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Pathogenesis of Temperature-Sensitive Mutants of Sindbis Virus in the Embryonated Egg

II. Control of the Infectious Process

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Viral infection in the embryonated egg was controlled through appropriate temperature shifts. Through such control of the infection, certain groups of key tissues were implicated in the pathogenic processes that lead to death of the embryo, whereas other tissues were eliminated as relatively unimportant. Temperature shift-down experiments suggested that some mutants could resume the infectious process after relatively long periods (ribonucleic acid-negative mutants, Sts-4 and Sts-17) at the nonpermissive temperature, whereas others (RNA-positive mutants, Sts-2 and Sts-10) could not resume infection, even after a relatively short period at the nonpermissive temperature.

In an accompanying paper, Schluter, Bellomy, and Brown (4) concluded that temperature-sensitive (ts) mutants of viruses possess potential for better understanding the basis of viral pathogenesis and immunity in an acute infection in the animal host than by the use of parent virus alone. By conventional methods (4), it was possible to outline a general pattern of virus dissemination. The results indicated that, in order to trace the spread of virus more accurately, control of the infectious processes leading to death would be necessary. Temperature shift experiments utilizing ts mutants of Sindbis virus and the parent in the embryonated egg were therefore conducted in an attempt to discover whether viral multiplication and dissemination could be controlled.

MATERIALS AND METHODS

Most of the materials and methods used in this study were the same as those described in the previous paper (4). These include embryonated eggs, viruses, cell culture and media, virus infection and assays in cell culture, egg inoculations (via the chorioallantoic membrane), collection of specimens, and preparation of tissues.

Temperature shift-lethality experiments. The median lethal dose (LD_{50}) titers (3) were determined in temperature shift experiments which were performed by inoculating a set of eggs (usually 10 eggs per dilution) by the chorioallantoic membrane (CAM) route (4). At various time intervals during incubation at the alternate temperature, a set of eggs was shifted to either the permissive (33 C) or the nonpermissive (38.5 or 40 C) temperature where they were maintained with each group of uninoculated and sham (diluent)-inoculated eggs as controls. Virus-inoculated eggs were also maintained constantly at both temperatures as additional controls in the temperature shift experiments.

Temperature shift-kinetic experiments. The CAM was chosen as the route for inoculation. Tissue samples pooled from three eggs were taken periodically after inoculation and at the time of temperature shift. These samples included the amniotic membrane, yolk sac, CAM, blood, brain, and torso. Collection and preparation of the tissue specimens have been described previously (4). Tissues from dead embryos were stored individually. Doses selected for inoculation of the ts mutants were 1/10 to 1/100 of the LD_{50}. Eggs inoculated with the heat-resistant parent strain (HR) received a dose that would kill the majority of the eggs (between 80 and 100% lethal). As a control of the infecting dose, an LD_{50} was determined on a parallel set of virus-infected eggs which was shifted simultaneously with those used in the kinetic studies.

RESULTS

Effect of temperature shift on lethality. Embryonated eggs were originally transferred from 33 to 38.5 C at various time intervals ranging from 2 to 18 h after inoculation. Because 40 C was later found to be a more optimal nonpermissive temperature (in giving somewhat sharper end points), experiments were repeated at that temperature which essentially confirmed and extended the results obtained at 38.5 C.

As one measure of the effect of the temperature shift-up, and because a shift was involved, the LD_{50} of the ts mutants was calculated for each time interval after a total incubation

period of 5 days, a period well beyond the time necessary for any of the mutants to kill the embryos at the nonpermissive temperature. Controls consisted of two sets of eggs infected with the mutant and maintained constantly at each temperature for the duration of the experiments. It should be noted that in all temperature shift experiments the percentage dead in all sham-inoculated and uninoculated controls was negligible in repeated experiments.

Table 1 shows the effect of temperature shift-up on the LD_{50} of the temperature-insensitive (ti) parent HR and its ts mutants. The temperature change had no effect on the virulence of HR. When compared to the LD_{50} controls at 33 C, it may be seen that Sindbis virus ts mutants Sts-10 and Sts-17 remained attenuated for the embryonated egg even if the shift-up was made as late as 24 h. This suggests that by a temperature shift the infectious and pathological processes which result in death can still be inhibited after 24 h of incubation at the permissive temperature when virus multiplication and dissemination was evident (see Table 4 of reference 4 and Table 3 of this paper). Similar results were obtained with Sts-2. There was only a slight increase in the LD_{50} after 24 h of incubation at 33 C before the shift in temperature. Some petechiae, hemorrhages, and changes in the brain were also evident in mutant-infected embryos by 24 h (Table 6 of reference 4), yet the embryos survived if the shift to a nonpermissive temperature was made by this time with unopened eggs in the same experiment. With Sts-4, the irreversible infectious processes leading to an increase in deaths at the nonpermissive temperature were already underway after 12 h of incubation at the permissive temperature. Although there was an increase in lethality, the LD_{50} titer did not reach that of the control at 33 C.

