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Selection of Temperature-Sensitive Mutants During Persistent Infection: Role in Maintenance of Persistent Newcastle Disease Virus Infections of L Cells

OLIVIA T. PREBLE AND JULIUS S. YOUNGNER

Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

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Virus mutants (NDV_{pi}) recovered from L cells persistently infected with Newcastle disease virus (NDV, Herts strain) are temperature-sensitive (ts) at 43 C, although the wild-type virus (NDV_o) which initiated the persistent infection replicates normally at that temperature. To study the relationship between the ts marker of NDV_{pi} and the other properties which distinguish this virus from NDV_o, NDV_{pi} ts^+ revertants were selected at the nonpermissive temperature and NDV_o ts mutants were generated by treating NDV_o with nitrous acid. Spontaneously-occurring ts mutants in the Herts NDV population were also isolated. The different virus populations were characterized with regard to plaque size, virulence for eggs, and thermal stability of infectivity, hemagglutinin, and neuraminidase. The NDV_{pi} ts^+ revertants, although no longer temperature-sensitive, retained NDV_{pi} properties, whereas both spontaneously-occurring and mutagen-induced ts mutants remained wild-type in their other properties. These findings showed that the properties which characterized NDV_{pi} were independent of the ts marker. However, the ts marker and the other markers of NDV_{pi} were coselected during the persistent infection, and the combination of those markers appeared to be important in the outcome of NDV infection of L cells. NDV_{pi} replicated productively in L cells, whereas NDV_o, the NDV_{pi} ts^+ revertants, and the spontaneously-occurring ts mutants all yielded covert infections in L cells. The role of the selection of ts mutants in persistent infection was confirmed as follows: L cells were persistently infected with NDV_{pi} ts^+ revertants and NDV_o ts mutants. Virus recovered from the persistently infected cultures after eight cell passages was always temperature-sensitive and of smaller plaque size than the parental virus in chicken embryo cell cultures. Similar results were obtained with virus recovered from L-cell cultures persistently infected with two other velogenic strains of NDV, the Texas-GB and Kansas-Man strains. These results strongly suggest that selection of ts mutants during the persistent infection was not random and played a role in establishment or maintenance of the persistent infection, or both.

Henle and his co-workers (7) demonstrated that persistent infection of mouse L cells could be readily established with the Victoria strain of Newcastle disease virus (NDV). Early experiments indicated that the virus re-isolated from the persistently infected cultures differed from the parental virus in both plaque type in permissive chicken embryo (CE) cells (7) and in virulence for L cells (15). Thacore and Youngner (23) found that stable small-plaque mutants could be isolated from mouse L cells (L_{NDV}) persistently infected with the Herts strain of NDV and these mutants (NDV_{pi}) differed from the parental Herts virus (NDV_o) in several respects: plaque size in CE cells, virulence for chicken embryos, virulence for L cells, and thermal inactivation profiles of several virion-associated activities (23-25). In addition, all of the Herts NDV_{pi} clones were shown to be temperature-sensitive (ts) mutants with defects associated with RNA synthesis at the nonpermissive temperature (10). Complementation

could not be detected between any NDV_{pi} clones primarily as a result of viral interference at an early step in replication at either the permissive or nonpermissive temperature (11).

The present report deals with the relationship between the temperature-sensitive marker of NDV_{pi} and the other properties which differentiate NDV_o and NDV_{pi}. The following types of virus were compared: NDV_{pi} ts^+ revertants selected at the nonpermissive temperature; NDV_o ts mutants generated by treatment with nitrous acid; and spontaneously-occurring ts mutants in populations of Herts NDV. The properties of each type of virus were characterized by the methods used previously to define NDV_{pi} and NDV_o (10, 23).

To determine if selection of ts mutants of NDV is involved in the establishment and maintenance of persistent infection, the ability of NDV_{pi}, NDV_{pi} ts^+ revertants, and NDV_o ts mutants to initiate persistent infection in L cells was tested. In addition, persistent infections established by using two other strains of NDV, the Texas-GB and Kansas-Man strains, yielded temperature-sensitive mutants and the properties of the viruses selected in these persistent infections are also reported.

MATERIALS AND METHODS

Cells. Primary CE cell cultures and a continuous line of mouse L (clone 929) cells were propagated and maintained in Eagle minimal essential medium (MEM) plus 4% calf serum (27).

