JOURNAL OF VIROLOGY, Jan. 1977, p. 161-167

Vol. 21, No. 1
Printed in U.S.A.

Effects of Temperature and Antibody on the Cyclic Growth of Vesicular Stomatitis Virus

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Received for publication 9 September 1976

To see the effects of temperature on the interrelated cyclic production of standard and defective interfering (DI) particles of vesicular stomatitis virus, a temperature-sensitive (*ts*) G114 mutant was passaged successively at different temperatures and the production of the two types of viral particles as well as the ability of Chinese hamster ovary cells to survive each passage was continuously monitored. When the temperature was nonpermissive for standard virus, the synthesis of both standard and defective interfering particles was inhibited. When revertants appeared in the population, their ability to take over the infection depended on the permissiveness of the temperature for the temperature-sensitive mutant. At permissive temperatures periodic inhibition of both types of standard viruses was maintained by the production of defective interfering particles. Revertants did not become a majority of the population due to this periodic inhibition. When the conditions were nonpermissive for the mutant, revertants became the major standard virus in the population within a few passages. These findings can be understood if conditions of high and low multiplicities are dissected out together with a thorough understanding of the individual properties of each of the viral particles and of the result of interactions between them. In the presence of antiserum which neutralized only 90% of the viral particles, cyclic production of standard virus occurred, with a decline in the total amount of virus produced after each cycle. Therefore, in the presence of limiting concentrations of antiserum, the virus appeared to be able to establish a persistent cyclic growth pattern.

Viral pathogenesis involves not only the direct interaction of a virus particle with its host cell but also depends on a large number of known and unknown host factors in the whole animal. One approach in extending the knowledge obtained on the molecular level to mechanisms of pathogenesis is to establish infected cultures in such a way as to permit almost continuous monitoring of viral and cellular populations over a period of time. Using a highly virulent strain of vesicular stomatitis virus (VSV) and diluting the infected culture daily with fresh uninfected cells, it is evident that, rather than continuous cell destruction, cells survive at some passages and not at others. In fact, virus production and cell survival alternate in a cyclic fashion—a balance which is maintained by the production of defective interfering (DI) particles in the viral population (11). The interfering ability, the dependence on helper standard virus, and the lack of cytotoxicity are the essential properties of VSV DI particles that result in the cyclic nature of these interactions (see reference 5).

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To see the effects of other parameters on this cyclic growth pattern and to mimic in vivo conditions that might lead to viral persistence, two other conditions were independently introduced. The first condition was temperature sensitivity of the virus itself, and the second involved the addition of anti-viral antiserum. The temperature-sensitive (*ts*) mutant used in these experiments was *ts* G114, which belongs to complementation group I (16, 17). Previous studies with this mutant in Chinese hamster ovary (CHO) cells has shown that it is a relatively stable mutant with a readily characterized lesion in transcription (12, 13). This lesion was chosen particularly for this study because carrier cultures persistently infected with negative-strand viruses readily yield temperature-sensitive viruses that contain lesions in RNA synthesis (9, 14, 15). The general purpose of these experiments was to see if the cyclic production of standard and DI particles still occurred under partially nonpermissive conditions caused by changing the temperature of incubation. The addition of neutralizing antibodies to such continuous infections by wild-type virus was to see whether cyclic production

of virus occurred in the presence of limiting amounts of antibodies. These conditions superimposed on the interactions between DI and standard particles may show underlying mechisms in virus-host interactions.

MATERIALS AND METHODS

Cells and viruses. Chinese hamster ovary (CHO) cells were grown in suspension as previously described (18). A temperature-sensitive mutant of the Indiana serotype of VSV, *ts* G114, was used in these experiments. This temperature-sensitive mutant belongs to complementation group I, as defined by Pringle (16, 17), and is temperature sensitive in both primary and secondary transcription but not in the replication of 40S virion RNA (12, 13). *ts* G114 VSV was grown in suspended CHO cells at the permissive temperature of 31°C and purified by rate zonal centrifugation in sucrose gradients (18). Unpurified virus preparations of *ts* G114 VSV were used in some experiments as indicated. VSV wild type, the Indiana serotype (18, 23), was prepared by the same procedures.

