Temperature-sensitive Mutants of Respiratory Syncytial Virus

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Received for publication 15 January 1969

Four conditional-lethal temperature-sensitive mutants of RS virus were detected among the progeny of 454 plaques derived from virus grown in the presence of 10^{-4} M 5-fluorouridine. These mutants were stable (reversion frequency, $10^{-5.0}$ or less and failed to produce plaques at 38 or 39 C. Plaquing efficiency was depressed 100-fold or more at 37 C. Variable suppression of growth at the restrictive temperature of 39 C was observed, ranging from 16-fold to complete suppression. The temperature-sensitive defect of three of the mutants appeared to affect functions which were expressed late in the replicative cycle. One of the mutants produced atypical non-syncytial plaques.

Considerably more is known about the ecological and clinical aspects of respiratory syncytial (RS) virus than its basic biological properties (2). This virus contains an inner helix (presumed to be ribonucleoprotein) which is surrounded by a spike-armed lipoprotein envelope (1, 11). RS virus resembles the paramyxoviruses in fine structure, although there is some dispute as to whether the diameter of the inner helix is 13 or 18 nm (1, 11).

The results of clinical and epidemiological studies performed in many different geographic areas clearly indicate that RS virus is the most important respiratory tract pathogen of infancy and early life. The failure of conventional parenteral inactivated vaccine to protect against RS virus disease in early life and the recent recognition of respiratory tract secretory antibody as the major determinant of resistance to viral respiratory disease suggested to us that a live attenuated intranasally administered RS virus might be required for effective immunoprophylaxis (10; Chanock et al., Academic Press, *in press*; Kapikian et al., *Am. J. Epidemiol.*, *in press*; Kim et al., *Am. J. Epidemiol.*, *in press*). Although a promising start in this direction has been made with a low-temperature (26 C)-adapted strain of RS, we believe that other approaches to attenuation should be explored (4).

In the few instances in which appropriate studies have been performed, temperature-sensitive (ts) mutants of Semliki Forest virus and reovirus were found to exhibit reduced virulence in experimental laboratory hosts (3, 6). For this reason, we attempted to produce conditional-lethal ts mutants of RS virus by using the chemical

mutagens 5-fluorouridine and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; reference 5, 8). We sought ts mutants of RS virus which would grow well at the temperature of the nasopharynx (32-33 C) but not at the temperature of the lower respiratory tract (37 C). In addition to their potential usefulness in immunization, ts mutants of RS virus may prove helpful in delineating the genetic basis of various biological properties of this virus.

MATERIALS AND METHODS

Virus. The A2 strain of RS virus was obtained from Australia where it had been recovered in human embryonic kidney (HEK) culture (7). After a total of six passages in HEK at 37 C, the virus was adapted to growth in bovine kidney (BK) cells at 37 C. Prior to exposure to chemical mutagens, the virus was purified by three successive plaque-to-plaque passages in BK cells at 37 C.

Cell culture. Primary BK cultures were prepared at Flow Laboratories (Rockville, Md.). These cells were grown in 60-mm plastic petri dishes or roller culture tubes in a medium which contained Eagle's medium with 10% fetal calf serum. The petri dishes were seeded with 10^6 cells and then incubated at 37 C in a humidified atmosphere of 5% CO₂ for 6 to 7 days. The tube cultures, which were seeded with 10^6 cells, were also incubated at 37 C for 6 to 7 days prior to use.

HeLa cell monolayers grown in 35-mm petri dishes, 8-oz prescription bottles, roller tubes, and Leighton tubes with cover slips were also obtained from Flow Laboratories. Maintenance medium for roller tube, Leighton tube, and bottle cultures contained Eagle's minimum essential medium (MEM), 2% inactivated agamma calf serum, and 0.002 M glutamine. Overlay medium for HeLa and BK petri dish cultures consisted

of Leibovitz's medium; 10% inactivated agamma calf serum, 0.002 M glutamine, and 0.5% agarose. The overlay medium for BK petri dish cultures was the same except the final concentration of agarose was 1%. Penicillin (100 units/ml), streptomycin (100 µg/ml), and amphotericin (50 units/ml) were added to all media.

