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Growth and Genetic Stability of the *ts*-1 Mutant of Respiratory Syncytial Virus at Restrictive Temperatures

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An in vitro study was performed to define in greater detail those factors which favored the growth of the *ts*-1 mutant of respiratory syncytial virus under restrictive conditions and the emergence of genetically altered virus with decreased temperature sensitivity. Replication of *ts*-1 occurred at each of the restrictive temperatures of 37, 38, and 39 C, even though plaque formation was not observed. The level of virus growth under restrictive conditions was inversely related to the incubation temperature and directly related to the multiplicity of infection. These relationships appeared to reflect the effect of restrictive temperature in reducing the quantity of virus produced and released from an infected cell. Under restrictive conditions the production of genetically altered virus which exhibited reduced temperature sensitivity was directly related to the multiplicity of infection and inversely related to temperature. Production of genetically altered virus was not observed under permissive conditions.

The *ts*-1 temperature-sensitive mutant of respiratory syncytial virus has been investigated extensively both in vitro and in vivo in conjunction with its development and subsequent evaluation as a candidate strain designed for use in a live virus vaccine (3, 5–7, 10, 18–20). *ts*-1 was shown in vitro to be markedly restricted in growth at 39 C and unable to form plaques at 37 C and above, whereas wild-type virus grew and formed plaques without restriction at 39 C (3). During clinical trials *ts*-1 virus was found to retain a small amount of residual virulence for seronegative infants and to occasionally exhibit genetic instability as indicated by the emergence of genetically altered virus with decreased temperature sensitivity during infection of young vaccinees (5, 7). As a result of these clinical findings, the present in vitro study was performed to define in greater detail those factors which favored the growth of the *ts*-1 mutant and the emergence of genetically altered virus under restrictive conditions. It was hoped that examination of the behavior of *ts*-1 under restrictive conditions would permit a more thorough understanding of the mechanism(s) of defectiveness of this virus and also prove useful in devising in vitro criteria for selection of new respiratory syncytial virus mutants which are more attenuated and stable.

MATERIALS AND METHODS

Viruses and cell cultures. The *ts*-1 mutant of respiratory syncytial virus was isolated from the progeny of wild-type virus grown in the presence of 10^{-4} M 5-fluorouridine (3). Studies concerned with genetic stability of the mutant were performed with virus grown in bovine embryonic kidney culture which had been evaluated previously in children (7) or with a higher titered bovine embryonic kidney-grown virus suspension which was initiated with the same seed as the vaccine preparation. In other studies a HEp-2 cell-grown suspension of virus initiated with the bovine embryonic kidney-grown vaccine virus was used. The three *ts*-1 virus suspensions exhibited the same degree of temperature sensitivity and differed only in their titer at 32 C.

HEp-2 monolayer roller tube cultures were used for growth of virus, whereas HEp-2 cell 96-well trays (Linbro, New Haven, Conn.) were used for assay of infectivity and HEp-2 Leighton tube cultures were used for immunofluorescence studies. These cultures were obtained from Flow Laboratories (Rockville, Md.) and maintained as previously described (3).

Viral infectivity assays. Details of methods for determining efficiency of plaque formation at different temperatures have been described previously (5).

Replication of ts-1 mutant at different temperatures. HEp-2 roller tube cultures were inoculated with 0.1 ml of virus. Virus was allowed to absorb for 90 min at room temperature, and then infected cultures were fed 1 ml of maintenance medium. Roller tubes were sealed by immersion in liquid paraffin and incubated at 32 C in a forced air incubator or at 37, 38, or 39 C in a water bath in which the temperature was regulated within 0.05 C by a Braun constant-temperature water circulator.

Immunofluorescence. Leighton tube cultures (2×10^5 cells/cover slip) were infected with 0.1 ml of virus, incubated 90 min at room temperature, and

then fed with 1 ml of maintenance medium. After an appropriate incubation period, cultures were fixed in acetone at −60 C for 20 min. Fixed preparations were treated with 0.1 ml of 1:10 dilution of human convalescent serum (respiratory syncytial virus plaque reduction neutralizing antibody titer of 1:1,024) for 30 min at room temperature, exposed for 30 min to goat anti-human-gamma-G-globulin conjugated with fluorescein isothiocyanate (Antibodies, Inc., Davis, Calif.) diluted in a 1:10,000 concentration (wt/vol) of Evans blue in phosphate-buffered saline, and then mounted in 80% buffered glycerol. Specimens were examined with a Zeiss microscope having a primary interference filter (Baltimore Instrument Co., Baltimore, Md.; FITC filter no. 910031), a 515-nm secondary filter, and a 60-W tungsten light source. The number of cells producing viral antigens in a population was quantitated by counting the number of antigen-positive cells in at least 2,000 cells/cover slip.