Viruses. The origins and properties of NDV_o and the NDV_{pi} clones derived from the Herts strain of NDV have been described in detail previously (10, 23). Two velogenic NDV strains, Texas-GB and Kansas-Man, were obtained from Samuel Baron, National Institutes of Health.

Isolation of NDV_{pi} ts^+ and NDV_o ts clones. NDV_{pi} ts^+ revertants were cloned as follows: Stock preparations of NDV_{pi} clones 20, 25, 29, and 38 were diluted in medium and assayed at 37 and 43 C. Well-isolated plaques obtained from plates incubated at the nonpermissive temperature were transferred to CE cell monolayers in 2-oz prescription bottles. After 24 to 48 h at 37 C, when cell destruction was complete, infected fluids were harvested and assayed for infectivity at 37 and 43 C. Clones with a high plaquing efficiency at 43 C (43 C titer/37 C titer $> 10^{-1}$) were plaque purified once again. Working stocks of five clones isolated by this procedure were prepared in CE cell monolayer cultures in 32-oz prescription bottles.

Mutagen-induced NDV_o ts mutants were isolated after treatment of NDV_o with $NaNO_2$ in acetate buffer, pH 4.7, according to the method described by Granoff (6). Well-isolated plaques were picked from terminal dilution plates of samples showing about 10^{-5} survival, transferred to CE cell monolayers in 2-oz bottles, and the virus progeny were tested for plaquing ability at 37 and 43 C. Working stocks of four clones with low plaquing efficiencies ($< 10^{-2}$) at 43 C were prepared in CE cell monolayers in 32-oz bottles.

Spontaneous NDV ts mutants were isolated from uncloned Herts NDV. Infected allantoic fluid was serially diluted and plaqued at high dilutions on CE cells at 37 C. The agar over well-isolated plaques was removed, transferred to 1.0 ml of MEM, and the virus was allowed to elute from the agar at 4 C overnight. Herts clones (351) were isolated and screened by the procedure described below.

Screening of NDV clones for temperature-sensitivity. CE cell monolayers were grown in plastic trays (disposo-trays, Linbro Chemical Co.) containing 24 wells of 24-mm diameter. Each well was seeded with 1.5 ml of a standard dilution of primary CE cells; about 10^6 to 2×10^6 cells were present in each well when the monolayers were confluent. The cells in each well were infected with 0.1 ml of undiluted virus suspension from isolated plaques. CE cells in control wells were mock-infected or infected with NDV_o or NDV_{pi} clone 35. Replicate welled-trays were incubated at 37 and 43 C. The infected monolayers were examined under a microscope for cytopathology (CPE) which correlates well with virus replication (Youngner, unpublished data); cultures with CPE always showed increased virus production, whereas cultures without CPE were usually found to have produced no virus progeny. When CPE was complete in wells incubated at 37 C and absent in the replicate wells incubated at 43 C (usually 24–48 h after infection), fluids were harvested from both wells and assayed at 37 C. Virus clones with replication efficiencies of 10^{-2} or less at 43 C were considered potential ts mutants and working stocks of virus were prepared in CE cells.

Infection of CE or L cells. The procedures for infecting cells in monolayer cultures and for harvesting released (RV) and cell-associated virus (CAV) used in the experiments reported here have been described in detail elsewhere (10). Briefly, virus inoculum was adsorbed to CE or L-cell monolayers for 1 h at 37 C. The infected monolayers were washed twice, fed with fresh medium, and incubated at the appropriate temperature. At various times, culture fluids (RV) or infected cells were harvested and virus infectivity was determined by plaque assay in CE cells at 37 C.

Characterization of NDV clones. The characterization of the NDV_{pi} ts^+ clones and the NDV_o ts mutants was performed by methods used previously to differentiate NDV_{pi} and NDV_o (10). The thermal inactivation of viral infectivity, hemagglutinin, and neuraminidase activities was determined by diluting stock virus preparations into phosphate-buffered saline (PBS, 0.1 M, pH 7.2), and heating at the appropriate temperature (10, 23, 25).

