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Further Characterization of the Complementation Group B Temperature-Sensitive Mutant of Respiratory Syncytial Virus

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ts-2, a temperature-sensitive and plaque morphology mutant of respiratory syncytial virus and sole representative of complementation group B, was compared with members of the other complementation groups of respiratory syncytial virus (group A [*ts*-1] and group C [*ts*-7]). *ts*-2 was found to be 10- to 1,000-fold more restricted in growth and ability to spread at restrictive temperatures (37, 38, and 39°C) than at the permissive temperature (32°C). In temperature shift-up experiments, the *ts* defect of *ts*-1 and other members of complementation group A was found to affect a late function that was required for at least 13 h in the replicative cycle. The *ts* lesion of *ts*-7 affected a function early in the replication cycle. In contrast, *ts*-2 was not temperature sensitive when studied by the shift-up technique. The discrepancy between the *ts* plaque property and failure to detect temperature sensitivity during the shift-up experiment was resolved when it was shown that *ts*-2 had a defect in adsorption or penetration or both at the restrictive temperature. Clonal analysis of revertant *ts*-2 showed a coordinate restoration of ts^+ phenotype and syncytium-forming capacity. It appears that *ts*-2 has a defect in a protein that is involved in adsorption and/or penetration of virus and is also responsible for cell fusion activity.

Seven temperature-sensitive (*ts*) mutants of the A2 strain of respiratory syncytial (RS) virus were described and partially characterized previously (2, 4, 9, 10). Each was selected on the basis of a 100-fold or greater reduction in plaque formation at the restrictive temperature of 39°C compared with the permissive temperature of 32°C. Further characterization revealed that the efficiency of plaque formation at 39°C was reduced at least 10^5-fold with each of the mutants, except *ts*-5, which was a "leaky" mutant. Genetic analysis of these mutants defined three complementation groups: group A contained *ts*-1, *ts*-3, *ts*-4, *ts*-5, and *ts*-6; groups B and C contained single mutants, *ts*-2 and *ts*-7, respectively (10). In addition, *ts*-2 was noted to be a plaque morphology mutant that did not fuse cells to form syncytia during plaque formation at the permissive temperature (2). In this communication we report on further studies of the complementation group B mutant, *ts*-2, and compare its properties to those of group A and C mutants.

MATERIALS AND METHODS

Procedures for growth of virus and assay of infectivity, as well as the indirect immunofluorescence technique, have been described previously (9).

Viruses and cell cultures. The development of the seven RS *ts* mutants was described previously in detail (2, 10). Six mutants, *ts*-1 to *ts*-6, were produced after exposure of RS virus to either 10^{-4} M 5-fluorouridine (*ts*-1 to *ts*-4) or $10^{-2.5}$ M 5-fluorouracil (*ts*-5, *ts*-6) in the growth medium. *ts*-7 was isolated by Louis Potash after treatment of RS virus with ethyl methane sulfonate. HEp-2 cell cultures were obtained from Flow Laboratories (Rockville, Md.) and maintained as described (2).

Growth of *ts*-2. The *ts*-2 mutant was inoculated into a set of HEp-2 roller tube tissue cultures at a multiplicity of infection (MOI) of 0.1 or 0.001. Incubation was carried out at 32, 37, 38, or 39°C. Daily for 5 days, a pair of tissue cultures from each experimental group was harvested and assayed for infectious virus at 32°C as previously described (9).

Temperature shift analysis. Each *ts* virus was inoculated at an MOI of 0.1 into a set of HEp-2 roller tube cultures that were incubated at room temperature for 90 min. The cultures were then washed three times with medium, refed with medium, and allowed to incubate at 32°C. At various times, two cultures were shifted from 32 to 39°C. After 24 h, each of the cultures was harvested, and the combined cells and fluid medium were assayed at 32°C for infectious virus by the plaque technique.

Adsorption-penetration experiments. The ability of a representative of each *ts* complementation

group to adsorb and penetrate HEp-2 cells at the permissive or restrictive temperature was examined using two indexes of infection, namely, production of infectious virus and production of viral antigens.