Infectious center assay. After the fluid medium had been removed, centrifuged at 1,000 rpm (International PR-J centrifuge), and quick frozen at −70 C, the cell monolayer was washed three times with Eagle minimal essential medium, twice with 0.02% EDTA-0.05% trypsin, and incubated in the latter solution at 32 C until the cells had detached from the glass. The cells were then washed twice, and the final cell pellet was suspended and diluted in Tris-buffered saline. The diluted material was then inoculated onto HEp-2 cell monolayers in 96-well Linbro trays, methyl cellulose overlay medium was added 30 min later, and the trays were incubated at the permissive temperature of 32 C. Plaques were counted after 7 days of incubation. An aliquot of the cell suspension was also quick frozen and stored at −70 C for later assay of cell-associated infectivity.

RESULTS

Growth of ts-1 virus at restrictive temperatures. Previously the *ts*-1 mutant was shown to be completely restricted in plaque formation at temperatures from 37 to 39 C; growth of virus, however, still occurred at 39 C but was markedly reduced when compared to replication at 32 C (3). To define more precisely the growth of the *ts*-1 mutant at restrictive temperatures, replicate HEp-2 cell cultures were inoculated with virus (multiplicity of infection [MOI] 0.1 PFU/cell), incubated at 32, 37, 38, or 39 C, and sampled daily for 7 days, and the amount of virus produced was quantitated by plaque assay at 32 C. Replication occurred at each of the restrictive temperatures, but the amount of virus produced was inversely related to temperature and was significantly reduced compared either to peak titer of wild-type respiratory syncytial virus at the same temperature (data not shown) or the peak titer attained by the *ts*-1 mutant at permissive temperature (Fig. 1). The effect of restrictive temperature upon growth of *ts*-1 virus was confirmed in a series of other experiments, one of which is shown in Table 1.

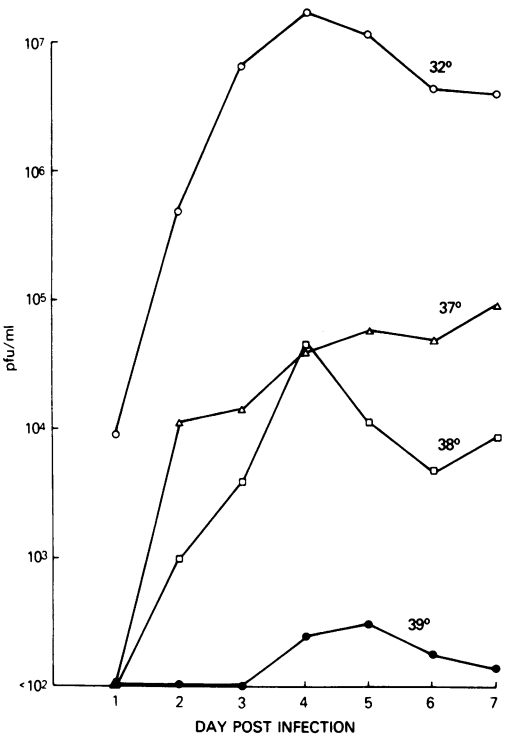

FIG. 1. *Growth of ts-1 virus at permissive (32 C) and restrictive temperatures (37, 38, or 39 C). HEp-2 cell cultures inoculated with ts-1 virus (MOI = 0.1 PFU/cell) were incubated at 32, 37, 38, or 39 C. Cells and fluid were harvested together daily, quick frozen at −70 C, and stored at that temperature until assayed at 32 C for infectious virus by the plaque technique.*

Effect of MOI on viral growth at restrictive temperatures. In the past, growth of *ts*-1 virus at 39 C was quite variable and the cause of this variation was not understood. The basis for this variability became clear when the effect of input multiplicity was investigated. As shown in Table 1 and Fig. 2, growth of the *ts*-1 mutant at restrictive temperatures was a direct function of MOI. Titers attained at 6 days after inoculation of replicate cell cultures with different amounts of virus were found to be directly and linearly related to the amount of input virus; this relationship was seen at each of the restrictive temperatures but was not observed at the permissive temperature. At the permissive temperature of 32 C, lowering the MOI delayed the time of onset but not the magnitude of the peak viral titer (Fig. 2A). At 38 or 39 C (Fig. 2C and D), however, maximal level of replication was achieved by day 3 to 4, and this level was then