Virus assay. The determination of efficiency of plaque formation of parent virus and ts mutants at different temperatures was carried out at 28, 30, 37, 38, and 39 C. Confluent HeLa monolayers grown in 35-mm petri dishes were used for such plaque assays. The virus inoculum of 0.1 ml per plate was allowed to adsorb for 1.5 hr at room temperature, after which overlay medium was added. Cultures were incubated for various periods which ranged from 4 days at 39 C to 6 days at 28 C. The petri dish cultures were then fixed with 10% neutral Formalin and stained with hematoxylin and eosin. The stained plaques were counted with the aid of a dissecting microscope at a magnification of 15X.

Exposure to chemical mutagens. Thrice plaque-purified virus (HEK passage six, BK passage nine) was treated by one of two methods. (i) 5-Fluorouridine (5-FU): BK cultures infected with A2 virus at low multiplicity [0.02 plaque-forming units (PFU) per cell] were incubated at 28 C in the presence of 10^{-4} M 5-FU in the maintenance medium. The supernatant fluid and cells were harvested separately at the end of 8 days, when the cytopathic effect, which was delayed by 3 days compared to untreated infected cultures, was at its maximum. (ii) NTG: Virus was diluted in a freshly prepared solution of NTG in 0.05 M maleic buffer (pH 9), which had a final concentration of 100 µg/ml and a final pH of 7.2. The mixture (2 ml) was incubated at room temperature for 15 min, and the treated virus was then dialyzed at 4 C for 6 hr against 2 liters of cold phosphate-buffered saline (PBS). Since RS virus is relatively unstable and is completely inactivated by overnight dialysis at 4 C, a rapid method of dialysis was employed in which PBS was changed every hour during a 6-hr interval.

Evaluation of treated virus for presence of ts mutants. Mutagen-exposed virus was appropriately diluted in tris(hydroxymethyl)aminomethane (Tris) buffer, and 0.1 ml was adsorbed to BK cultures at room temperature for 1.5 hr. The cultures were then washed once with Hanks' salt solution (HSS) and overlaid with agar medium without neutral red. After the plates were incubated at 28 C for 6 days, a second agar overlay containing neutral red (0.01%) was added; the plates were then incubated at 28 C for an additional 24 hr. Single plaques were picked by aspiration of cell debris through the overlay, by use of a siliconized capillary pipette. To minimize the possibility of contamination of plaque progeny with virus from other plaques, only those petri dish cultures which contained one to three plaques were used (9). The plaques were stored at -60 C as suspensions in 1 ml of maintenance medium.

The virus clones grown at 28 C were then passaged in BK tube cultures at 28 C. These cultures were harvested when maximal cytopathic effect was observed, i.e., about 3 weeks, and virus was stored at

-60 C. To detect potential ts mutants, the harvest of this material was titrated on HeLa cell and BK monolayers at 39 and 28 C. The clones which yielded a 100-fold or higher titer at 28 C, as compared to 39 C, were scored as potential ts mutants. The BK tube-culture-grown potential ts mutants were then subjected to plaque purification at 28 C in BK petri dish cultures. Virus stocks for definitive studies of the ts property were then prepared in BK and HeLa cultures from the progeny of these single plaques. Thus, prior to final evaluation, each mutant was cloned twice. The twice-cloned mutants were evaluated for plaquing efficiency at 39 and 28 C; those mutants found to possess the ts property were sequentially numbered in order of isolation and stored at -60 C.