Production of infectious virus after adsorption at 32 or 39°C was tested in the following manner. HEp-2 roller tube tissue cultures were maintained at either 32 or 39°C throughout the adsorption-penetration period. Virus was warmed to 32 or 39°C and then inoculated onto the HEp-2 cells, which were incubated at the same temperature. After 90 min of adsorption, unattached virus was removed by washing the culture three times with medium warmed to 32 or 39°C; the culture was then overlaid with prewarmed medium containing 1% human convalescent serum containing a high level of RS virus antibody (plaque reduction titer of 1:1,024) and then again washed three times with prewarmed medium. At no time were the 39°C cultures allowed to cool during the adsorption-penetration period. After being overlaid with prewarmed maintenance medium, the cultures were incubated at either 32 or 39°C. After 24 h of incubation, cells and fluid media were assayed for infectious virus by the plaque technique.

Production of viral antigens after adsorption-penetration at 32 or 39°C was assayed with Leighton tube HEp-2 tissue cultures. Leighton tube cultures were inoculated with virus, adsorbed, washed, and incubated at either 32 or 39°C in the same manner as described above for roller tube tissue cultures. After 24 h of incubation, cells were fixed and assayed by indirect immunofluorescence for production of viral antigen. Under these conditions, the viral antigen-containing cells were predominantly those infected with the input virus (8).

Clonal analysis of revertant *ts*-2. Revertant *ts*-2 virus was generated by incubating replicate-infected HEp-2 cultures for 6 days at 35, 36, 37, 38, or 39°C. Each culture was subpassaged twice, from culture to culture, at the original temperature of incubation. The second and third passages were initiated by inoculation of undiluted, pooled cells and medium to maximize the input multiplicity and hence the emergence of altered virus with the ts^+ phenotype (9). Syncytial cytopathic effects were observed in some of the second-passage cultures infected with *ts*-2, and ts^+ virus was also detected in these cultures. To characterize the relationship between these two events, i.e., restoration of syncytial cytopathic effect and restoration of ability to produce plaques at 38°C (ts^+ property), a clonal analysis of virus derived from 10 separate cultures was performed. Clones were selected by picking plaques produced at 32°C and subsequently passaging the plaque-purified virus one time in HEp-2 roller tube cultures to increase the quantity of virus for analysis (3). A total of 57 clones from the 10 cultures were examined for their ability to form syncytia and for efficiency of plaque formation at 38°C.

RESULTS

Effect of temperature on growth of *ts* mutants. Growth occurred at each of the restrictive temperatures (37, 38, or 39°C); however, the level of replication was inversely related to temperature (Fig. 1). In each instance, growth at the restrictive temperature was reduced compared with growth at the permissive temperature of 32°C. At the higher MOI (0.1), growth of *ts*-2 was much less restricted at 39°C (10-fold reduction) than was the growth of *ts*-1 (100,000-fold reduction [9]) or *ts*-7 (1,000-fold reduction, data not shown). The effect of input

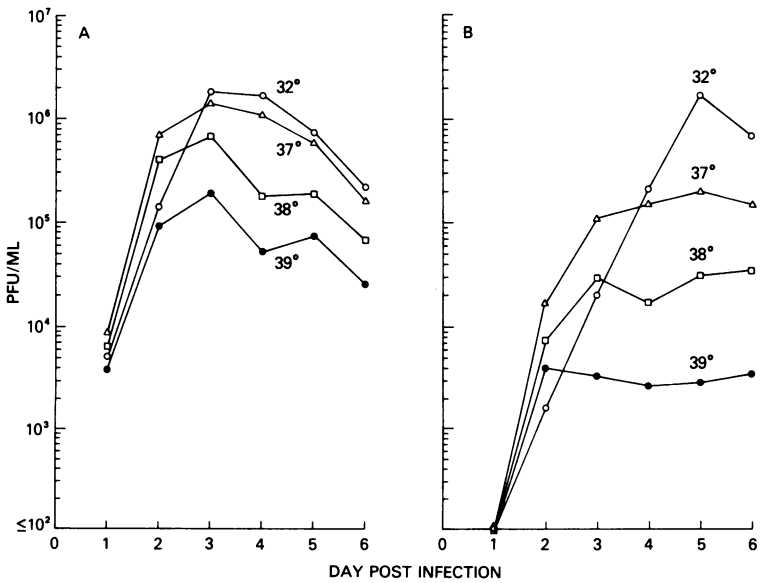

FIG. 1. *Growth of ts-2 at permissive (32°C) and restrictive (37, 38, 39°C) temperatures. (A) MOI = 0.1; (B) MOI = 0.001.*