TABLE 1. *Effect of MOI upon growth of ts-1 virus and emergence of genetically altered virus after incubation of cultures at restrictive temperature*

Temp of incubation (C)	Titer of virus produced at indicated MOI[a] (log_{10} PFU/ml)			Proportion of inoculated HEp-2 cultures which yielded virus with ability to produce plaques at indicated restrictive temp (C)								
				37			38			39		
	0.1	0.01	0.001	0.1[b]	0.01	0.001	0.1	0.01	0.001	0.1	0.01	0.001
37	5.0	4.2	3.1	12/12	3/12	3/24	9/12	2/12	2/24	8/1	2/12	1/24
38	4.1	3.0	1.8	9/11	1/10	1/24	8/11	1/10	1/24	7/11	1/10	0/24
39	2.3	<1.0	<1.0	6/24	0/24[c]	0/24[c]	6/24	0/24[c]	0/24[c]	5/24	0/24[c]	0/24[c]

[a] Geometric mean of virus titer of 10 to 24 replicate ts-1-infected HEp-2 cell cultures after 6 days of incubation.
[b] MOI.
[c] Viral growth was not detected in any of the inoculated cultures.

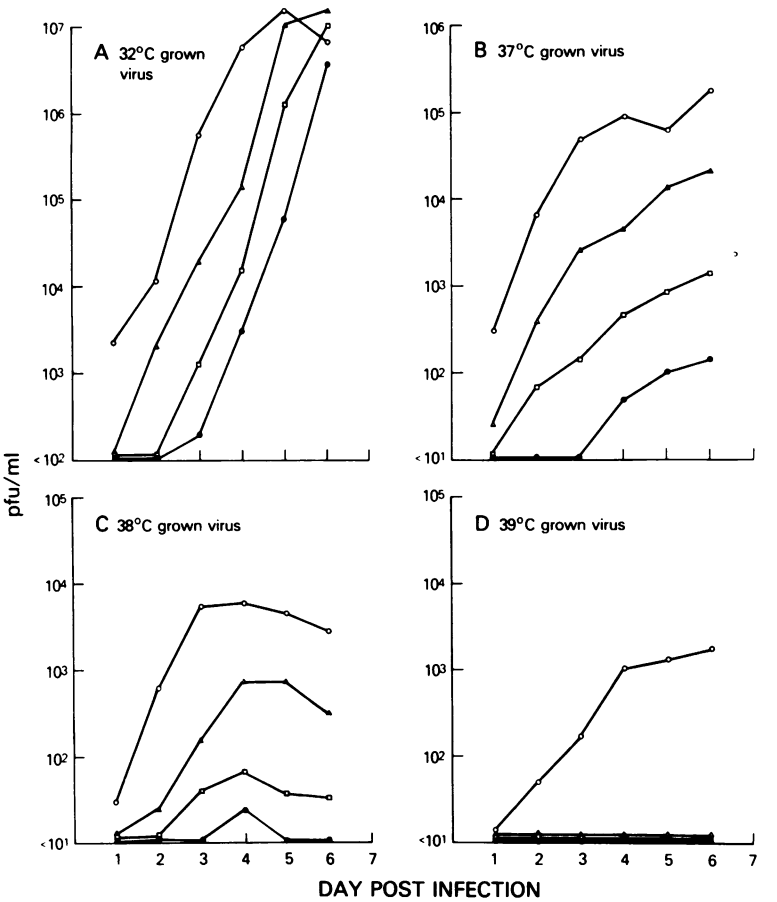

FIG. 2. *Effect of MOI on growth of ts-1 virus at permissive (32 C [A]) and restrictive temperatures (37 C [B], 38 C [C], or 39 C [D]). HEp-2 cell cultures were infected with ts-1 virus at a MOI of 10^{-1} (○), 10^{-2} (△), 10^{-3} (□), or 10^{-4} (●), and incubated at 32, 37, 38 or 39 C. Cells and fluid were harvested together daily, and infectious virus was quantitated by plaque assay at 32 C.*

maintained for the duration of the experiment, i.e., an additional 2 to 3 days. The plateau titer attained was directly related to the size of the input inoculum. Infected cultures incubated at 37 C (Fig. 2B) failed to show a definite peak in viral growth, and the titer was still rising at the end of 6 days. Nonetheless, the viral titer attained on any given day was still directly related to the initial MOI.