Preparation of ts mutant stocks in HeLa cells. Since the supply of BK cell cultures was limited, most studies of ts mutants were performed in HeLa cells. The stocks with high infectivity at 28 or 30 C and low efficiency of plating at nonpermissive temperature, i.e., 39 C, were prepared by infecting HeLa cell monolayers grown in 8-oz bottles with undiluted virus suspension. The cultures were adsorbed at room temperature for 1.5 hr, fed with 10 ml of maintenance medium, and then incubated at 30 C. Since it was found that the majority of the infectious virus produced at 28 to 30 C is cell-associated (*unpublished data*), only the cells were harvested in a small quantity of the supernatant fluid when the cytopathic effect was at its maximum. In order to release intracellular virus, cells were sonically treated in a Raytheon sonic oscillator (model DF 101) at 0.8 to 0.9 amp for 1 min in two 30-sec intervals. The stocks were stored at -60 C.

Single-step growth curves at 30 and 39 C. HeLa roller tube cultures containing approximately 5×10^5 cells per tube were used for the growth curve experiments. The monolayers were infected at a multiplicity of infection which varied from 5 to 10 PFU per cell. Virus adsorption was allowed to proceed at room temperature for 1.5 hr. The monolayers were then washed once with HSS, incubated for 0.5 hr with Eagle's medium containing a 1:100 dilution of human RS convalescent serum (RS plaque reduction neutralizing antibody titer of 1:1,024). The tubes were then washed three times with HSS, fed with 1.5 ml of maintenance medium, and incubated at 30 or 39 C. The cells and the fluid medium were harvested separately after various periods of incubation. Cells were scraped from the glass in 1 ml of fresh maintenance medium and disrupted by sonic oscillation for 30 sec. The tissue culture fluids and the cellular suspensions were quick-frozen in a dry ice-alcohol bath and stored at -60 C until titrated for virus content.

Fluorescent-antibody techniques. The indirect immunofluorescence technique was used to detect the presence of RS-specific antigen in HeLa cover-slip cultures inoculated with the ts mutants or their parent strain. Cover slips contained 2×10^6 cells and were infected at an input multiplicity of 5 to 10 PFU/cell. In these studies, two sets of pre- and post-RS infection sera from young children were used to confirm the specificity of the revealed antigens. Preinfection sera lacked detectable plaque-reduction neutrali-

zation antibody (<1:20) and complement-fixing (CF) antibody (<1:4), whereas postinfection sera had a high titer of CF antibody (1:128 or >). Cover slips were exposed to antisera for 30 min at room temperature and then washed in PBS and distilled water. They were then incubated for 30 min with a 1:10 dilution of anti-human γ -G-globulin conjugated with fluorescein (Antibodies, Inc., Davis, Calif.). A 1:16 dilution of lissamine rhodamine-conjugated bovine albumin was added to the fluorescein conjugate to minimize non-specific staining. The cover slips were washed as before and mounted in 80% buffered glycerine. They were then examined by use of a fluorescent microscope (American Optical) with a Corning 5970 exciter filter and a Kodak Wratten 2A barrier filter.

RESULTS

Temperature range. Prior to the search for ts mutants, it was determined that the efficiency of plaque formation of RS virus (A2 strain) in BK cells did not vary appreciably over the temperature range from 28 to 39 C. However, the time required for development of plaques varied from 7 to 8 days at 28 C to 4 days at 39 C. Based upon this information, 28 C was selected as the permissive temperature and 39 C as the restrictive temperature to be used in the search for ts mutants of RS virus.

Exposure to mutagens and recovery of ts mutants. Virus growing in BK cells at 28 C was exposed to 10^{-4} M 5-FU (Table 1). This resulted in a 300-fold suppression of growth and a 3-day delay in appearance of cytopathic effect. In another experiment, a virus suspension was exposed to 100 μ g of NTG per ml for 15 min at pH 7.2 (Table 1). This resulted in a 300-fold reduction in virus titer. Mutagen-exposed virus was plaqued in BK cells at 28 C, and 1,159 plaques were picked from culture plates which contained not more than three plaques per plate. Of these plaques, 703 (61%) yielded infectious virus which was then evaluated in BK cells for its capacity to produce plaques at the permissive (28 C) and restrictive (39 C) temperatures.