multiplicity on the peak titer attained by *ts*-2 at restrictive temperature is shown in Fig. 1A (MOI = 0.1 PFU/cell) and B (MOI = 0.001 PFU/cell). At each of the restrictive temperatures, the peak titer was higher with the higher MOI. However, an effect of MOI on level of virus replication was not seen at the permissive temperature. In cultures incubated at 39°C, the viral titer peaked after 2 days of incubation, and further growth was not observed. In contrast, growth was seen after 2 days when cultures were incubated at the permissive temperature (Fig. 1B, 32°C growth curve).

Temporal expression of the *ts* lesion. Temperature shift-up revealed that growth of the wild-type virus was not affected when cultures were shifted from 32 to 39°C (Fig. 2). *ts*-1 was previously found to have a late lesion, requiring about 13 h of incubation at the permissive temperature before completion of the *ts*-1 function (L. S. Richardson, T. J. Schnitzer, R. B. Belshe, E. Camargo, D. A. Prevar, and R. M. Chanock, Arch. Virol., in press). In the present study this was confirmed when it was shown that at least 13 h of incubation was required at the permissive temperature for infectious virus to be produced. When other complementation group A viruses were tested by temperature shift-up, each exhibited a pattern similar to *ts*-1 (data not shown). *ts*-7 had an early lesion and required 5 h of incubation at the permissive temperature for production of infectious virus (Fig. 2). An unanticipated finding was that the growth of *ts*-2 was not affected by a shift-up in temperature at any time in the 24 h of viral growth (Fig. 2).

ts-2 was temperature sensitive with regard to plaque formation, but the mutant was not *ts* when studied by temperature shift-up. Possibly, this contradiction reflected an early defect which restricted the initial stage of infection, but which did not affect virus replication once infection was initiated. To investigate this possibility, the ability of *ts*-2 to adsorb and penetrate cells was studied at 32 and 39°C. A defect in this property could explain the apparent discrepancy in our findings with *ts*-2; the temperature shift experiment measures only one cycle of growth, but formation of plaques involves multiple growth cycles (and therefore multiple cycles of adsorption and penetration). Wild-type RS virus, *ts*-1, *ts*-2, and *ts*-7 were tested for their ability to absorb and penetrate at 32 or 39°C (Tables 1 and 2). Wild-type virus adsorbed and grew equally well at 32 and 39°C. *ts*-1 and *ts*-7 adsorbed equally well at 32 and 39°C, but neither mutant grew well at 39°C (Table 1) nor produced as much antigen at 39°C as compared

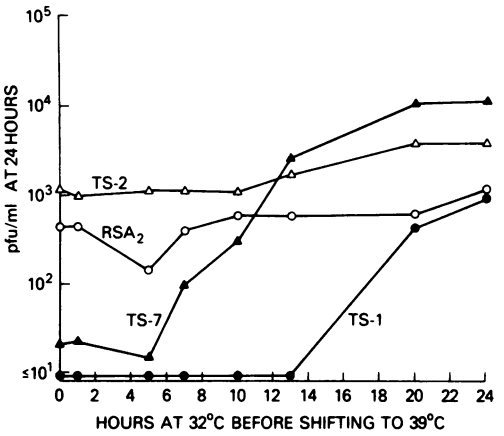

FIG. 2. *Effect of time of shift from 32 to 39°C temperature of incubation on the titer of wild-type RS* (○), *ts-1* (●), *ts-2* (△), *and ts-7* (▲) *at 24 h; MOI = 0.1.*

TABLE 1. *Effect of adsorption temperature on the growth of wild-type RS virus, ts-1, ts-2, and ts-7 incubated for 24 h at permissive or restrictive temperature*

Virus	Adsorption temp (°C)[a]	Quantity of virus produced after incubation at the indicated temp for 24 h	
		32°C	39°C
Wild type	32	4.6[b]	4.3
	39	4.6	4.5
ts-1	32	4.3	<1.0
	39	3.9	<1.0
ts-2	32	3.8	3.6
	39	2.5	1.6
ts-7	32	5.3	2.9
	39	5.3	2.5

[a] HEp-2 cells were inoculated with the indicated virus at an MOI of 0.1 (wild type, *ts*-1, and *ts*-2) to 0.3 (*ts*-7), incubated for 90 min at 32 or 39°C, and then washed with medium containing RS virus antibody, refed, and incubated at 32 or 39°C.