Establishment of persistently infected L cells. L-cell monolayer cultures (5×10^6 to 6×10^6 cells) in 2-oz prescription bottles were infected with 0.1 PFU/cell of the appropriate NDV clones. The cultures were washed twice, fed with 7 ml of warm MEM, and incubated at 37 C. This was considered "passage zero" (P_0). Cultures with no obvious cytopathology by 72 h were subcultured (P_1) by scraping the cells from

the glass into 2 ml of fresh MEM and transferring 0.3 to 0.4 ml of this cell suspension (5×10^5 to 10^6 cells) into new 2-oz bottles. The infected cultures and mock-infected control cultures were subcultured every 7 days after that (P_2, P_3, etc.). Both supernatant fluids and cells were assayed for infective centers at the time of subculturing. At P_0, P_1, P_4, and P_8, the interferon activity in supernatant fluids was also determined by the plaque-reduction method (27).

To confirm the establishment of persistent infections, cultures at P_8 were challenged with vesicular stomatitis virus (VSV) (MOI = 0.1 PFU/cell) or with NDV_{pi} clone 35 (MOI = 5 PFU/cell). Cultures were infected with challenge virus for 1 h at 37 C, rinsed, and fed. Culture fluids harvested 24 h later were assayed on CE cells. VSV plaques were counted at 2 days and were easily distinguishable from NDV plaques counted 3 to 4 days after infection.

The reisolation of clones from L_{NDV} cultures was also performed at P_8. Cultures were assayed for infective centers at the time of subculturing for P_9, the plaque size was measured, and the agar over well-isolated plaques from the assay plates was placed into 1.0 ml of MEM at 4 C overnight. The virus eluted from these plaques was screened for temperature-sensitivity by the "welled-tray" technique described above.

RESULTS

Isolation of NDV_{pi} ts^+ revertants and NDV_0 ts mutants. The objective of this set of experiments was to determine if the ts marker of NDV_{pi} was linked to any of the other properties characteristic of NDV_{pi}. To obtain NDV_{pi} ts^+ revertants, stock preparations of NDV_{pi} were plated on CE cells, overlaid with agar and incubated at 43 C. If NDV_{pi} plaques which appear at the nonpermissive temperature are due to revertants rather than to leakiness, then isolation of NDV_{pi} plaques formed at 43 C should result in selection of ts^+ clones. From cultures plated with clone 38, 6 plaques were isolated and from plates infected with clone 20, 10 plaques were isolated. Of these 16 plaques, 4 (NDV_{pi} ts^+ 20R3, 38R1, 38R4, 38R6) had replication efficiencies at 43 C greater than 10^{-1} and were kept for further study.

NDV_0 ts mutants were isolated after treating NDV_0 stocks with nitrous acid as described in Materials and Methods. The kinetics of virus inactivation by HNO_2 were very similar to those obtained by Granoff (6). In three experiments, 70 plaques were screened for temperature-sensitivity and 4 clones (NDV_0 ts 17, ts 29, ts 31, ts 43) had replication efficiencies of less than 10^{-2} at 43 C.

The plaquing efficiencies of cell culture preparations of the NDV_{pi} ts^+ revertants and the NDV_0 ts mutants are compared to the plaquing efficiencies of allantoic fluid preparations of parental NDV_{pi} and NDV_0 (Table 1). Revertants from both NDV_{pi} clones 20 and 38 were clearly no longer ts, whereas the nitrous acid-induced NDV_0 ts mutants were ts to varying degrees. Three of the NDV_0 ts mutants produced large plaques in CE cells at 37 C, whereas NDV_0 ts 31 produced small plaques under the same conditions. All of the NDV_{pi} ts^+ clones retained the small-plaque characteristic at both 37 and 43 C. Plaque size and temperature-sensitivity therefore appear to be independent characteristics.

The growth patterns of the NDV_{pi} ts^+ revertants and the NDV_0 ts mutants at 37 and 43 C were confirmed by one-cycle growth curves in CE cells. The growth curves of NDV_0 ts 17 are shown in Fig. 1; the results with the other NDV_0 ts mutants were essentially the same. Replication curves at 37 C were very similar to those previously described for NDV_0 (10, 23). At 43 C, however, no replication of NDV_0 ts 17 was detectable, although NDV_0 replicates normally at 43 C. The replication of NDV_{pi} ts^+ 38R6 in CE cells at 37 and 43 C is shown in Fig. 2. At 43 C, significant virus replication was observed, with peak virus production (~30% of the 37 C yield) occurring 12 h after infection. The results also suggest that the ts^+ revertants may be defective in a maturation step at 43 C since most of the virus remains cell-associated.