Growth of VSV in continuous culture. Infection of cells, virus growth, and VSV plaque assays on CHO cells have been described in detail (11), except that the medium that was used was maintained at pH 7.4 by the organic buffers HEPES (*N*-2-hydroxyethylpiperzine-*N'*-2-ethanesulfonic acid; 0.25 mmol/ml) and TES [*N*-Tris(hydroxymethyl)-2-aminoethane sulfonic acid; 0.1 mmol/ml]. Infections were carried out in mini-Spinner bottles immersed in water baths held at the indicated temperature ± 0.01°C.

Radioactive labeling of DI particles. Preparation and analysis of radioactively labeled nucleocapsids of DI particles in the extracellular medium and in cytoplasmic extracts of infected cells were as described previously (11).

Materials. All radioisotopes were obtained from New England Nuclear, Boston, Mass. Hyperimmune rabbit anti-VSV serum was prepared as previously described (7) and inactivated at 56°C prior to use. Normal rabbit serum was a kind gift from A. H. Coons. Actinomycin D was kindly supplied by Merck, Sharp and Dohme, Rahway, N.J.

RESULTS

Growth of mutant *ts* G114 in CHO cells at different temperatures. To determine the amount of *ts* G114 produced at permissive, nonpermissive, and partially permissive temperatures, CHO cells were infected with the mutant at 31, 34.5, 37, and 38°C. The infected cultures were assayed at intervals after infection for plaque-forming virus at 31°C. *ts* G114 grew to a maximum titer of 2.4×10^8 PFU/ml at 31 and 34.5°C (Fig. 1). At the partially nonpermissive temperature of 37°C, the production of infectious virus was inhibited by 2 logs. At 38°C, virus growth was completely inhibited. The titer at 37°C was higher than previously published

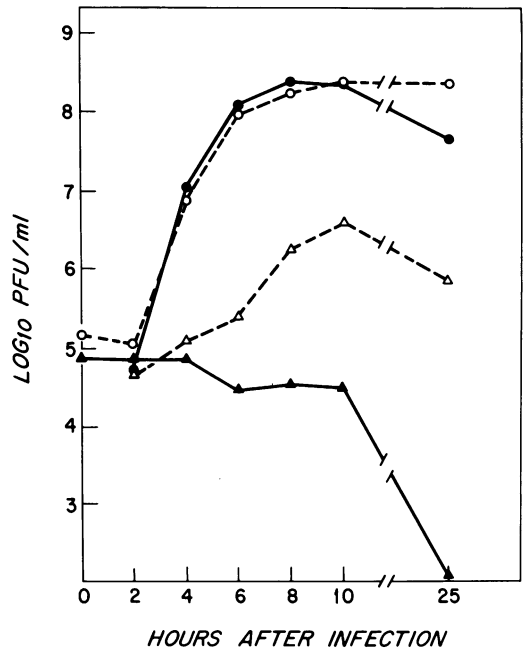

FIG. 1. *Growth of ts G114 in CHO cells at different temperatures. Portions of CHO cells (2.4×10^7) were incubated with purified B particles of ts G114 at a multiplicity of 10 for 30 min at 4°C and then washed three times with medium. The infected cells were suspended in 20 ml of organically buffered medium held at 31, 34.5, 37, or 38°C. Portions were taken at the indicated time after infection and plaque assayed at 31°C. Symbols: ○, 31°C; ●, 34.5°C; △, 37°C; ▲, 38°C.*

observations on *ts* G114 from this laboratory (13). This difference can be explained by the incorporation of organic buffers for the maintenance of an optimal pH for virus growth at the different temperatures (7). Therefore, in these experiments the temperature of 31 and 34.5°C are permissive temperatures; 37°C is partially permissive; and 38°C is nonpermissive for *ts* G114.