From the data presented in Table 2 for a shift-down experiment, it was concluded that infection resulting in death by the ts mutants can resume at the permissive temperature if the interval of incubation at the nonpermissive temperature is not too long. These results confirmed data obtained in previous experiments carried out at 38.5 C as the nonpermissive temperature. For three of the mutants, the resumption of virus multiplication and death of the embryos at the permissive temperature was sharply curtailed after incubation for 24 h at the nonpermissive temperature. Sts-4 apparently required the longest period of incubation at the nonpermissive temperature before inhibition of embryo deaths at the permissive temperature was evident. Both ribonucleic acid-negative (RNA^-) mutants could resume infection at the permissive temperature with relatively little inhibition after 12 h at the restrictive temperature. In contrast, both RNA-positive (RNA^+) mutants (Sts-2, Sts-10) were significantly inhibited after only 2 h at the restrictive temperature. For Sts-10 particularly, incubation for as little as 2 h at the nonpermissive temperature was enough to prevent an increase in lethality after shift-down to the permissive temperature. The correlation of the RNA^+ character with inability to resume infection may be significant and not merely coincidental. One can hypothesize, for example, that the ability to synthesize RNA may allow a greater or more rapid induction of interferon than might be expected for RNA^- mutants. This has not yet been tested.

The general conclusions from this experiment may be stated as follows. The ability of the ts mutants to resume the infectious processes

TABLE 1. *Effect of temperature shift-up (33 to 40 C) on the LD_{50} of HR and Sts mutants*

Incubation (h)[a]	LD_{50}/ml (log_{10})[b]				
	Sts-2 (+)[c]	Sts-4 (−)	Sts-10 (+)	Sts-17 (−)	HR
2	4.3	5.5	5.3	5.3	8.3
6	4.6	5.8	5.1	5.3	8.2
12	4.3	7.0	5.0	5.4	8.4
24	5.3	7.2	5.4	5.5	7.8
Control, 33 C	7.4	8.4	7.2	8.4	8.8
Control, 40 C	4.4	5.5	5.0	5.2	8.2

[a] Embryonated eggs were incubated at 33 C for the number of hours indicated before shift-up to 40 C. Controls were maintained at the temperatures indicated.

[b] After 5 days of incubation.

[c] (+) = RNA^+; (−) = RNA^-.

TABLE 2. *Effect of temperature shift-down (40 to 33 C) on the LD_{50} of HR and Sts mutants*

Incubation (h)[a]	LD_{50}/ml (log_{10})[b]				
	Sts-2 (+)[c]	Sts-4 (−)	Sts-10 (+)	Sts-17 (−)	HR
2	6.0	7.9	5.8	7.8	8.6
6	5.9	7.4	5.5	7.5	8.2
12	5.7	7.7	6.0	7.0	8.4
24	4.7	6.4	5.9	5.8	8.2
Control, 33 C	7.0	8.5	7.0	7.9	8.7
Control, 40 C	<4.7	<5.7	5.1	5.5	8.6

[a] Embryonated eggs were incubated at 40 C for the number of hours indicated before shift-down to 33 C. Controls were maintained at the temperatures indicated.

[b] After 5 days of incubation.

[c] (+) = RNA^+; (−) = RNA^-.

leading to death after incubation at the nonpermissive temperature varies with the mutant and is dependent on the interval of incubation at that temperature. As discussed earlier, the RNA$^+$ mutants (Sts-2 and Sts-10) are least able to resume the lethal process of infection which may be the result of inactivation of virus, or virus induction of interference, or some other mechanism.

