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Virulence of Temperature-Sensitive Mutants of Sindbis Virus in Neonatal Mice

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The virulence in neonatal mice of temperature-sensitive (ts) mutants of Sindbis virus was determined by measurements of mean survival time and 50% lethal dose after intracerebral injection. For 11 ts mutants, mean survival time was determined by the ribonucleic acid (RNA) phenotype, RNA^+ mutants killing the mice sooner than RNA^- mutants for the same titer of virus injected. Mortality caused by seven ts mutants was, with one exception, correlated with the proportion of revertants recovered after death. A82, a presumed double mutant showing low reversion, showed no detectable lethality. The pathogenicity of this mutant could be detected by inhibition of weight gain, which was proportional to the titer of virus injected. A low-level persistence, independent of the titer injected, occurred up to 7 days after injection. This was followed by complete clearance. It is concluded that the virulence of Sindbis virus may be considerably altered by mutation, and that this is related to events occurring at the cellular level.

Temperature-sensitive (ts) mutants have been used extensively to study the multiplication of animal viruses. Recently, it has become apparent that these mutants may show altered pathogenicity in experimental animals. These properties may be used to study the relationship between the genotype of the virus and the disease process. For example, several ts mutants of vesicular stomatitis virus have been shown to produce a protracted neurological disease in experimental animals, unlike the rapid disease produced by the wild-type virus (9, 10, 13). Some measles virus ts mutants are capable of inducing hydrocephalus, unlike the encephalitis induced by the wild-type virus and some other ts mutants (7). For foot-and-mouth disease virus ts mutants, reduced manifestation of the disease in steers has been shown to be correlated with reduced virulence for mice (11). Several ts mutants of dengue virus have reduced virulence in mice, and in two cases this was associated with inability to produce viremia in monkeys (14). For Sindbis virus, most ts mutants were less virulent for embryonated eggs than the wild type; some pathological features were apparent after infection with ts mutants that were not seen with the wild type (12). In general, the virulence of the ts mutants varied greatly in comparison to the wild-type; in some cases it has been postulated that virulence is determined by the genotype of the virus (7), by reversion and leak (12-14), or by a locus independent of the ts locus (10, 11).

In this report, we describe experiments designed to determine the virulence in neonatal mice of Sindbis virus ts mutants. These mutants are part of a series originally described by Atkins et al. (5) and have been used to study virus multiplication (5, 6) and virus-cell interactions (1-4). The results indicate that the time of death of infected mice is determined by the genotype of the virus, but that in most cases development of a lethal infection per se is determined by the ability of the mutant to revert to wild type.

MATERIALS AND METHODS

Virus. All ts mutants were from the AR339 strain of Sindbis virus and were described by Atkins et al. (5). Stocks of virus were grown by first picking plaques formed at 30°C on chicken embryo fibroblasts (CEF). Virus from these plaques was used to infect a culture of CEF, and this virus was again passaged in CEF. The fluid from these cells was frozen at −70°C in aliquots, and after thawing was plaque assayed at 30°C (the permissive temperature) and 39°C (the restrictive temperature). The efficiency of plating of the virus at 39°C compared with 30°C was taken as a measure of the revertant content of the preparation. Mutants making more than 10% of the wild-type level of ribonucleic acid (RNA) at 39°C (as measured by Atkins et al. [5]) are defined as having RNA^+ phenotype, whereas those making less than this level are defined as RNA^-.

Methods of preparing chicken embryo cells, growth of BHK cells, and the method of plaque assay were as described previously (5).

Mice. Inbred BALB/c mice were used in all experiments. Litters were adjusted to contain six mice before use and were injected before 3 days after birth. Injection was by the intracerebral route, and the virus was contained in a volume of 0.02 ml. Control mice,

injected with cellular growth medium alone, survived this procedure. We determined the titer of virus in brain tissue after removing the brains and storing them at −70°C in 1 ml of medium 199 per brain. After thawing, brains were homogenized to a fine suspension in a glass homogenizer, debris was removed by centrifuging at 3,000 × *g* for 5 min, and the supernatant plaque was assayed. Other organs were extracted as a 10% (wt/vol) suspension and treated similarly.