Effect of restrictive temperatures on

spread of infection. Two sets of experiments were performed to evaluate the effect of restrictive temperature on the spread of infection. First, *ts*-1-infected Leighton tube cultures (MOI = 0.1 PFU/cell) were incubated at permissive (32 C) or restrictive temperature (37, 38, or 39 C) for 5 days, and cover slip preparations were fixed daily and examined for the number of fluorescent foci present by indirect immunofluorescence. At 24 h after inoculation approximately the same percentage of cells contained respiratory syncytial virus antigen in each of the experimental groups, indicating that the early steps of infection up to and including antigen production were intact at each of the incubation temperatures (Table 2). With longer incubation times, however, there was an increase in the percentage of cells expressing respiratory syncytial virus antigens at both 32 and 37 C, and this increase was more rapid in the former than the latter. With continued incubation at 38 and 39 there was no increase in the percentage of cells expressing viral antigens. On the other hand, there was no decrease in antigen-containing cells, suggesting that virus persisted in such cells in a manner which allowed expression of some viral functions without destruction of the host cell.

Spread of infection under restrictive conditions was also examined by the infectious center technique. Replicate cell cultures were infected with *ts*-1 virus (MOI = 0.001 PFU/cell) and incubated at 32, 37, 38, or 39 C. Duplicate cultures were harvested at 6 h postinoculation and then daily for the duration of the experiment, and the number of infectious centers, corrected for cell number, was determined for each culture. There was an increase in the number of infectious centers with time in cultures incubated at 32 C until, by day 6, most cells were capable of giving rise to infectious virus (Fig. 3). Infected cultures incubated at 37 C showed a similar, although less rapid, increase in the number of infectious centers with time. At 38 and 39 C there was no increase in the relative number of cells able to produce infectious virus, confirming the results of the fluorescent focus assay. In addition, the number of infectious centers detected in cultures incubated at 38 or 39 C approximated the input titer of *ts*-1 virus. These observations suggest that the *ts*-1 mutant was able to initiate infection at each of the restrictive temperatures as efficiently as it did at permissive temperature. In addition, although *ts*-1 virus was able to induce production of antigens but not infectious virus at the restrictive temperatures of 38 and 39 C, it was able to persist in infected cells at restrictive temperature in a manner permitting full expression of all viral functions, with the resultant production of infectious progeny after shift down to permissive temperature.

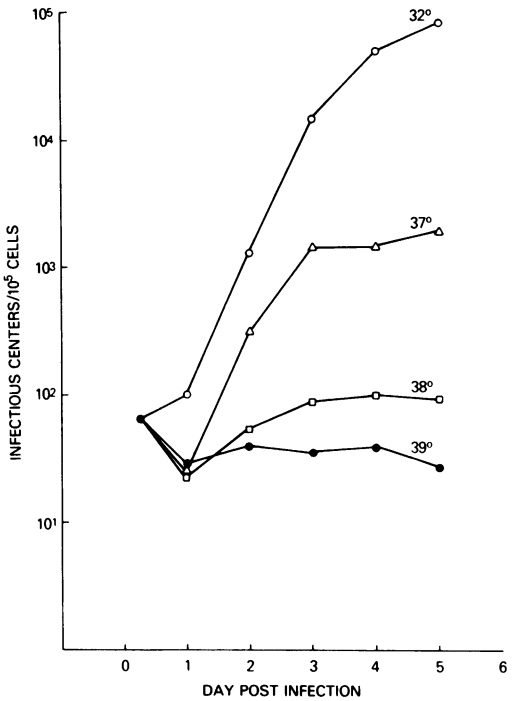

FIG. 3. *Time course of increase of infectious centers in ts-1-infected cultures incubated at permissive and restrictive temperatures. HEp-2 cell cultures were inoculated with ts-1 virus at a MOI of 0.001 PFU/cell. The cultures were incubated at 32, 37, 38, or 39 C, and at daily intervals the number of infectious centers per 10⁵ cells in each culture was determined at 32 C as described.*

TABLE 2. *Proportion of HEp-2 cells with antigens of the ts-1 mutant detectable by indirect immunofluorescence*

Incubation temp (C)	Cells positive by indirect immunofluorescence on indicated day after infection[a] (%)				
	1	2	3	4	5
32	4	15	90	90	NT[b]
37	6	7	15	25	90
38	3	6	5	6	5
39	4	3	2	3	2

[a] Input MOI, 0.1.
[b] NT, Not tested.

