneal route. Resistance to intracerebral challenge with ts+ VS virus was only minimal in mice vaccinated intraperitoneally with ts+ or ts mutants and only moderate in mice vaccinated intranasally with three ts mutants. Intranasal vaccination, particularly with group IV mutants, resulted in solid immunity within 3 days to intranasal challenge with ts+ virus. VS viral neutralizing antibody was present in the bronchial secretions of mice by 12 h after intranasal inoculation of mutant ts IV44; the bronchial antibody titers declined to undetectable levels between 3 and 7 days after vaccination. Neutralizing antibody was detected in the serum of mice by the third day after intranasal vaccination with ts IV44 and persisted at high level for at least 11 days. Certain classes of ts mutants would appear to be promising candidates for use as attenuated, live virus vaccines.

An important approach to the problem of development of viral vaccines is the use of live, attenuated viruses capable of synthesizing proteins to achieve high antigenic mass but restricted in viral replication to the point of producing no disease manifestations. Familiar examples of host range mutants of proven prophylactic effectiveness are attenuated viruses of smallpox, yellow fever, and poliomyelitis, but little knowledge is available about the genetic and biochemical characteristics of these vaccine viruses, with the possible exception of poliovirus (7). Recent studies have opened up avenues of research in the genetics and related biochemistry of, among others, togaviruses (1), reoviruses (6), and rhabdoviruses (16), but the possibility of using conditional lethal mutants as vaccines has barely been explored. One particular class of conditional lethal mutants, the temperaturesensitive (ts) mutants, would seem to have potential value as attenuated vaccines because their growth should be restricted at the body temperature of mammalian hosts.

This report presents preliminary evidence for the immunization potential of ts mutants of vesicular stomatitis (VS) virus (8, 16). Many of these mutants grow quite normally and to high titer at the permissive temperature of 31 C, but their reproduction is almost completely restricted at 39 or 37 C (15). Moreover, reversion frequencies of ts VS viral mutants are relatively low for some and extremely low for others. All ts mutants of Indiana serotype VS virus studied to date fall into five genetic complementation groups (8). Mutants of at least two of these groups, I and IV, have a phenotype which results in deficient synthesis of ribonucleic acid (RNA-) at nonpermissive temperatures. Although agreement is not universal, ts mutants of group I appear to be defective in transcription (15, 24) at the level of virion transcriptase (11) and, therefore, are presumably defective in protein synthesis (17). On the other hand, ts mutants of complementation group IV are probably restricted at the level of ribonucleic acid replication (2a) and should, therefore, be capable of synthesizing proteins at restrictive temperature (30). With the possible exception of group II, VS virus ts mutants of complementation groups II, III, and V do not exhibit significant defects in ribonucleic acid and protein synthesis at restrictive temperature (17, 30).

Representative ts mutants of groups I, II, and IV were chosen for comparison of their vaccine
potential. It was postulated that the transcriptase-defective group I mutants would serve as inferior vaccines compared with the presumably competent transcription-translation mutants of groups II and IV. This hypothesis turned out to be at least partially incorrect, probably because of the leakiness of the mutants.

**MATERIALS AND METHODS**

**Viruses.** The Indiana serotype of VS virus was used throughout. The wild-type (ts +) was the Charlotte-ville strain. Mutants ts I5 and ts II52 were isolated in Orsay (8) and were kindly supplied by P. Printz; mutants ts I11, ts IV41, and ts IV44 were isolated in Glasgow and were kindly supplied by C. R. Pringle (16). All mutants were derived from clones directly picked from purified plaques and grown on L cells at 31 C. The ts + virus was grown at 37 C on L cells. All stocks were free of defective T virions. Titration assays were performed by plaque assay on monolayers of L cells incubated at 31 C (permissive) or 39 C (restrictive), and the results were recorded as plaque-forming units (PFU) of virus. Media, infection of cells, growth of virus, and storage have been described previously (17).