Of the 5-FU-treated plaque progeny, 0.8% were found to be ts conditional-lethal mutants,

whereas none of the 249 survivors of NTG treatment exhibited this property. The rate of spontaneous ts mutation was not determined; however, the failure to recover ts mutants from NTG-treated survivors suggests that it must be quite low.

Plaquing efficiency, stability, and reversion of ts mutants. After the isolation and recognition of ts mutants in BK cultures, the subsequent characterization of these mutants was performed by using HeLa cell cultures. Comparative titrations of the parent virus and the ts mutants at 28, 30, and 32 C indicated that efficiency of plaque formation was the same at these three temperatures. Because plaque formation occurred earlier at 30 or 32 C (5 days) than at 28 C (7 days), the former were selected as the permissive temperatures for plaque assay in HeLa cells.

The four ts mutants exhibited $10^{5.0}$ to $10^{7.6}$ or greater efficiency of plaquing at permissive (30 or 32 C) as compared to restrictive (39 C) temperature (Table 2). This difference was still observed after six to eight passages in tissue culture. These findings indicate that the mutants were stable and had a low reversion frequency (PFU at 39 C/PFU at 30 or 32 C) which was less than $10^{-5.0}$ to $10^{-7.6}$. In one instance, a $10^{-3.1}$ reversion frequency was detected with ts2 (fourth BK passage after second plaque purification); however, subsequent passages of this mutant in HeLa cells exhibited a $10^{-6.9}$ or lower reversion frequency (Table 2). Failure to detect revertants was not due to interference by the mutant viruses, since 10^5 to 10^6 PFU of ts3 or ts4 (as determined at the permissive temperature of 32 C) did not interfere significantly with plaque formation by 16 or 53 PFU of parent virus at 39 C.

In addition, plaquing efficiency of the mutants was also studied at 37 and 38 C to determine if these temperatures are also restrictive (Table 2). None of the mutants produced plaques at 38 C. Ts2 and ts4 similarly failed to produce plaques at 37 C, whereas ts1 and ts3 exhibited an approximate 100-fold decrease in plaquing efficiency, and those plaques which developed were approxi-

TABLE 1. Recovery of ts mutants from RS virus exposed to mutagen

Mutagen	Titer in PFU/ml ^a		Plaques produced by treated virus		No. of plaque progeny exhibiting 10 ² -fold or greater plaquing efficiency at 28 vs 39 C
	Treated	Nontreated	No. examined	No. viable ^b	
5-FU (10^{-4} M) in growth medium....	3.8×10^2	1.3×10^5	709	454	4
NTG (100 μ g/ml), 15 min, pH 7.2....	1.1×10^4	3.4×10^6	450	249	0

^a Determined at 28 C.

^b Viability established by passage of plaque suspension into BK roller tube culture at 28 C; those cultures which exhibited cytopathic effect were harvested and progeny were examined for ts property.

TABLE 2. *Plaquing efficiency of parental virus and ts mutants of RS virus (A2 strain)*

Virus		Temp. of propagation	No. of passages after second plaque purification	PFU/ml ($\times 10^3$) at assay temp of			PFU at 30 or 32 C/PFU at 39 C
Treat ment	Designa- tion			30 or 32 C	37 C	39 C	
5-FU	ts2	30	BK 1	2,400		<0.001	$10^{5.4}$ or >
			BK 4, HeLa 4	2,600	<0.001 ^a	<0.001 ^b	$10^{6.4}$ or >
	ts3	30	BK 1	560		<0.001	$10^{5.7}$ or >
			BK 4, HeLa 5	5,800	14 ^c	<0.001 ^b	$10^{6.8}$ or >
	ts1	30	BK 1	1,600		<0.001	$10^{6.2}$ or >
			BK 2, HeLa 5	42,000	39 ^c	<0.001 ^b	$10^{7.6}$ or >
	ts4	30	BK 1	100		<0.001	$10^{6.0}$ or >
None	Parent virus	37	BK 2, HeLa 5	13,000	<0.001	<0.001 ^b	$10^{7.1}$ or >
			BK 11	480		300	1.6
			BK 11, HeLa 5	3,400	2,900	3,300	1.0

^a Plaques not seen; however, cell sheet on plates inoculated with 10^0 and 10^{-1} dilutions of virus appeared abnormal in that cells were smaller and less confluent than on control culture plates.