Effect of temperature on infectious virus-to-infected cell ratio. The number of infectious virions present in an infected cell was estimated in an attempt to quantitate more fully the suppression of viral growth at restric-

tive temperature. Growth of virus at 32 C yielded a cell-associated infectious virus-to-infectious center ratio which varied from 1 through 5 at different times in the growth cycle. Shown in Table 3 is the ratio observed on day 5. At incubation temperatures of 37 and 38 C this ratio was depressed for the duration of the experiment to a level of approximately 0.1. At 39 C the infectious virus-to-infectious center ratio was 0.01 or lower. It appeared that at restrictive temperatures production of infectious virus occurred in only a small proportion of infected cells at any one time and this "leakiness" was temperature dependent. In addition, at 38 and 39 C free virus was not detectable in the supernatant fluid, suggesting that a second temperature-dependent mechanism, inhibition of viral release, was also operative at these restrictive temperatures. However, the inactivating effect of higher temperature (38 or 39 C) on free virus cannot be ruled out as being responsible for this effect.

Production of altered virus able to produce plaques at restrictive temperatures. In recent vaccine trials of the *ts*-1 mutant in infants and young children a small proportion of the virus recovered exhibited a partial or complete loss of temperature sensitivity, i.e., change from *ts* to ts^+ (5). Since in many instances this type of virus retained some temperature sensitivity we preferred to designate it as genetically altered virus rather than revertant virus. It is possible that virus which exhibited complete loss of temperature sensitivity was revertant, but the possibility of suppressor mutation must also be considered for such virus as well as the intermediate forms. Subsequently, the emergence of genetically altered virus in cell cultures incubated at restrictive temperature was also noted. Genetic alteration in vitro, however, occurred irregularly and unpredictably. This in vitro phenomenon was investigated further after more was learned about factors which influenced growth of *ts*-1 at restrictive temperature.

TABLE 3. *Estimate of quantity of infectious virus recoverable per infectious center after 5 days of incubation at indicated temperature*

Temp of incubation (C)	Cell-associated infectivity (PFU/infectious center)[a]	Ratio of cell-associated infectivity to cell-free infectivity[b]
32	3.0	0.1
37	0.1	0.1
38	0.1	<0.001
39	<0.01	<0.001

[a] HEp-2 cells were inoculated with input multiplicity of 0.001; after 5 days of incubation the cells were washed three times with Eagle medium and removed from the glass surface with trypsin (0.05%) and EDTA and tested for infectious centers. After an aliquot of the cells was frozen and thawed the resulting suspension was assayed for infectious virus by the plaque assay.

[b] Supernatant fluid from cultures harvested after 5 days of incubation was also tested for infectious virus by plaque assay, and the ratio of cell-associated to cell-free infectivity was determined.

Virus produced by infected cultures which had been incubated at 32, 37, 38, or 39 C was titrated not only at 32 C (Fig. 1) but also at temperatures restrictive for *ts*-1 plaque formation to detect virus having an altered efficiency of plaquing compared to the input *ts*-1 virus. Virus produced at 32 C failed to produce plaques at 38 or 39 C; at 37 C small, abortive plaques that could not be easily quantitated were occasionally seen (data now shown). Growth of *ts*-1 at 37, 38, and 39 C did, however, result in the production of virus having an altered efficiency of plaquing (Table 1 and Fig. 4A–C). After replication of *ts*-1 at restrictive temperature a spectrum of altered viruses was detected (Table 1). Some of the altered virus resembled wild-type virus in producing plaques at each of the restrictive temperatures, whereas in other instances some degree of temperature sensitivity was retained, i.e., virus produced plaques at 37 C but not at 38 or 39 C or produced plaques at 37 and 38 C but not at 39 C. Plaques detected at restrictive temperature represented genetic alteration and not leakiness since analysis of 10 plaques produced at 38 C indicated that each contained virus which was not restricted in efficiency of plaque formation at 38 C. Altered virus first appeared early after infection (though not before day 2) in some experiments and at a later time in other experiments, suggesting that the genetic changes which led to alteration in efficiency of plaquing were a consequence of events which occurred at random during the growth cycle.