Effects of temperature on the cyclic production of *ts* G114 during continuous passage in CHO cells. To study the effects of temperature on the cyclic production of *ts* G114, a preparation of *ts* G114 containing standard and DI particles in a ratio of 20:1 was passed successively at the four different temperatures and a detailed analysis was made on: (i) the production of standard virus, (ii) the production of DI particles and (iii) the ability of cells to survive each 24-h period of infection. At 31 and 34.5°C, *ts* G114 grew in a cyclic fashion over a range of 4 logs, as had been found with wild-type VSV (Fig. 2A and 2B; cf. Fig. 6C). Maximum synthesis of standard and DI particles appeared to be the same at both temperatures. The only ob-

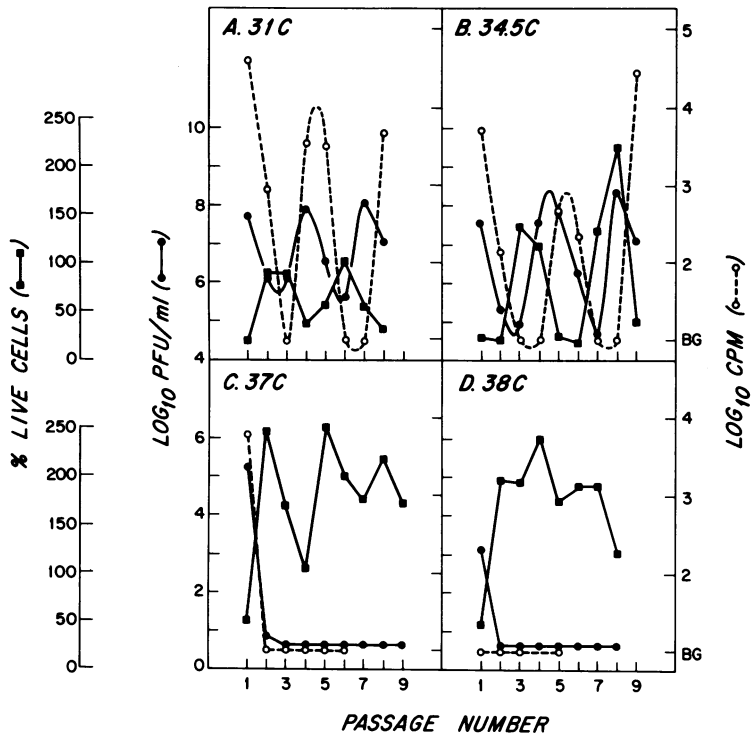

FIG. 2. *Continuous growth of standard and DI particles of ts G114 and survival of infected CHO cells at 31, 34.5, 37, and 38°C. At the first passage, 2.4 × 10⁷ CHO cells were infected with mixture of B and T particles of ts G114 at multiplicity of 24 for B particles. After 30 min of attachment at 4°C, the cells were quickly warmed to each of the incubation temperatures and diluted to 20 ml with the addition of prewarmed, organically buffered growth medium and then incubated at the same temperature for 22 h. Successive passages were made by resuspending fresh CHO cells with 2 ml of infected cell suspension from the previous passage, and a portion was taken and plaque assayed on CHO cells at 31°C. Also, portion of cells was stained with 0.2% trypan blue, and the nonstained live cells were expressed as a percentage of live cells found at the beginning of each passage. At each passage, a second portion was passed and actinomycin D was added at 5 μg/ml during the attachment period and [³H]uridine, at a final concentration of 10 μCi/ml, was added to cells at the beginning of each incubation period. After 22 h the extracellular labeled viral particles were pelleted from the medium of the infected culture by centrifugation at 78,500 × g for 30 min at 4°C in a Beckman type 30 rotor. The pellet was resuspended in buffer containing detergents, and the nucleocapsids were isolated by rate zonal centrifugation as previously described (11). Total counts per minute in the DI nucleocapsids sedimenting at 60S represents extracellular DI particles. These counts per minute are plotted together with viable cells and plaque-forming standard virus at each passage. Symbols: ■, percentage of live cell; ○, DI nucleocapsids; ●, titer of ts G114 in CHO cells. BG represents the background limit of detection in the plaque assays.*

servable difference was that cell killing by VSV was slightly less effective at the higher temperature (Fig. 2). At 34.5°C, cells even multiplied well during passages three and eight, whereas infected cells at 31°C showed some cytopathic effect during all passages.