[b] PFU per milliliter ($\log_{10}$).

with 32°C (Table 2). *ts*-2, however, did not adsorb and/or penetrate cells efficiently at 39°C. Adsorption of *ts*-2 to HEp-2 cells was approximately 20- to 100-fold less efficient at 39°C than at 32°C (Tables 1 and 2). The decrease in viral growth or production of viral antigens when adsorption-penetration was allowed to occur at 39°C cannot be ascribed to heat lability of the *ts*-2 mutant, since this virus was shown to be as stable as wild-type virus at this temperature

TABLE 2. *Effect of temperature of adsorption and incubation on production of viral antigens in HEp-2 cells by RS virus and its ts mutants*[a]

RS virus	Cells containing viral antigens (%)[b] (32°C adsorption for 90 min; then 32°C incubation for 24 h)	Fold reduction in percentage of cells containing viral antigens		
		32°C adsorption for 90 min; then 39°C incubation for 24 h	39°C adsorption for 90 min; then 32°C incubation for 24 h	39°C adsorption for 90 min; then 39°C incubation for 24 h
Wild type	11.0	0	0	0
ts-1	15.0	75.0	1.9	37.5
ts-2	10.4	1.9	20.8	52.0
ts-7	15.0	6.3	1.7	21.4

[a] HEp-2 cells were inoculated with an MOI of approximately 0.1, incubated for 90 min at 32 or 39°C, washed with medium containing RS virus antibody, refed, and incubated at 32 or 39°C.

[b] Tested by indirect immunofluorescence with human convalescent serum.

(4). Once *ts*-2 was permitted to adsorb to HEp-2 cells at 32°C, transfer to 39°C for subsequent incubation did not reduce virus yield or antigen production as markedly as with the *ts*-1 or *ts*-7 mutant.

***ts* phenotype and plaque morphology of revertant *ts*-2.** Serial passage of *ts*-2 at 35 to 39°C yielded some virus that produced plaques at 38°C (i.e., ts^+) and induced formation of syncytia. This phenomenon was observed during the second and third passages. In the third-passage cultures, most of the virus appeared to be wild type since these virus suspensions produced plaques at 38°C with an efficiency equivalent to wild-type virus, and most plaques were syncytial. In contrast, viruses in the second-passage cultures were intermediate between the *ts*-2 mutant and wild-type virus with respect to plaquing efficiency at 38°C and plaque morphology. To determine whether all of the virus particles in the second-passage suspensions were intermediate in ts^+ and plaque morphology phenotype or whether a mixed population of *ts* and ts^+ viruses and nonsyncytial and syncytial viruses were produced, a clonal analysis was performed. Fifty-seven clones derived from 10 separate cultures, 5 incubated at 35°C and 5 incubated at 37°C, were selected by picking individual plaques at 32°C. After one passage in HEp-2 roller tube cultures at 32°C, these clonal populations were evaluated for ts^+ and syncytial properties (Fig. 3). Thirty-four clones that did not produce syncytia plaqued with greatly reduced efficiency at 38°C or not at all (plaque titer at 38°C/titer at 32°C was 10^{-4} to 10^{-5}). Nineteen clones, derived from six of the second-passage cultures, formed wild-type syncytia and formed plaques at 38°C as efficiently as the wild-type virus. In addition, four clones that formed syncytia smaller than those of wild-type virus were intermediate in their efficiency of plaque formation at 38°C. Thus, there was coordinate restoration of the ts^+ and syncytial properties. Furthermore, viruses that were intermediate in plaquing efficiency at 38°C also exhibited an intermediate type of syncytial cytopathology.