Kinetics of growth of ts mutants after temperature shift. Knowing that infection and lethality could be controlled, temperature shift kinetics were done in a preliminary effort to better follow viral multiplication and dissemination and to discover which tissues were primarily involved in pathogenesis. In the first preliminary experiment, eggs were shifted to the nonpermissive temperature (38.5 C) after 6 h at 33 C. These results for all of the mutants showed limited virus multiplication in the CAM only (up to 3.5 $\log_{10}$ plaque-forming units [PFU]/g) with virtually no dissemination to the other tissues examined. Almost all of the mutant-infected embryos survived. The two embryos that succumbed were found to have ti, virulent revertant populations (4) in their tissues. Embryos infected with HR at a dose well below that of the mutants (120 PFU/egg) showed significant multiplication and dissemination beginning at 12 h. Death occurred before 48 h in almost all of the embryos. In a repeat experiment, an interval of 24 h of incubation at 33 C was chosen before shift-up to 40 C. The results are presented in Table 3. At the time of shift-up (24 h), parent HR had already undergone several cycles of multiplication and was well disseminated. In direct contrast, viral recovery from the mutant-infected eggs was limited after shift-up. Although virus was recovered from all of the tissues of eggs infected with Sts-2 and Sts-17 after 24 h at 33 C, no virus was recovered 12 h after shift-up to 40 C. Virus populations recovered at 48 h after infection by Sts-2 and Sts-17 proved to be revertants by subsequent testing. Mutant virus had apparently multiplied in the membranes of eggs inoculated with Sts-10 at the time of shift-up. After 3 h at 40 C, the titer of Sts-10 dropped, and after 12 h at 40 C a ti revertant was recovered from the CAM and the torso. Death (one embryo) occurred 96 h after inoculation with this mutant; a ti revertant was recovered from the embryo. These results suggest that early virus replication per se in the membranes does not appear sufficient to cause death, and that, as a minimum requirement, virus replication in the torso is necessary to cause death.

TABLE 3. *Kinetics of virus growth after temperature shift-up (33 to 40 C) at 24 h*

Hours after inoculation	Tissue	PFU/g of tissue				
		Sts-2 (3.8)[a]	Sts-4 (1.7)[a]	Sts-10 (3.4)[a]	Sts-17 (2.8)[a]	HR (1.5)[a]
12	Amnion	0[b]	0	0	2.7	5.1
	Blood	5.0	0	0	3.7	3.7
	Brain	0	0	0	2.1	—[c]
	CAM	5.0	—	2.9	5.7	6.0
	Torso	0	0	0	2.0	—
	Yolk sac	2.9	0	0	3.3	—
24 (time of shift)	Amnion	7.7[d]	0	2.1	2.6	8.5
	Blood	6.7	0	0	3.7	7.3
	Brain	7.1	0	0	2.5	7.8
	CAM	7.5	—	5.0	4.9	8.3
	Torso	7.5	0	0	3.5	9.3
	Yolk sac	6.1	0	3.0	3.1	9.0
27	Amnion	4.8	0	0	4.2	7.3
	Blood	2.4	0	0	6.0	8.6
	Brain	2.8	0	0	3.4	7.8
	CAM	4.7	2.2	3.9	6.5	8.2
	Torso	3.7	0	—	3.8	9.4
	Yolk sac	3.7	0	0	4.5	9.1
36	Amnion	0	—	—	0	7.6
	Blood	0	2.7	0	0	8.2
	Brain	0	—	0	0	8.5
	CAM	0	3.4	3.5	0	8.9
	Torso	0	3.0	2.7	0	9.2
	Yolk sac	0	—	—	0	7.0
48	Amnion	7.1	0	0	7.7	ND[e]
	Blood	6.9	0	0	5.9	ND
	Brain	7.2	0	0	5.8	ND
	CAM	8.2	0	0	6.6	ND
	Torso	8.2	0	0	7.5	ND
	Yolk sac	7.2	0	0	5.4	ND

[a] $\log_{10}$ PFU/egg inoculated.

[b] 0 = no virus recovery; less than 10^2 PFU/g of tissue.

[c] — = virus recovered; less than 10^2 PFU/g of tissue.

[d] Revertant (ti) found in this sample.

[e] Not done; embryos dead.

Table 4 shows virus multiplication and dissemination after incubation at 40 C for 12 h followed by a shift-down to 33 C. Again the temperature change had no effect on the multiplication and dissemination of HR. Compared to the RNA$^-$ mutants, virus was recovered earlier in tissues taken from eggs infected with RNA$^+$ mutants (Sts-2 and Sts-10), perhaps because the latter (RNA$^+$) were "primed" for maturation. Death occurred within 48 h after inoculation. There was a greater delay in the resumption of the infectious process in eggs

TABLE 4. *Kinetics of virus growth after temperature shift-down (40 to 33 C) at 12 h*