Passaging *ts* G114 at 37 and 38°C showed a rapid loss of infectious standard virus (Fig. 2C and 2D). DI particles were detected at 37°C only during the first passage; none could be detected at any time when passed at 38°C. The rapid drop in infectivity (about 6 logs) between passage 1 and 2 at 37°C cannot be accounted for solely by the partially nonpermissive condition, which would reduce virus growth only by 99% (see Fig. 1). Therefore, DI particles, although undetectable by our assay procedures, must play an additional inhibiting role. By shifting the cell cultures from 37 to 31 or 34.5°C, standard virus was readily rescued from passages 1 to 3 but not from subsequent passages (data not shown). Thus, the inhibiting effects of temperature and DI particles appear to result in curing the infected cultures of the presence of virus. A similar observation on curing has been made by Youngner and his colleagues, although DI particles were not implicated in their system (21).

During each passage of the experiment shown in Fig. 2, total intracellular DI nucleocapsids were also measured, and the amount

was compared to that of extracellular nucleocapsids (Fig. 3). More DI nucleocapsids accumulated in the infected cells than in the extracellular medium during most of the passages. The levels of intracellular DI nucleocapsids varied in the same cyclical fashion as the extracellular DI nucleocapsids, reflecting a direct relationship between the production of intracellular nucleocapsids and DI particles (Fig. 3). Thus intracellular accumulation of antigens such as nucleocapsids indicate the production of DI particles.

The temperature of incubation affects the production of both standard and DI particles of *ts* G114 in the same way, showing, as had been shown with wild-type (11), that cyclical production of the two virus particles is an interrelated phenomenon. These experiments also demonstrate that persistence of temperature-sensitive virus production cannot be maintained under nonpermissive conditions. Some synthesis of the virus, either by its inherent leakiness or by partial permissiveness, is necessary for continued viral presence.

Rate of reversion of *ts* G114 at different temperatures. To measure the relative reversion frequencies of *ts* G114 at 31, 34.5, 37, and 38°C, the progeny from each of the passages shown in Fig. 2 was also plaque assayed at 38°C. Figure 4 shows that revertants were present at 34.5°C and not at any of the other temperatures (data from 37 and 38°C not shown). It is not clear if the occurrence of revertants at 34.5°C is a rare event or not. Temperatures of 37 and 38°C barely allow for enough replication of *ts* G114 for the generation of revertants (see Fig. 2).

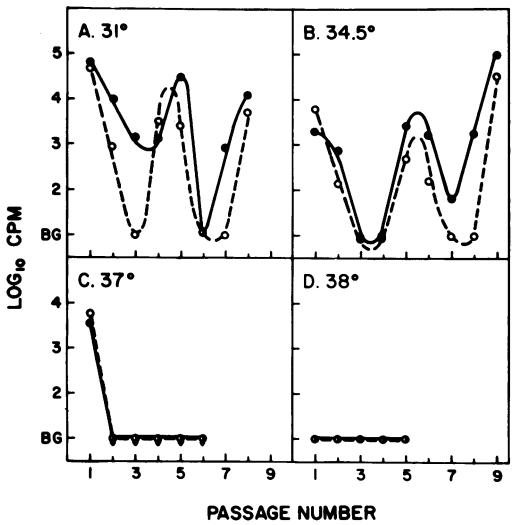

FIG. 3. *Distribution of [^{3}H]uridine-labeled DI nucleocapsids in and outside the cell during undiluted passages at 31, 34.5, 37, and 38°C. To detect intracellular nucleocapsids, the [^{3}H]uridine-labeled, infected cells used for Fig. 2 were analyzed for intracellular nucleocapsids on sucrose gradients exactly as described (11). The total counts per minute sedimenting at 60S represent intracellular DI nucleocapsids. For comparison, extracellular DI nucleocapsids shown in Fig. 2 are also plotted. Symbols: ●, cell-associated DI nucleocapsids; ○, extracellular DI nucleocapsids.*

Of particular interest is the cyclic growth of the revertants themselves at 34.5°C once they were generated. It is apparent that the temperature-sensitive virus remained in excess of the revertant virus during each burst of production of standard virus. Both revertant and temperature-sensitive mutant were inhibited by DI particles, which reduced the revertants proportionately to the temperature-sensitive mutants. Thus, the revertants were kept in the minority during each cycle. Because 34.5°C is permissive for *ts* G114, the cyclic synthesis of revertant and mutant resulted in the inability of revertants to become a majority of the population. It is possible, however, that upon infinite passaging the revertants would eventually become the dominant standard virus in the population.