Effect of MOI and temperature on production of virus with altered efficiency of plaquing. Earlier it was shown that the number of cells infected and the amount of infectious virus produced during growth of *ts*-1 virus at restrictive temperature was a direct function of the input inoculum. This suggested that MOI might also have an effect on production of genetically altered virus. Replicate cultures were inoculated with *ts*-1 virus at MOIs of 0.1, 0.01 and 0.001 PFU/cell, incubated at 37, 38, or 39 C for 6 days, and then harvested and assayed for quantity of plaque-forming virus at permis-

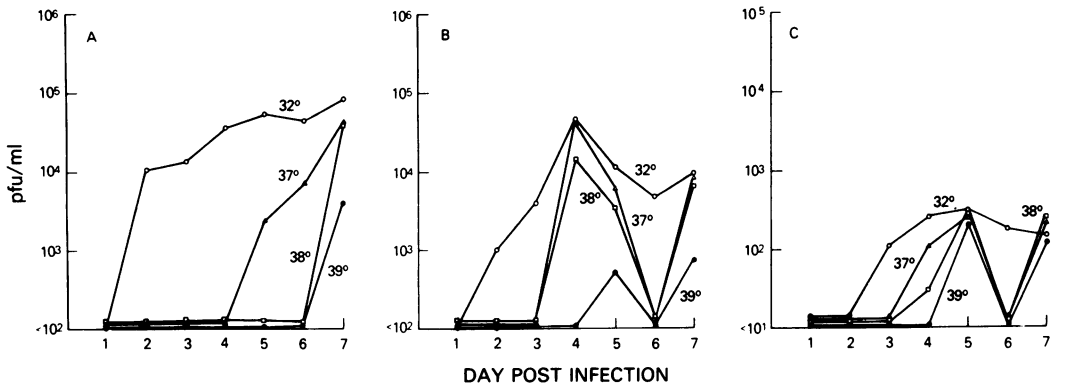

FIG. 4. *Detection of progeny virus with an altered temperature sensitivity of plaque formation during growth of ts-1 at 37 C (A), 38 C (B), and 39 C (C). Virus grown at restrictive temperature in the experiment shown in Fig. 1 was tested for capacity to produce plaques at 32 C (○), 37 C (△), 38 C (□), and 39 C (●) in an attempt to detect virus with an altered ts phenotype. Virus with an altered ts phenotype was not detected among the progeny of ts-1 grown at 32 C (data not shown).*

sive and restrictive temperatures. These results (Table 1) demonstrated that at restrictive temperatures the emergence of virus with an altered efficiency of plaquing was a direct function of the MOI. At the restrictive temperatures of 37 and 38 C, 90 to 100% of cultures infected at an MOI of 0.1 PFU/cell produced altered virus, whereas only 10 to 25% of cultures infected at an MOI of 0.01 PFU/cell yielded such virus. Importantly, altered virus was never detected in the growth yield of virus grown at permissive temperature, although the quantity of virus produced was considerably greater than that generated under restrictive conditions. Reconstruction experiments indicated that a large excess of *ts* virus (input MOI = 1) did not interfere significantly with detection of ts^+ virus at restrictive temperature. In three separate experiments the presence of *ts* virus at an MOI of 1 reduced the plaquing efficiency of wild-type virus at 38 C only two- to fivefold. Thus, if ts^+ virus were present in the 32 C growth yield it should have been detectable at restrictive temperature.

Emergence of virus with altered temperature sensitivity was consistently more frequent in cultures incubated at 37 C than in cells kept at 38 C and occurred least often at 39 C, indicating that the height of the restrictive temperature also exerted an effect which was independent of input multiplicity.

DISCUSSION

It has been suggested that the failure of the *ts*-1 mutant of respiratory syncytial virus to form plaques at 37 C and above (restrictive temperatures) was a reflection of its inability to replicate at these temperatures (3). However, the pattern of growth of the mutant was studied in detail only at 39 C, and the small amount of infectious virus detected in various harvests was interpreted as a residual of the inoculum rather than evidence of viral replication. In the present study, growth of *ts*-1 virus was examined in detail at the restrictive temperatures of 37, 38, and 39 C using different-sized inocula. Replication occurred at each of these temperatures, even though plaque formation was not observed. At restrictive temperatures, the peak viral titer was inversely related to temperature, due, in part, to an effect of temperature on reduction in the amount of infectious virus produced per infected cell.