Hours after inoculation	Tissue	PFU/g of tissue				
		Sts-2 (3.8)[a]	Sts-4 (1.7)[a]	Sts-10 (3.4)[a]	Sts-17 (2.8)[a]	HR (1.5)[a]
12	Amnion	0[b]	0	0	0	0
	Blood	0	0	0	0	0
	Brain	0	0	0	0	0
	CAM	—[c]	0	0	0	4.1[d]
	Torso	0	0	0	0	2.0
	Yolk sac	0	0	0	0	0
15	Amnion	6.8	0	0	0	5.6
	Blood	6.0	0	0	0	5.3
	Brain	5.2	0	0	0	5.0
	CAM	6.9	0	2.0	0	5.8
	Torso	6.5	0	0	0	6.4
	Yolk sac	5.4	0	0	0	2.1
24	Amnion	0	0	0	0	7.9
	Blood	0	0	0	0	7.4
	Brain	0	0	0	0	7.8
	CAM	—	0	2.5	—	8.2
	Torso	0	0	0	0	9.0
	Yolk sac	0	0	0	0	7.4
48	Amnion	0	0	7.6	4.0	ND[e]
	Blood	0	0	6.4	5.3	ND
	Brain	0	0	6.4	3.9	ND
	CAM	0	0	5.6	5.1	ND
	Torso	0	0	7.0	6.0	ND
	Yolk sac	0	0	5.7	2.3	ND

[a] Log_{10} PFU/egg inoculated.

[b] 0 = no virus recovery; less than 10^2 PFU/g of tissue.

[c] — = virus recovered; less than 10^2 PFU/g of tissue.

[d] Log_{10} PFU/g of tissue.

[e] Not done; embryos dead.

infected with the RNA⁻ mutants. Death occurred 69 to 72 h after inoculation. A ti revertant was recovered from all of the tissues at the time of death.

The results obtained from the shift-down experiment with the RNA⁺ mutants appear to indicate that limited virus multiplication resumed during the intervals tested, but the corresponding LD_{50} data (Table 2) indicated that deaths did not increase significantly over controls held at 40 C when eggs were shifted down to the permissive temperature. One of several explanations possible is that an (auto) interference mechanism is induced by the infecting RNA⁺ virus during incubation at the nonpermissive temperature which inhibits resumption of virus multiplication after shift-down.

DISCUSSION

By the use of temperature shifts, it was possible to control viral multiplication in the embryonated egg. This is somewhat analogous to the temperature shift studies done in cell culture by Burge and Pfefferkorn in which they were able to determine when in the course of infection the particular function defective in a given ts mutant was expressed (2). According to Burge and Pfefferkorn (1), Sts-4 and Sts-17 belong to the same complementation group and should therefore have similar functional defects. However, the results obtained from the in vivo studies presented in this and the accompanying paper (4) were often different for these two mutants. This may be the result of subtle intracistronic differences not detectable by the complementation tests.

Temperature shifts had no effect on the virulence of HR. The data from the shift-up experiments with the ts mutants suggest that the infectious and pathological processes which result in death can still be inhibited after 24 h of incubation at the permissive temperature (Table 1). At this time, virus multiplication and dissemination and several pathophysiological signs were evident with some mutants (Table 6 of reference 4). With Sts-4, the irreversible infectious processes leading to death at the nonpermissive temperature after the shift were already underway after the initial 12 h of incubation at the permissive temperature.

It was concluded from the shift-down experiments that infection by the ts mutants which culminates in death can resume if the interval of incubation at 40 C is not too long (Table 2). With the RNA⁺ mutants, resumption of virus multiplication and dissemination leading to death of the embryo was limited when compared to the controls at both 33 and 40 C. However, infection was significantly resumed with the RNA⁻ mutants even after 12 h of incubation at 40 C. It is not known at the present whether the greater inability to resume the lethal process of infection particularly by the RNA⁺ mutants is the result of virus inactivation or virus induction of interference, or of some other mechanism of resistance. It was found that ti revertants almost always eventually appeared at 33 C in shift experiments, but only after an initial incubation at the nonpermissive temperature; this happens only rarely when the embryos are incubated at 33 C throughout the incubation period. The latter is probably due to a rare chance selection. The former may be due to some change in the physiology of the embryo, when previously incu-

bated at the high temperature, which selects a ti revertant even though the temperature is subsequently lowered.

It is obvious that a finer control of the infectious process than employed here (e.g., closer temperature shift intervals), plus a wider sampling of tissues for assay of viruses and for histopathological studies (including use of specific fluorescent antibody), will be necessary to focus on the critical events leading to death. Nevertheless, it is believed that these experiments suggest that ts mutants offer greater potential usefulness for analyzing the pathogenesis of viruses than the use of their virulent parents alone. Furthermore, the use of ts mutants may offer deeper insights into their relative avirulence than is superficially obvious from their ts property. For example, it is conceivable that their ts property may permit them to replicate (virus or viral RNA) enough to induce resistance (or interference) to continued autologous infections, or to superinfection by a virulent parent, or to superinfection by an unrelated virus.

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