**Mice.** Male Swiss mice (Webster strain) 3 to 4 weeks of age and weighing 10 to 12 g were used in all experiments. Intracerebral inoculation was performed by injecting 0.03 ml through a 25-gauge needle inserted under light ether anesthesia. Intranasal infections were performed using light ether anesthesia by placing 0.05 ml of the inoculum on the external nares; proper anesthesia resulted in rapid and complete inhalation of the inoculum. All virus suspensions for inoculation were diluted in nutrient broth containing 100 U of penicillin per ml and 5 mg of streptomycin per ml (28).

**RESULTS**

Comparative lethality of ts + and ts mutants of VS virus inoculated by different routes. Sabin and Olitsky (19) and Miyoshi et al. (14) have described in considerable detail the neuropathology, virus growth and dissemination, and comparative susceptibility of mice to encephalitis after intracerebral and intranasal inoculation of VS virus. The present experiments compare the susceptibility of weanling mice to infection with wild-type (ts+) and three ts mutants of VS virus. Groups of 7 to 10 mice were injected with 10-fold dilutions of each virus preparation by the intracerebral, intraperitoneal, or intranasal route. The 50% lethal dose (LD 50) was determined by the Reed-Muench method.

Table 1 compares the amounts of virus required to cause death from infection with ts + or each ts mutant by each route of inoculation. As noted, all virus preparations were most virulent by the intracerebral route, whereas no mice died from intraperitoneal injection of ts + or the mutants at doses of 10 7 PFU or greater. Intermediate susceptibility was noted by the intranasal route; about 10 times as much ts + virus was required to produce fatal infection by the intranasal as compared to the intracerebral route. Only mutants ts I5 and ts II52 caused any deaths by the intranasal route. Among the three ts mutants tested, ts IV41 was completely avirulent even by intracerebral infection, whereas ts I5 and ts II52 required >10,000 times more infectious virus to induce encephalitis and death by the intracerebral route than did ts + virus. Manifestations of infection with either ts + or ts mutants were characteristic of encephalitis as evidenced by paralysis and convulsions.

Differences in lethality after intracerebral infection with ts + and ts mutants were also reflected in the times of death. Onset of paralysis and death began 2 days after infection with ts + virus, and almost all mice were dead by the third or fourth day after intracerebral infection with 50 to 500 PFU. In contrast, mice that did not survive infection with 10 5 or 10 6 PFU of mutants ts I5 or ts II52 died after 5 to 8 days; only an occasional mouse succumbed later to infection with 10 7 PFU of ts IV41. Mice 2 weeks older (16 to 20 g) were more resistant to infection by intracerebral and intranasal infection with ts + and ts mutants; 10 to 50 times more virus was required to produce equivalent mortality among older mice.

**Table 1. Lethality of VS virus wild-type (ts +) and ts mutants for weanling mice infected by different routes of inoculation**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titer on L cells (PFU/ml)</th>
<th>LD 50 (PFU/mouse)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>31 C</td>
<td>39 C</td>
</tr>
<tr>
<td>ts +</td>
<td>3.5 x 10^4</td>
<td>3.6 x 10^4</td>
</tr>
<tr>
<td>ts I5</td>
<td>2.2 x 10^4</td>
<td>1.5 x 10^2</td>
</tr>
<tr>
<td>ts II52</td>
<td>1.6 x 10^4</td>
<td>1.0 x 10^2</td>
</tr>
<tr>
<td>ts IV41</td>
<td>2.7 x 10^4</td>
<td>&lt;10^4</td>
</tr>
</tbody>
</table>

*The LD 50 for each route of inoculation was calculated from the mortality among groups of 7 to 10 mice injected by each route with 10-fold dilutions of virus titered by plaque assay at 31 C.
The cause of death after intracerebral (or intranasal) infection with VS virus ts mutants has not been determined. The data shown in Table 1 reveal that the reversion frequency of mutant ts IV41 is \( <10^{-4} \) as determined by plating efficiency on L cells at 31 and 39°C. Only ts I5 and ts II52 showed any significant presence of revertants in cloned stocks. A somewhat higher reversion frequency had been reported in previous studies of uncloned mutants (17). A more likely explanation than revertants for the sporadic deaths occurring at high intracerebral infectious doses of ts I5 and ts II52 is the leakiness of these mutants.