Properties of NDV_{pi} ts^+ and NDV_0 ts clones. The new virus isolates were characterized to determine if reversion of the ts mutation of NDV_{pi} or introduction of a ts mutation into

TABLE 1. *Plaquing efficiencies of NDV_{pi} ts^+ revertants and NDV_0 mutants in CE cells at 37 and 43 C*

Virus	PFU/ml		Plaquing efficiency (43 C/37 C)
	37 C	43 C	
NDV_0[a]	4.7×10^9	1.7×10^9	0.36
NDV_{pi} Cl 20[b]	4.0×10^8	7.7×10^4	1.8×10^{-4}
NDV_{pi} Cl 38	1.5×10^9	1.0×10^7	6.7×10^{-3}
NDV_{pi} ts^+ 20R3[c]	1.5×10^6	6.1×10^5	0.41
NDV_{pi} ts^+ 38R1	6.6×10^6	5.1×10^6	0.78
NDV_{pi} ts^+ 38R4	1.0×10^7	6.1×10^6	0.61
NDV_{pi} ts^+ 38R6	7.9×10^6	6.4×10^6	0.84
NDV_0 ts 17[d]	3.2×10^7	3.3×10^2	1.0×10^{-5}
NDV_0 ts 29	1.4×10^7	1.0×10^3	7.2×10^{-5}
NDV_0 ts 31	3.4×10^6	3.5×10^4	1.0×10^{-2}
NDV_0 ts 43	1.1×10^7	1.5×10^5	1.4×10^{-2}

[a] Wild-type cloned Herts NDV, infected allantoic fluid.

[b] ts Mutant from persistent infection, infected allantoic fluid.

[c] ts^+ Revertants isolated from NDV_{pi} clones, infected CE cell culture fluid.

[d] ts Mutants from NDV_0 treated with nitrous acid, infected CE cell culture fluid.

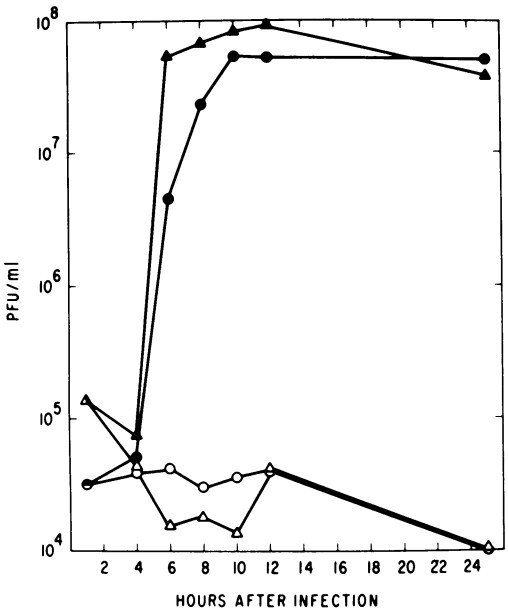

FIG. 1. *Single cycle yield of NDV_o ts 17 in CE cells at 37 and 43 C. CE cell cultures were infected and incubated at 37 and 43 C. At various times, RV (●, ○) and CAV (▲, △) were determined. Results with NDV_o were very similar to those obtained previously (10). Filled symbols, 37 C; open symbols, 43 C.*

NDV_o altered the other physical or biological properties of the virus. Previous experiments (10, 23) have shown that NDV_o is more lethal for fertile eggs than NDV_{pi}. As shown in Table 2, the NDV_{pi} ts^+ clones tended to behave like NDV_{pi} and the NDV_o ts mutants tended to behave like NDV_o, although the correlation was not absolute (e.g., clones ts^+ 38R1 and ts 31). This suggests that virulence for eggs may not be related to temperature-sensitivity. Also, no absolute correlation exists in this case between plaque size of the infecting virus and its lethality for eggs, although this correlation has been reported for other NDV strains (13, 16).