Weight gain. Mice were injected intracerebrally with the virus within 24 h of birth and then weighed at daily intervals. Control mice were injected with 0.02 ml of medium 199. The percent weight gain is expressed as [(weight on day X − weight on day 0 for mutant)/(weight on day X − weight on day 0 for control)] × 100. The significance of the results was assessed by comparing the weight gains of virus-infected mice with those of control mice injected at the same time, using Student's t test.

RESULTS

Mean time of death. As a preliminary screening procedure, groups of 6 to 12 mice were injected with 10^5 plaque-forming units (PFU) of a series of 11 mutants and the wild type. The mean times of death were 1.9 days for the wild type, 1.8 to 4 days for RNA^+ mutants, and 4.5 days or more for RNA^- mutants (Table 1). There was therefore a distinction in mean time of death between mutants exhibiting different RNA phenotypes. Mean time of death did not correlate with the titer of virus recovered from brain tissue at death, which was always lower than that recovered from wild-type-infected mice, nor did it correlate with the proportion of revertants recovered. However, these findings do not exclude the possibility that eventual death was due to reversion. For one RNA^- mutant, A82, all mice survived infection, but mortality for the other mutants was 100%.

LD_{50}. The 50% lethal dose (LD_{50}) was determined for seven mutants and the wild type (Table 2). All mutants examined showed a higher LD_{50} than the wild type. However, the LD_{50} values varied greatly, and there was no correlation with RNA phenotype. With the exception of one RNA^+ mutant, N32, the LD_{50} correlated with the proportion of revertants recovered from dead mice. Again, mutant A82 showed no detectable lethality.

Pathogenicity of mutant A82. Since mutant A82 was apparently avirulent, we decided to further investigate its properties. Two series of experiments were performed. Groups of 25 to 30 mice were injected with 10^3, 10^4, and 10^5 PFU of A82, as assayed at 30°C, and others were mock infected. The weights of the mice were then monitored daily over a period of 40 days (Fig. 1). Also, duplicate mice injected with the same quantities of virus were sacrificed at 2-day intervals, the brain extracts were pooled, and the presence of infectious virus was monitored by plaque assay (Table 3).

The initial amount of virus injected had a significant effect on subsequent weight gain. For 10^5 PFU injected, the weight difference between infected and mock-infected controls was significant ($P < 0.05$) up to 18 days after injection. The corresponding times for 10^3 and 10^4 PFU were 2 and 5 days. Thus there was an initial inhibition of weight gain which was dependent on the titer of virus injected.

A low-level persistence at 10^2 to 10^3 PFU recovered per brain occurred up to 7 days after infection, irrespective of the titer injected (Table 3). This probably resulted from virus replication, since thermal inactivation of A82 at 37°C resulted in a 10^{-3} reduction in titer in 2 days (data

TABLE 1. *Mean times of death of mice injected with 10^5 PFU of virus*[a]

Mutant	RNA phenotype	EOP[b] (39°C/30°C) of injected virus	Virus recovered from brain[c]		Mean time of death (days)[d]
			Titer (PFU/brain)	EOP (39°C/30°C)	
F104	−	$<10^{-7}$	3×10^4	0.66	8.8 (±0.5)
N2	−	1.6×10^{-4}	8×10^5	0.13	6.8 (±0.1)
H98	−	$<10^{-7}$	7×10^5	4.5×10^{-2}	4.5 (±0.3)
F346	−	1.0×10^{-7}	6×10^5	0.70	4.8 (±0.2)
A82	−	$<10^{-7}$	All mice survived infection		
A120	+	1.5×10^{-5}	8×10^4	5.3×10^{-2}	2.3 (±0.2)
A93	+	$<10^{-7}$	2×10^6	$<10^{-6}$	1.9 (±0.2)
F127	+	$<10^{-7}$	6×10^3	0.26	3.5 (±0.3)
N32	+	$<10^{-7}$	9×10^4	2.3×10^{-2}	4.0 (±0.2)
F36	+	2.0×10^{-5}	5×10^4	8.0×10^{-2}	1.8 (±0.2)
H18	+	$<10^{-7}$	1×10^4	0.14	3.3 (±0.3)
Wild type		1.0	3×10^7	1.0	1.9 (±0.1)