Vaccination against intracerebral challenge. The capacity of wild-type (ts\(^+\)) or ts mutants to induce protection against intracerebral challenge with virulent VS virus was tested in mice vaccinated by either the intraperitoneal or intranasal route.

In this series of experiments groups of 10 mice were vaccinated by each route with varying concentrations of each virus preparation and challenged 10 days later by intracerebral injection with 100 LD\(_{50}\) (~10\(^4\) PFU/mouse) of ts\(^+\) VS virus. This dose of challenge virus caused the death of all unvaccinated control mice within 5 days. Survival of mice from VS viral encephalitis was calculated on the basis of the PFU dose (at 31°C) of vaccine virus required for 50% protection of test mice.

Table 2 shows that intraperitoneal vaccination required very large and equivalent doses of ts\(^+\) or ts I5, ts II52, and ts IV41 virus for even minimal (50%) protection of mice against intracerebral challenge. It can be assumed from these data that live ts\(^+\) as well as ts mutant viruses are poor vaccines when administered by the intraperitoneal route, presumably because of limited growth even of ts\(^+\) virus at permissive temperature. In all likelihood, the slight immunity produced by intraperitoneal injection of these viruses is attributable to the inoculated antigenic mass.

Also shown in Table 2 is the greater effectiveness of intranasal vaccination with the three ts mutants. In each case considerably less virus was required to produce a level of immunity equivalent to that of intraperitoneal vaccination. The somewhat greater protection afforded by ts I5 vaccine may be a function of the leakiness of this mutant (11). In any case the superior immunizing potential of the ts mutants by the intranasal route is presumably attributable to their capacity to synthesize more antigen in susceptible respiratory cells.

Vaccination against intranasal challenge. If ts mutants of VS virus are capable at restrictive temperature of undergoing an abortive cycle of infection in respiratory mucosa, local immunity might result. This hypothesis was tested by intranasal vaccination with ts mutants followed by ts\(^+\) challenge by the same route.

Two series of experiments were performed in which groups of 10 mice were inoculated intranasally with serial 10-fold dilutions of VS virus mutants ts I5, ts II11, ts II52, and ts IV41, as well as ts\(^+\) inactivated by heating at 56°C for 1 h. Fourteen days later these mice were challenged by intranasal injection of 10 or 100 LD\(_{50}\) of ts\(^+\) VS virus, as were equivalent numbers of controls. The data were again calculated as the 50% protective dose in terms of PFU required to protect half the mice against intranasally induced VS viral encephalitis.

Table 3 reveals somewhat greater effectiveness of intranasal vaccination with live ts mutants against intranasal challenge as compared with controls.

### Table 2. Resistance to intracerebral challenge with 100 LD\(_{50}\) of ts\(^+\) VS virus induced by intraperitoneal or intranasal vaccination of mice with ts\(^+\) or ts mutant viruses

<table>
<thead>
<tr>
<th>Vaccine virus</th>
<th>PD(_{50}) (PFU/mouse)(^a)</th>
<th>Intraperitoneal</th>
<th>Intranasal</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts(^+)</td>
<td>3.5 × 10(^4)</td>
<td>1.2 × 10(^5)</td>
<td></td>
</tr>
<tr>
<td>ts I5</td>
<td>2.2 × 10(^4)</td>
<td>5.0 × 10(^5)</td>
<td></td>
</tr>
<tr>
<td>ts II52</td>
<td>7.7 × 10(^4)</td>
<td>3.2 × 10(^5)</td>
<td></td>
</tr>
<tr>
<td>ts IV41</td>
<td>2.7 × 10(^4)</td>
<td>1.2 × 10(^5)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The 50% protective dose (PD\(_{50}\)) was calculated as the amount of each virus (in terms of plaque-forming units at 31°C) required to promote the survival of half the mice challenged intracerebrally with 100 LD\(_{50}\) of ts\(^+\) VS virus 10 days after vaccination. Calculations were made by the Reed-Muench method for groups of 10 mice vaccinated at each 10-fold dilution of virus.