^b Identical results obtained at 38 C.

^c Tiny plaques.

mately one-half the size of the plaques produced at the permissive temperature.

Growth of ts mutants. The efficiency and rate of growth of ts mutants at permissive and restrictive temperatures was determined and compared to that of the parent virus. After infection of HeLa cells at a multiplicity of 5 to 10 PFU per cell, the ts mutants showed varying degrees of growth restriction at 39 C, ranging from 16- to 7,000-fold lower titer at 39 C compared to that seen at 30 C (Table 3; Fig. 1). The parent virus showed equivalent growth at the two temperatures at 48 hr, although newly formed virus was detected earlier at 39 C (8 hr) than at 30 C (12 to 24 hr). Growth of two of the mutants (ts1 and ts4) was not detected at 39 C. At the restrictive temperature, ts2 grew as rapidly as the parent virus; newly formed virus was first detected at 8 hr with both of these viruses. Despite the rapid growth of ts2 at 39 C, the titer attained at 48 and 72 hr was 14- to 16-fold lower than that at the permissive temperature. The pattern seen with ts3 was intermediate between that shown by ts2 and that of ts1 and ts4.

The extent of growth restriction at 39 C permits an estimate of the degree of "leakiness" of the mutants. Ts2 appeared to be most "leaky," while there was no evidence that ts1 and ts4 were able to overcome their ts defect. Although appreciable yields could be recovered from cells multiply infected with ts2 or ts3 at the restrictive temperature, the level of viral growth was apparently not sufficient for development of plaques initiated by a single virus particle.

Although ts2 and ts3 grew to a limited extent at the restrictive temperature, the resulting progeny did not produce plaques at 39 C. These findings are

TABLE 3. *Efficiency of virus production at restrictive temperature—comparison of parental virus and ts mutants*

Virus	Quantity of virus (PFU) produced per cell after 48 hr of incubation at		Virus produced at 30 C/virus produced at 39 C
	30 C	39 C	
Parent	2.3	1.6	1.4
ts2	28	1.7	16.4
ts3	3.6	0.035	102.8
ts1	7	0.001 ^a	7,000 or >
ts4	0.35	0.002 ^a	175 or >

^a No evidence of viral replication at 39 C; observed value does not differ significantly from the residual of the virus inoculum.

consistent with our previous failure to detect reversion of these mutants.

Plaque morphology. Three of the ts mutants (ts1, ts3, and ts4) produced syncytial plaques which were indistinguishable from those of the parent virus. In contrast, ts2 produced a non-syncytial plaque which characteristically consisted of a circumscribed area of pyknotic, presumably dead, cells (Fig. 2).

Synthesis of viral antigens. Two techniques were used to study the synthesis of viral antigens by ts mutants at the restrictive temperature. Three of the mutants (ts2, 3, and 1) produced RS viral antigens which were revealed by immunofluorescence (Table 4). Immunofluorescent antigen was not detected in cells inoculated with ts4 (5 to 10 PFU per cell) and incubated at 39 C. Ts2 produced almost as much antigen(s) at 39 C as did the parent virus, whereas ts1 and ts3 produced

FIG. 1. Growth of *ts* mutants and parent virus in HeLa cells infected at a multiplicity of 5 to 10 PFU per cell and incubated at 30 or 39 C.

significantly less antigen. The specificity of the immunofluorescent reactions was indicated by (i) the failure of human preinfection sera (which lacked neutralizing antibody) to stain infected cover slips, and (ii) the absence of staining in cover slips harvested 3 hr after infection and tested with human sera containing RS antibody.

The development of CF antigen(s) was measured in HeLa cells inoculated with *ts* mutants and incubated for various periods at 39 C (Table 5). Ts2 produced as much antigen at the restrictive temperature as did the parent virus. Ts3 produced less antigen under these conditions, whereas antigen was not detected in cells inoculated with the other two mutants. The general pattern of CF antigen development resembled that seen with immunofluorescent antigen.