On occasions, revertants of *ts* G114 have been detected after passaging at 37 and 38°C. Figure 5 demonstrates one such example at 37°C, where standard virus titers increased after the third passage instead of dropping to undetectable levels. Revertants gradually became a major part of the viral population on continuous passaging. Several factors play a role in this infection. At 37°C, *ts* G114 has only a 1% leak. If a revertant did arise early during the passaging, it would multiply readily to detectable levels. However, from passage 5 through 7 the

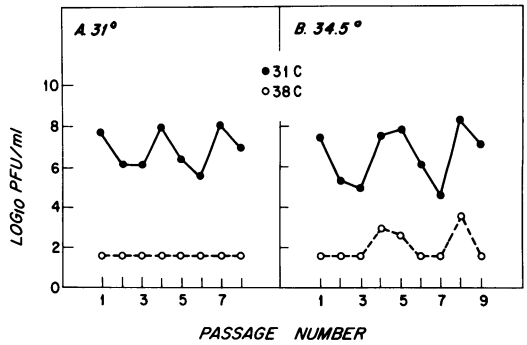

FIG. 4. *Plaque assay at permissive and nonpermissive temperatures of ts G114 grown under conditions of continuous culture. ts G114 passaged at 31 and 34.5°C identically to that shown in Fig. 2 was plaque assayed on CHO cells at the two different temperatures. Symbols: ●, 31°C; ○, 38°C.*

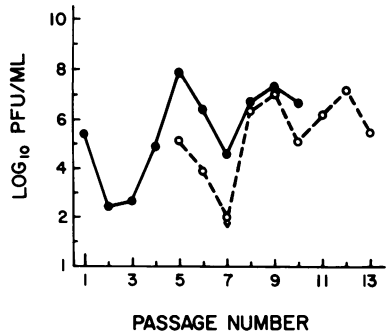

FIG. 5. *Appearance of revertants during continuous passaging of ts G114 at 37°C. Passage of ts G114 was done in the same continuous manner as that shown in Fig. 2. Revertants were not detected prior to passage 4, and after passage 9 there were no significant differences between plaque assays done at 31 or 38°C. Symbols: ●, titer of ts G114 at 31°C; ○, titer of ts G114 at 38°C.*

proportion of revertants unexpectedly remained lower than *ts* G114. This can be understood from the observations of Youngner and Quagliana (22), who noted that revertants complemented temperature-sensitive mutants of the same complementation group at the nonpermissive temperature and, in turn, were inhibited by the temperature-sensitive mutant. However, in this particular case (Fig. 5) the temperature-sensitive mutant was so reduced by passage 7 that not enough cells were coinfected with *ts* G114 and revertant to permit rescue of *ts* G114 by the revertant at this passage. Due to the cumulative inhibiting effects of nonpermissiveness and DI particles, the revertants became dominant in the culture and developed their own cyclic pattern of growth.

Therefore, two examples are given where revertants arise during an infection initiated by a temperature-sensitive mutant. In one case, because the temperature was permissive for the mutant, revertants were kept in the minority by the overall cyclic nature of virus growth (Fig. 4). In the second example, the rare revertant arising at the relatively nonpermissive condition did eventually become the dominant type of standard virus in the culture (Fig. 5).