### Table 3. Resistance of mice to intranasal challenge with ts\(^+\) VS virus 14 days after intranasal vaccination with ts mutants

<table>
<thead>
<tr>
<th>Mutant vaccine</th>
<th>PD(_{50}) (PFU/mouse)(^a)</th>
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<tbody>
<tr>
<td></td>
<td>Versus 10 LD(_{50})</td>
</tr>
<tr>
<td>t5</td>
<td>1.0 × 10(^4)</td>
</tr>
<tr>
<td>ts I11</td>
<td>5.0 × 10(^4)</td>
</tr>
<tr>
<td>ts II52</td>
<td>3.3 × 10(^4)</td>
</tr>
<tr>
<td>ts IV41</td>
<td></td>
</tr>
<tr>
<td>Δts(^+), b</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Groups of 10 mice were vaccinated by the intranasal route with 10-fold dilutions of infectious ts mutants or heat-inactivated ts\(^+\). The 50% protective dose (PD\(_{50}\)) level of resistance was calculated as in Table 2.

\(^b\) Heated at 56°C for 1 h prior to intranasal injection.
with intracerebral challenge shown in Table 2. Not unexpectedly, resistance was greater against a smaller challenge dose. These data do not permit any meaningful quantitative comparison of the vaccination potential of the ts mutants. Once again, ts I5 seemed to afford somewhat greater protection. Quite clearly, however, each ts mutant was far more effective as a vaccine than was heat-inactivated ts+ virus administered by the intranasal route.

The onset of immunity to intranasal challenge with VS virus was determined by intranasal vaccination with two of the ts mutants. In these experiments groups of 10 mice were each inoculated by the intranasal route with 107 PFU of either ts I11 or ts IV41 at times varying from 12 h to 14 days before intranasal challenge with \(~100 \text{ LD}_{50}\) of ts+ VS virus. All vaccinated mice and 15 unvaccinated controls (day 0) of the same age were challenged at the same time.

Figure 1 shows the rapid rate at which each group of mice developed immunity to intranasal infection with VS virus. Mice inoculated intranasally with mutant ts IV41 were completely resistant by the third day after vaccination and remained so for 2 weeks. The results with ts I11 vaccination were somewhat more erratic but immunity was complete by 9 days. The evidence for some degree of immunity 12 h after vaccination with ts IV41 is quite impressive. This effect could, of course, be attributable to interferon production (31).

Similar results demonstrating rapid onset of immunity to intranasal challenge were obtained with another group IV mutant, ts IV44.

Neutralizing antibody in bronchial secretions and blood after intranasal vaccination. It seemed logical to postulate that the rapid induction of local immunity by intranasal inoculation of group IV ts mutants is due to production and secretion of specific antibody capable of neutralizing the infectivity of challenge ts+ VS virus.