DISCUSSION

There were several technical difficulties encountered during the characterization of RS virus and the selection of *ts* mutants in BK tissue culture. In part, these difficulties were self-imposed. The aim of the investigation was the recovery of *ts* mutants which could be tested for virulence in man, with the ultimate objective of

developing a live, attenuated RS virus vaccine. For this reason, we were limited to those tissue culture systems which are acceptable or potentially acceptable for production of virus to be administered to man. In BK cells, RS virus produced small irregular plaques from which it was often not possible to recover viable virus. After *ts* mutants had been selected in BK tissue and stocks of virus prepared in these cells, further characterization was performed in a heteroploid cell line (HeLa) in which RS virus produced larger plaques and higher yields.

The parent virus stock from which the *ts* mutants were derived was purified by three plaque-to-plaque passages at 37 C so as not to favor the selection of any possible naturally occurring *ts* mutants. Whether the *ts* mutants recovered after exposure to 5-FU were induced by this chemical could not be determined with certainty, since nonmutagenized virus was not studied in parallel. The failure to detect *ts* mutants among 249 survivors of NTG treatment suggests that naturally occurring *ts* mutants were extremely rare and that the mutants recovered in this study were probably induced by 5-FU.

The extent of the genetic change which oc-

FIG. 2. Morphology of plaques produced by *ts* mutants in *HeLa* cells at 32 C. $\times 40$. (A) *Ts2*; dark areas represent syncytia which are partially retracted. (B) *Ts2*; light areas are plaques in which cell loss and pycnosis have occurred.

curring in the *ts* mutants cannot be inferred from the available data. The low frequency of *ts* mutants in the 5-FU-treated virus stock (0.8%) and the low concentration of 5-FU employed (10^{-4} M) favor the view that single-step (single nucleotide sequence) mutants were produced. On the other hand, stability of the mutants, i.e., low

frequency of reversion to wild type, suggests that each of the *ts* mutants had undergone more than one change in nucleotide sequence, either in the same or different cistrons. In the procedure used for characterization of mutagenized virus, plaque progeny were subjected to a passage in roller tube cultures of BK prior to being tested for plaqueing

TABLE 4. Production of RS virus antigen at permissive and restrictive temperatures as detected by indirect immunofluorescence

Virus	Patient no. ^a	Proportion of HeLa cells in which cytoplasmic staining was detected at indicated time ^b					
		Cells incubated at 30 C			Cells incubated at 39 C		
		3 hr	24 hr	48 hr	3 hr	24 hr	48 hr
Parent	1	0	++	++++	0	++	++++
	2			+++ to ++++			+++ to ++++
ts2	1	0	+++ to ++++	+++ to ++++	0	++++	+++ to ++++
	2			++ to +++			++ to +++
ts3	1	0	+++	++++	0	+ to ++	++ to +++
	2			++++			++
ts1	1	0	++	+++ to ++++	0	± (<1%)	+ to ++
	2			+++			+
ts4	1	0	++	+++ to ++++	0	0	0
	2			++			0

^a Serum obtained from patients convalescing from RS virus illness. Preinfection serum of patient no. 1 failed to stain any of the infected cover slips harvested at 24 hr; preinfection serum of patient no. 2 did not react with any of the 48-hr cover slip preparations.

^b Values: + = 25%, ++ = 50%, +++ = 75%, and ++++ = 100%.