[a] Groups of 6 to 12 mice were injected with each mutant.
[b] EOP, Efficiency of plating.
[c] On death of infected animals.
[d] Arithmetic mean (± standard error).

not shown). Subsequent clearance of virus was complete by 11 days after infection.

The possibility that A82 was persisting in an infectious form in other organs after elimination from the brain was also investigated. Six weeks after injection with 10^3, 10^4, and 10^5 PFU of A82, groups of eight mice were sacrificed, and brain, liver, kidneys, lung, spleen, and blood were extracted and pooled. The supernatant of each homogenate and a 0.5-ml serum sample were added to monolayers of subconfluent BHK cells, which were incubated at 30°C. Mock-infected animals were treated similarly to determine the toxicity of the organ extracts. Samples of homogenates from mock-infected animals containing 10^3 PFU of A82, added before homogenization, were also added to BHK monolayers; these cells showed a cytopathic effect (CPE) after 3 days of incubation at 30°C. Monolayers treated with tissue extracts from test animals showed no signs of CPE, even after they had been split one-fifth and regrown to confluence. Thus there was no evidence for the persistence of infectious virus in any of the organs tested.

TABLE 2. *LD_{50} for ts mutants*[a]

Mutant	RNA phenotype	LD_{50} (PFU)	EOP (39°C/30°C) of virus recovered[b]
F104	−	56	0.4
N2	−	5	1.0
A82	−	$>10^6$	All mice survived
A120	+	27	0.3
A93	+	220	2×10^{-3}
F127	+	2	0.9
N32	+	30	$<10^{-6}$
Wild type		0.5	1.0

[a] Groups of six animals were injected with 10-fold dilutions of the virus from undiluted to 10^{-9} and were observed over a 2-week period.

[b] Recovered from the highest dilutions of virus which resulted in the death of all mice injected. EOP, Efficiency of plating.

DISCUSSION

Our results show that the mean time of death of neonatal mice infected with Sindbis virus ts mutants is dependent on the RNA phenotype of the virus, RNA^- mutants generally killing the mice later than RNA^+ mutants for the same titer of virus injected. That the eventual death of the mice was probably due to reversion of the mutants in most cases is shown by the LD_{50} determinations, which show a correlation between the proportion of revertants recovered from dead mice and the LD_{50} titer. An exception is mutant N32, which is an RNA^+ mutant able to kill mice without reversion to wild type. These results confirm those obtained by Haspel et al. (7) for measles virus; they postulated that time of death was determined by the genotype of the virus, whereas mortality was determined by reversion.