To test this hypothesis mice inoculated by the intranasal route with 107 PFU of ts IV44 were examined for the presence of neutralizing antibody in bronchial secretions and circulating blood. At intervals after intranasal vaccination, blood was collected by severing the axillary arteries or hearts of ether-anesthetized mice. Serum separated from clotted blood was inactivated at 56 C for 1 h. As previously described in some detail (28), bronchial washings were collected from the same exsanguinated mice by cannulating the trachea with a 19-gauge needle through which 0.5 ml of antibiotic broth was injected into the lungs; 0.3 to 0.5 ml of these bronchial washings could be aspirated from the lung. Paired bronchial aspirates and sera from each control or vaccinated mouse were tested individually for their capacity to neutralize the infectivity of ts+ VS virus. Virus neutralizing activity was determined for duplicate samples of 100 \(\mu\)l of each bronchial washing or each serum mixed with 100 \(\mu\)l of 100 PFU of virus in one tube or 1,000 PFU of virus in another tube. After incubation for 1 h at room temperature, each virus—bronchial or virus—serum mixture was plated on L-cell monolayers and covered with agar; plaques were scored after incubating the infected monolayers at 37 C for 48 h. Residual infective ts IV44 virus present in the bronchial washings was also sought by plating the aspirate alone on L cells which were incubated at 31 C.

Figure 2 shows the plots of neutralizing activity in the bronchial secretions and blood of mice at intervals after intranasal vaccination with ts IV44 virus. The data represent average values for individual determinations on three mice at each time point. Bronchial secretions and sera of control unvaccinated mice showed minimal or no plaque neutralization. Bronchial washings obtained from mice 12 to 48 h after vaccination completely neutralized 100 and 1,000 PFU of VS virus. Thereafter, the neutralizing activity of bronchial washings declined, and none could be detected at 7 or 11 days after vaccination. In sharp contrast, serum of the identical vaccinated mice had no neutralizing activity until

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**Figure 1. Development of immunity to intranasal infection with virulent VS virus.** Groups of 10 mice were vaccinated by the intranasal route with 107 PFU of ts I11 or ts IV41 at intervals before intranasal challenge with 100 \(\text{LD}_{50}\) of ts+ virus. All vaccinated mice and 15 unvaccinated controls of the same age were challenged on the same day with a single batch of VS virus (5 \(\times\) 104 PFU/mouse). Day 0 = unvaccinated controls, all of which died within 6 days after challenge. The experiment was terminated 14 days after challenge.
the third day, but by day 5 and thereafter the sera of vaccinated mice completely neutralized 100 or 1,000 PFU of VS virus.

These data clearly illustrate that an antiviral substance, presumably neutralizing antibody, rapidly appears in the bronchial secretions of mice vaccinated with avirulent VS mutant ts IV44. This local immune response is short lived and is replaced later by production of circulating antibody presumably in response to the bronchial infection. More quantitative determination of bronchial and serum levels of neutralizing antibody are difficult to obtain, albeit technically feasible, and would require a more prolonged study.

Similar results were obtained by neutralization of VS virus on monolayers of chicken embryo cells which rules out interferon as a significant factor, particularly in early stages of vaccination.

**DISCUSSION**

Attenuated, live viruses have attracted considerable attention as candidates for vaccines with high immunogenic potential and relative ease of production and administration. Most of the mutant viruses used as vaccines have not been characterized genetically, which raises serious questions of genetic stability and safety. Few attempts have been made to determine the biochemical phenotypes of vaccine mutants. To be suitable as a vaccine a mutant should be highly infectious, capable of synthesizing abundant protein antigen, be restricted in virion assembly and pathogenicity, and exhibit low reversion frequency. Except for the empirical live virus vaccines for smallpox, yellow fever, and poliomyelitis, few, if any, mutant viruses currently under investigation as potential vaccines would appear to meet these criteria.

The VS virus ts mutants tested in this study would seem to possess at least some of the characteristics desirable for use as vaccines. These mutants, particularly those of group IV, are genetically stable (2a, 16), and revertants should not be of great concern although more tests must be done under stringent in vivo conditions. Leakiness of the mutants should not pose a serious problem and, in fact, may be an advantage. Leakiness is probably the explanation for the vaccination efficacy of ts 15 and ts 111 which should have been restricted in protein synthesis because of their defective transcriptase phenotype (11, 24). This finding refutes our hypothesis that ts mutants of complementation group I should not serve as vaccines. It is possible, as predicted, that the group IV ts mutants, which are not restricted in transcription and translation (2a, 30), possess the greatest immunogenic potential; however, the comparative data are far from conclusive. More detailed studies, including testing ts mutants of complementation groups III and V, must be done before a decision can be made about the best candidate for a vaccine.