Effects of antibody on cyclical growth of wild-type VSV during continuous passage in CHO cells. To study the effects of anti-VSV antiserum on production of standard and DI particles, several concentrations of antiserum were incorporated into the medium at each passage of wild-type VSV. At concentrations of antiserum that would neutralize 99.9% of VSV, cyclic growth of the virus was prevented; after the initial passage, virus particles were no longer detectable (data not shown). Figures 6A and 6B demonstrate the effect of neutralizing only 90% of the virus. When VSV was only partially neutralized at each passage, cyclic growth was able to proceed for sometime. Although the data are somewhat variable, it is clear that cyclic growth can continue to occur in the presence of antiserum. The maximal amount of inhibition became greater after each cycle of growth. Finally, virus production became undetectable.

If both standard and DI particles were neutralized to 90% by antiserum during each passage, such a cyclic growth pattern would not be expected. An explanation for the burst in synthesis of standard VSV in the presence of limiting amounts of antiserum is likely to be found in the synthesis of both DI particles and virus-specific soluble antigens that can combine and inactivate antibody. Evidence for this comes from experiments where conditions of complete interference are established by DI particles. These cultures carry on primary translation by standard VSV with some replication of DI particles. At the same time, considerable soluble virus-specific antigens are released by the infected cells (S. Little and A. S. Huang, manuscript in preparation).

DISCUSSION

Cyclic production of animal viruses due to the production of DI particles has been reported for several animal viruses (1, 8, 10, 19). Such cyclic viral growth patterns have important implications for the outcome of virus infections. In our model system with VSV and its DI particles, it is shown that the response of individual host cells varies over a wide range depending on the total amount of virus particles and the relative ratios of standard to DI particles in the infected culture (11).

The addition of another parameter such as temperature or anti-VSV antisera to such an analysis of cyclic growth yields several interesting and sometimes unexpected observations. The effects of temperature on the growth of a temperature-sensitive mutant results is an overall inhibition of standard and DI particles as the temperature becomes less permissive. Why revertants do not always become the major standard virus during such infections is apparent in the analysis of *ts* G114 grown at 34.5°C. At this temperture, which is permissive for the mutant, revertants, once they arise in the population, are readily detected during every passage. Whether revertants arise more frequently at this temperature than at 31°C is not known. The revertant is kept from becoming the majority of the standard virus popula-