The results obtained in these experiments

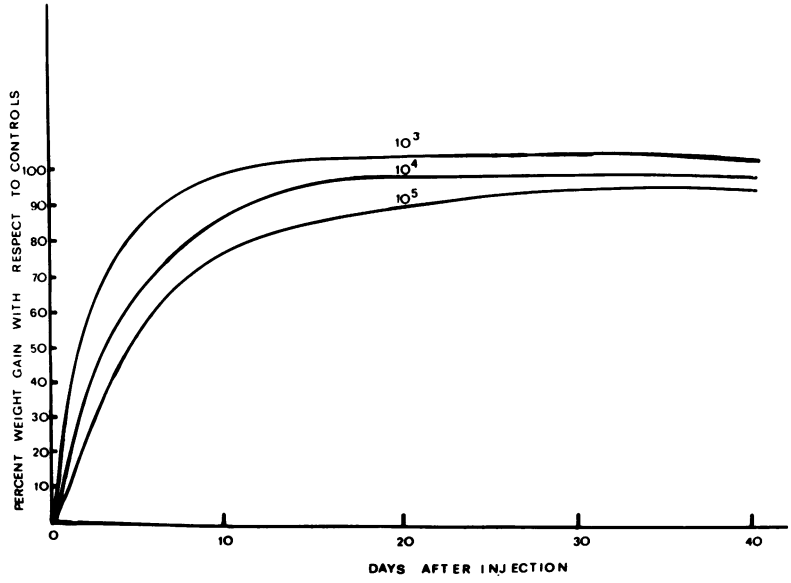

FIG. 1. *Percent weight gain of A82-injected mice as compared with controls. Groups of 25 to 30 mice were injected with the titers of virus indicated within 24 h of birth. Individual mice were weighed at daily intervals. Weight gains are expressed as a percentage of that shown by mock-infected controls, as described in Materials and Methods.*

TABLE 3. *Clearance of A82 from brains of injected mice*

Days after injection	Titer recovered (PFU/brain at 30°C)[a]		
	10^{3}[b]	10^{4}	10^{5}
1	9×10^{2}	8×10^{2}	8×10^{3}
3	2×10^{2}	3×10^{2}	3×10^{2}
5	5×10^{2}	1×10^{2}	4×10^{2}
7	2×10^{2}	2×10^{2}	7×10^{2}
9	None	6×10^{1}	None
11	None	None	None

[a] Assays were also carried out at 39°C; no plaques were observed in any assay at this temperature.
[b] Titer injected (PFU/0.02 ml).

may be compared with those obtained earlier for the cytopathogenicity of the mutants in BHK cells. RNA^+ mutants inhibited protein synthesis in infected cells (1), whereas RNA^- mutants did not. The CPE produced by RNA^+ mutants was different qualitatively in its early stages from that produced by the wild type, but eventually the cells disintegrated in a manner similar to that produced by wild-type infection (2). In several instances RNA^+ mutants were able to kill cells without reversion to wild type. The CPE produced by RNA^- mutants was delayed, and if it did occur was correlated with reversion to wild type. Taken together, these sets of data indicate that the ability of mutants to inhibit protein synthesis, to cause CPE, and to kill neonatal mice is dependent on their ability to make virus-specific RNA inside infected cells.

This study has also shown that one RNA^- mutant, A82, is avirulent. This mutant is, however, able to inhibit weight gain in infected mice, and this inhibition is dependent on the titer of virus injected. The mice did not show neurological symptoms and eventually recovered completely from the disease. Thus the disease produced by A82 was different from that produced by the wild type, and this difference was due to mutation. The inability of A82 to establish a lethal infection is probably due to its nonreverting properties. This mutant synthesizes uncleaved proteins characteristic of both RNA^+ and RNA^- mutants on shift from permissive to restrictive temperature, and is probably therefore a double mutant (G. J. Atkins, unpublished data).

The mechanisms underlying the low-level persistence of A82 in the brains of infected mice up to 7 days after infection are at present unclear. The level of persistence is unrelated to the titer of virus injected, and it is probable that 10^2 to 10^3 PFU per brain represents the maximum multiplication potential of A82 in the neonatal mouse brain. However, the inhibition of weight gain by A82 does depend on the titer injected, and so probably results from tissue damage during the early stages of infection, which is manifested later. Eventual complete clearance of the virus may be due to immunological mechanisms. Further experiments are in progress to compare the neuropathology of A82 infection with that of the wild type.

In view of the isolation of ts mutants from cells persistently infected with many viruses in tissue culture (8), we thought it important to determine whether the virus persisted in organs other than the brain. We could obtain no evidence for the long-term persistence of A82 in any of the organs tested.

This work has therefore shown that the disease produced by Sindbis virus may be modified by mutation. Further experiments are currently in progress in our laboratory to isolate and characterize avirulent mutants which are not selected for temperature sensitivity.

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