TABLE 5. Development of RS CF antigen by HeLa cells infected at a multiplicity of 5 to 10 PFU/cell with parent virus or ts mutants

Virus	Reciprocal of CF antigen titer of infected cell suspension ^a after indicated hours of incubation at											
	30 C						39 C					
	0 hr	8 hr	12 hr	24 hr	36 hr	48 hr	0 hr	8 hr	12 hr	24 hr	36 hr	48 hr
Parent	<1	<1	<1	2	4	4	<1	<1	<1	8	8	4
ts2	<1	<1	<1	4	8	8	<1	<1	2	4	8	8
ts3	<1	<1	<1	4	8	16	<1	<1	<1	<1	2	2
ts1	<1	<1	<1	2	2	8	<1	<1	<1	<1	<1	<1
ts4	<1	<1	<1	4	8	8	<1	<1	<1	<1	<1	<1

^a HeLa cells (5×10^5) harvested into 1 ml of Eagle's medium with 2% agamma calf serum.

efficiency at permissive and restrictive temperatures. Because of this intermediate step, which was necessitated by the poor yields of viable virus from BK plaques, single-step mutants with a high frequency of reversion may well have gone unrecognized. In this situation, only those mutants which were stable could be detected.

The properties of the ts mutants are summarized in Table 6. At the restrictive temperature, ts2 produced as much immunofluorescent and CF antigen as the parent virus. One or both of these antigens were also produced during ts1 and ts3 infection at 39 C, but in lesser amounts. This suggests that the ts defects of these mutants affected functions which were expressed relatively

late in the replicative cycle. Preliminary complementation studies suggest that the four ts mutants fall into one of two complementation groups—one group consists of ts1, 3, and 4; the other is represented by ts2 (P. F. Wright, M. A. Gharpure, and R. M. Chanock, *in preparation*). Although these studies suggest that ts4 shares one defect in common with ts1 and ts3, it was not possible to demonstrate expression of certain late functions (immunofluorescent and CF antigen production) in ts4-infected cells incubated at the restrictive temperature. Possibly, ts4 may produce a structural protein whose defect renders it nonreactive with RS virus antibodies or, alternatively, ts4 may have two defects, one which it shares with ts1 and ts3 and the other which blocks replication at an early stage.

Ts2 was of special interest since, at the permissive temperature, it produced plaques which differed in morphology from those of the parent virus and the other ts mutants. It remains to be determined whether the ts and plaque morphology properties of ts2 are expressions of a single defect, or if this mutant has two defects, one which is ts and the other which affects plaque morphology independent of temperature. In either case, ts2, which does not produce syncytia, should prove useful in studies designed to elucidate the mechanism of cell fusion by RS virus as well as in complementation and recombination studies in which this mutant can be scored separately from the other ts mutants and wild-type virus.

The ts mutants of RS virus may prove to be

TABLE 6. *Summary of properties of ts mutants*

Virus	Reversion frequency	Efficiency of virus production at 39 C ^a ("leakiness")	Production of antigen at 39 C ^b		Plaque morphology
			FA ^c staining	CF	
ts2	10 ^{-3.1} to 10 ^{-6.4}	10 ^{-1.2}	++++ ^d	++++	Circular, clear, nonsyncytial
ts3	10 ^{-5.7} or <	10 ^{-2.0}	+++	+	Syncytial
ts1	10 ^{-6.2} or <	10 ^{-3.8} or <	++	0	Syncytial
ts4	10 ^{-5.0} or <	10 ^{-2.2} or <	0	0	Syncytial

^a Referable to virus titer attained at 30 C after 48 hr.^b Referable to 100% antigen production by parent virus.^c Fluorescent antibody.^d See footnote b, Table 4, for values.

useful in the prevention of lower respiratory tract disease caused by this important pathogen of infancy and early childhood (Chanock et al., *in press*). The temperature range of the mutants, which permits efficient growth at 32 C but not at 37 C, may restrict infection in man to the nasopharynx (temperature of 32 to 34 C) and thus prevent spread to the site of major pathology, the lower tract. The ultimate success of this approach to immunoprophylaxis depends upon retention by the mutants of their capacity to infect the upper respiratory tract of man and to grow to a level which provides adequate stimulation of the respiratory tract secretory antibody system.

ACKNOWLEDGMENTS

We thank Sherry Braheny, Joanne Broyhill, and William Woodend for expert technical assistance, and W. E. Scott of Hoffman-LaRoche, Inc., for the kind gift of 5-fluorouridine.

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