The observation that production of infectious virus proceeded without observable cytopathic effect at restrictive temperatures suggested that under these conditions the *ts*-1 virus was capable of completing its normal replicative cycle, including budding from the cell membrane, without expression of those factors responsible for syncytium formation (fusion factor) and cell lysis as has also been described for other viruses (1, 13, 17), or that some late block occurred in the replicative cycle which inhibited virus release, thus limiting viral replication to those cells originally infected. The fact that growth of *ts*-1 was found to be a direct and linear function of the input inoculum at restrictive temperatures and that peak viral titer tended to plateau in cultures incubated at 38 or 39 C suggested that the spread of infection was inhibited under these conditions. This was indeed shown to be the case when infected cultures incubated at 38 or 39 C were examined by the fluorescent focus and infectious center assays.

Study of the course of infection at restrictive

temperatures by the fluorescent focus and infectious center assays also suggested that the *ts*-1 mutant was able to induce infection at each of the restrictive temperatures as efficiently as it did at permissive temperature. Although about 50% of infected cells produced viral antigen, only a minority (10% at 38 C, <1% at 39 C) produced infectious virus at these restrictive temperatures. However, when infected cells were "stepped down" to permissive temperature, each infected cell was able to produce infectious virus, indicating that *ts*-1 virus was able to persist in these cells at restrictive temperature in a noninfectious state, its maturation blocked by the elevated temperature. Similar findings have been reported with other viruses during establishment of persistent infections in vitro (4, 8, 9). Under appropriate conditions, partial inhibition of viral function effected by mechanisms such as elevated temperature (with *ts* viruses), restrictive host cells, or interferon has resulted in persistence of virus (16). Interestingly many (8, 11, 12, 14), although not all (M. linuma and R. W. Simpson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S197, p. 246), of the viruses recovered from persistent in vitro infections have been shown to be temperature-sensitive, conditional lethal mutants. Recently it has been suggested that the genetic material of several RNA viruses, including respiratory syncytial virus, exists in persistently infected cells in the form of complementary DNA (21, 22; Iinuma and Simpson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S197, p. 246). This aspect of persistence of the *ts*-1 mutant at restrictive temperature should be evaluated.

Genetic instability of the *ts*-1 virus, as indicated by a partial or complete loss of temperature sensitivity, was first seen during evaluation of the mutant in infants and young children (5). In the present study this type of genetic alteration was shown to occur in vitro subsequent to viral growth at each of the restrictive temperatures. Indeed, the frequent appearance of altered virus in cultures infected with the highest MOI of *ts*-1 made it difficult to determine if the pattern of growth was attributable to the *ts*-1 virus itself or to the development of altered virus which might have complemented the *ts* virus population. At lower input multiplicities, however, altered virus was detected infrequently, and the patterns of growth which were seen paralleled those observed with the highest MOI.

The frequency at which *ts*-1-infected cultures gave rise to virus with altered temperature sensitivity was a function of both the incubation temperature and the size of the input inoculum, the same two factors which influenced the extent of viral replication at restrictive temperatures. Since genetic change in *ts*-1 was favored by those conditions which facilitated virus growth, i.e., high MOI and low restrictive temperature, it appeared that genetic alteration of *ts*-1 was a random event which occurred with a fixed frequency during viral replication under restrictive conditions. The fact that altered virus was never detected among the progeny of *ts*-1 grown at permissive temperature, although the quantity of virus produced was considerably greater than that generated under restrictive conditions, suggested that elevated temperature itself might play a role in increasing the frequency of mutation (i.e., *ts* to ts^+), possibly through an action on RNA-dependent polymerase. Valentine et al. have commented upon the infidelity of the RNA polymerase of the RNA phage Q beta (15). Approximately 8% of clones of wild-type virus exhibited the *ts* phenotype. Similarly, in unpublished studies approximately 5% of plaque progeny of wild-type rhinovirus 1 was found to be temperature sensitive (Perkins et al., unpublished observations). Possibly the infidelity of the respiratory syncytial polymerase is increased at elevated temperature. This suggestion has been made previously to explain the increased mutation rate of vesicular stomatitis virus at 39.3 C (2).

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