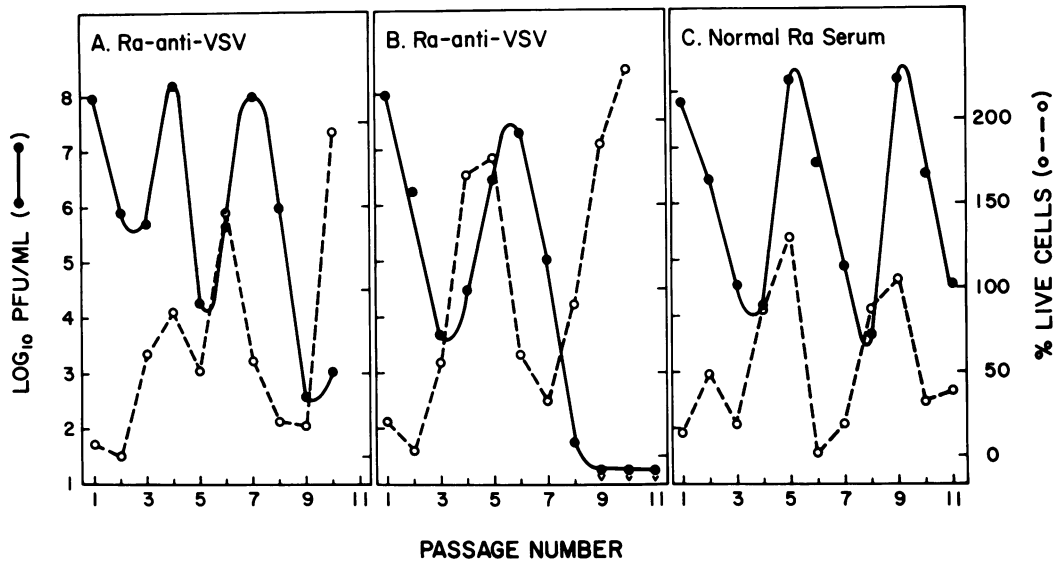

FIG. 6. *Cyclic growth pattern of VSV in the presence of antibody. At the first passage, three portions of CHO cells (1.2 × 10⁷) were infected with a preparation of wild-type VSV containing standard and DI particles both at a multiplicity of 35. After 30 min of attachment at room temperature, two portions of infected cells were diluted to 10 ml with prewarmed, organically buffered medium containing rabbit anti-VSV serum at a concentration known to neutralize 90% of standard VSV. A control aliquot of infected cells was diluted in medium containing the same concentration of normal rabbit serum. All samples were incubated at 37°C for 22 h. Further successive passages were similarly made with 1 ml from the previous passage by mixing it with fresh CHO cells (1.2 × 10⁷) in the presence of the same amount of immune or normal rabbit serum. At the end of each passage, a portion was taken and plaque assayed at 37°C. The percentage of live cells from each passage was determined as described in the legend to Fig. 2. Passages shown in (A) and (B) are duplicate cultures done in the presence of antiserum capable of neutralizing 90% of the virus at each passage. Passages shown in (C) are made in the presence of normal antiserum. Symbols: ●, titer of standard virus; ○, live cells.*

tion by a periodic reduction of both types of standard viruses. Interference by DI particles can account for this periodic inhibition. The situation becomes more complex as the temperature is raised and relatively nonpermissive conditions exist for the mutant. Here, the nonpermissiveness inhibits the mutant so that eventually the revertant dominates the infection.

Under somewhat similar circumstances Youngner and Quagliana (22) reported that the rescue of another temperature-sensitive mutant by revertants and the subsequent interference against the revertant kept the revertant population at a low level. Continuous monitoring in our system indicates that the degree of permissiveness controls the eventual outcome. Rescue of the mutant by revertants can occur at nonpermissive temperatures only when the multiplicities are high; at low multiplicities coinfections would not occur and the infection becomes cured of the mutant. Under conditions of nonpermissiveness the revertant will rapidly take over the infection.

These results with our model system at different temperatures confirm and add to previously published analysis of VSV growth in persistently infected cell cultures (3, 14, 15, 21, 22). Some general conclusions are as follows. (i) To establish persistent infections, virulent viruses need to be inhibited to some degree so that cell killing is ameliorated; this can be accomplished by DI particle, by temperature sensitivity, by the addition of antisera, or by other factors. (ii) Once established, maintenance of persistence requires some virus growth. (iii) Virus growth results in concomitant DI particle synthesis, despite the inability to detect them under some circumstances. (iv) Dissecting these interactions between virus particles depends on the identification of each of the particles present and on the knowledge of their individual capabilities or defects. Total nonpermissiveness for temperature-sensitive virus growth results in "curing" cells of VSV unless a revertant arises.

Growth in the presence of a limiting concentration of antisera yields the surprising result of continued cyclic production despite the presence of antisera. Therefore, in compartments of the whole animal where the antibody level may be lower than in serum, virus replication in a

cyclic fashion may occur, thus allowing persistence of the virus. These results can be explained in terms of our detailed knowledge of the VSV-cell interaction, where cell-associated and extracellular viral antigens are produced in the absence of genome replication (Little and Huang manuscript in preparation). Such antigens can tie up antibody and thereby permit the spread of infectious progeny. Therefore, model systems such as the VSV-CHO cell system, on the cellular level, provide a useful tool in the dissection of the relevant parameters that regulate the final outcome of the interactions between viruses and hosts. The outcome of these interactions depends on a thorough understanding of the properties of individual viruses, their capabilities, and their defects. Only then, with the knowledge that interactions among virus particles occur at high multiplicities and not at low multiplicities, can extrapolations be made to studies on VSV in the whole animal (2, 4, 20).

ACKNOWLEDGMENTS

This study was supported by research grant VC-63A from the American Cancer Society and by Public Health Service grant AI-10100 from the Natioinal Institute of Allergy and Infectious Diseases. P.S. was a postdoctoral fellow of the Rockefeller Foundation, and A.S.H. was a Public Health Service Research Career Development Awardee (grant no. K04 AI 70413) from the National Institute of Allergy and Infectious Diseases.

We thank Norma Hewlett for excellent technical support and Gail Clinton for critically reading the manuscript.

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