Detailed genetic analyses have not been performed on mutants of viruses currently in use or being tested for potential use as attenuated, live vaccines. It is of interest that Sabin vaccine strain of poliovirus type I has a thermosensitive block (7). More recent ts mutants of poliovirus have not completely lost their pathogenicity for monkeys (9). Among the myxoviruses, some fowl plague ts mutants exhibit diminished pathogenicity for chickens, whereas others do not, and revertant ts+ phenotypes appear frequently in the host (9). Moreover, these latter studies revealed no correlation between pathogenicity and genetic complementation group. Influenza viruses considered for human use by Russian investigators have generally been attenuated by growth at low temperature with the objective in mind of producing mutant lines that grow well in human nasal mucosa but cause a low incidence of febrile responses after
IgA and to (18). Similar evidence for the such immunity lumen (25). known collectively overwhelming 50 rated by ended by infected cell. Reovirus plaque on infected mice, whereas infected neuron (4). Another way to invade by neuronal pathways (14).

mals; the source of the IgA precursors has been found in Peyer's patches of the rabbit intestine (3). No detailed studies of the onset and duration of IgA synthesis in response to specific immunogens have come to my attention. Few attempts seem to have been made to detect antibody in mucosal secretions before 1 week after vaccination with viruses; such antibodies could be detected by the exquisitely sensitive technique of viral neutralization (26, 27).

The data reported here suggest extremely rapid antibody response to local respiratory infection with VS virus ts mutants. No attempt has yet been made to quantitate the level of antibody, but it could be detected in bronchial washings by 12 h. The short duration of synthesis of bronchial antibody is consistent with a primary immune response. The rapidity of this evanescent response is reminiscent of the findings reported by Uhr et al. (26) that neutralizing antibody to bacteriophage could be detected in the circulation soon after injection of immunologically virginal guinea pigs. The doubling time of serum 19S antibody was estimated to be 16 to 18 h (27).

The level of immunity to infection with VS virus is undoubtedly determined to some extent by route of infection and pathogenetic mechanisms. Intracerebral inoculation of VS virus causes acute, diffuse cerebritis, whereas intranasal infection progresses more slowly with lesions and antigen first appearing in olfactory bulbs, pyriform lobes, and hippocampus (14). Dissemination of VS virus from the intranasal site of infection to the brain appears to occur by way of primary neurons supplying the inoculation site (14, 19). Therefore, this mode of dissemination of VS virus from nasal mucosa to brain could readily account for the greater degree of immunity to intranasal challenge compared with intracerebral challenge. Circulating antibody might well provide less protection against rhabdoviruses, such as vesicular stomatitis and rabies, which appear to invade by neuronal pathways (14).

The host can develop resistance to infection with VS virus by mechanisms other than those mediated by neutralizing antibody. VS virus is not considered to be a good inducer of interferon synthesis (29), but mice will produce interferon in response to intravenous injection of VS virus (31). Induction of interferon synthesis by synthetic polyanions was also found to protect mice against intranasal infection with VS virus for at least 10 days and even after infection when the virus had reached the brain (4). Another mode of resistance, albeit slight, can result from interference by simultaneous injection of ho-
mologous defective T particles and small infecting doses of infectious VS virus (10).

ACKNOWLEDGMENTS

Ruth M. Snyder rendered invaluable assistance particularly in the neutralization assays. This research was supported by Public Health Service grant AI-11112 from the National Institute of Allergy and Infectious Diseases, by grant GB-35257X from the National Science Foundation, and by grant VC-88 from the American Cancer Society.

LITERATURE CITED