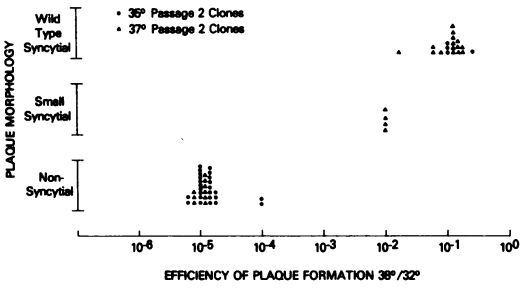

FIG. 3. *Plaque morphology of clones of ts-2 compared with efficiency of plaque formation at the restrictive temperature (38°C). The clones were selected from ts-2 virus passaged twice at 35°C* (○) *or 37°C* (△).

DISCUSSION

Little is known of the structure and function of RS viral proteins, because the virus does not grow to high titer in tissue culture and it is difficult to purify the virus to permit biochemical analysis of viral polypeptides. Despite these difficulties, two groups have presented evidence for the existence of two to three viral glycoproteins (5, 11). These glycoproteins presumably are the spikes that project from the viral envelope. The function of the spikes of RS virus has not been defined, but it is reasonable to assume that they are involved in attachment to the host cell and in cell fusion, which is a characteristic effect of this virus in a number of cell cultures. The paramyxoviruses, which resemble RS virus in possessing a limiting envelope with spikelike projections and in causing cell fusion, have two distinct surface glycoprotein spikes. One of these glycoproteins functions as a hemagglutinin and also has neura-

minidase activity (1). The other glycoprotein causes cell fusion and appears to be involved in penetration of virus into the host cell (7). RS virus has not been shown to possess hemagglutination or neuraminidase activity (6) and thus cannot be considered strictly comparable to the paramyxoviruses. However, RS virus must attach to the host cell, and this function could be considered comparable to hemagglutination. The prominent property of syncytium formation exhibited by RS virus suggests that it possesses a protein that causes cell fusion.

Against this background of relative ignorance of RS virus structure and function, the group B *ts* mutant, *ts*-2, was studied in an attempt to describe the *ts* defect more precisely with respect to the time and nature of the function affected. Although *ts*-2 was temperature sensitive with respect to plaque formation, it was not temperature sensitive when studied by shift-up. In this procedure, the adsorption of virus was allowed to occur at room temperature before incubation at 32 or 39°C. The discrepancy between the *ts* phenotype of *ts*-2 when studied by the plaque technique and its lack of temperature sensitivity when characterized by shift-up suggested that this mutant might have a defect that affected the initial stage of infection, namely, adsorption and penetration. Subsequently, this was shown to be the case. At this time a distinction between adsorption and penetration cannot be made, since 24°C, a temperature close to that which prevailed in the laboratory, is permissive for growth of RS virus, and thus both adsorption and penetration could have occurred at this temperature.

ts-2 is of special interest since it is a plaque morphology mutant. It does not produce syncytial plaques under permissive conditions (2). It is tempting to speculate that the *ts* defect of *ts*-2 affects the protein responsible for cell fusion and, presumably, the protein for penetration as well. If this is the case, a single defect could be invoked to explain the nonsyncytial plaque property at the permissive temperature and the failure of adsorption-penetration at restrictive temperature. Such a unitary hypothesis necessarily implies that the *ts*-2 defect is expressed at the permissive temperature (i.e., nonsyncytial plaques) and becomes progressively more restrictive with increasing temperature. It is also possible that the *ts*-2 virion fusion protein, although defective at permissive temperature, is still sufficiently active to permit penetration to occur, whereas the activity of the affected protein is not sufficient at the membrane of the infected cell to allow cell recruitment to occur by fusion of membranes.

An alternate explanation for the defectiveness of *ts*-2 is that the mutant contains two separate lesions, one that is *ts* and affects adsorption-penetration and another that is not *ts* and affects cell fusion. This is not likely the case, however, since cell fusion is probably a manifestation of the same activity responsible for cell penetration as has been shown for the paramyxoviruses (7). In addition, coordinate restoration of syncytium formation and the ability to form plaques at restrictive temperature was observed in clones selected from six independently derived virus populations containing revertant *ts*-2. This suggests that a single lesion was responsible for both the aberrant plaque morphology and the *ts* property.

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