Dead and live eggs were harvested and the virus in the infected allantoic fluid was assayed at 37 and 43 C. With all clones except NDV_o ts 31, virus from infected eggs was of the expected plaque size and temperature-sensitivity, and the allantoic fluids were used as a source of virus for subsequent experiments. NDV_o ts 31 was not stable and was not used in further experiments.

NDV_o and NDV_{pi} also differ markedly in thermal inactivation profiles of virion-associated activities. To determine the thermostability profiles of NDV_{pi} ts^+ revertants and NDV_o ts mutants, stock virus preparations diluted in PBS were heated at the appropriate temperature and assayed for residual activity compared to unheated controls. The NDV_{pi} ts^+ revertants remained "NDV_{pi}" despite reversion to wild-type with regard to replication at 43 C; virus infectivity was not stable to heating, whereas both hemagglutinin (HA) and neuraminidase activities were stable under conditions that completely destroyed the HA and neuraminidase activity of NDV_o (Table 3). In a separate experiment with the NDV_o ts mutants, the results showed that these mutants remained "NDV_o" even though they could no longer replicate at 43 C. Clearly, the ts mutation of NDV_{pi} can revert independently from the mutation(s) which result in altered thermal stability of virion-associated activities. Conversely, introduction of a ts mutation into NDV_o was not sufficient to change these physical properties of the virion.

Replication of NDV_{pi} ts^+ and NDV_o ts clones in L cells. To determine if temperature-sensitivity was linked to the ability of NDV_{pi} to

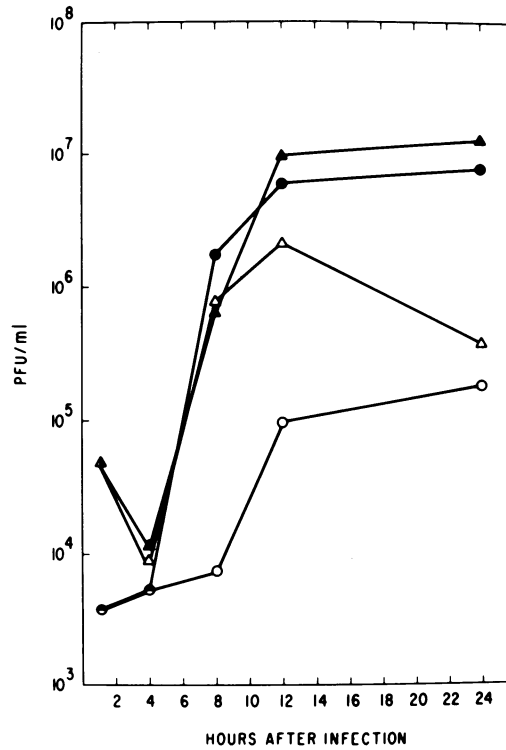

FIG. 2. *Single cycle yield of NDV_{pi} ts^+ 38R6 in CE cells at 37 and 43 C. CE cell cultures were infected with NDV_o or NDV_{pi} ts^+ 38R6 and incubated at 37 or 43 C. At various times, RV (●, ○) and CAV (▲, △) were harvested and assayed on CE cells at 37 C. The results with NDV_o were very similar to those obtained previously (10). Filled symbols, 37 C; open symbols, 43 C.*

replicate efficiently in mouse L cells, single-cycle yield experiments with the ts$^+$ revertants were performed in mouse L cells infected with 1 to 5 PFU/cell. The results with NDV_{pi} ts$^+$ 38R4, NDV_o, and NDV_{pi} clone 38 are shown in Fig. 3 A,B,C, respectively. The results with the other ts$^+$ clones were not significantly different from those obtained with ts$^+$ 38R4. The data shown for NDV_o and NDV_{pi} were very similar to those previously published (10, 23). The growth curves of the ts$^+$ clones were intermediate between those of NDV_o and NDV_{pi}, with the overall shape of the curve more similar to that of NDV_o. The large accumulation of CAV, characteristic of the covert infection of L cells by NDV_o, was also seen in L cells infected with ts$^+$ 38R4. Less than 5% of the CAV present in infected cells at 8 to 12 h after infection was released into the medium by 24 h after infection. With NDV_{pi} clone 38, however, peak virus

TABLE 2. *Lethality of NDV_{pi} ts$^+$ revertants and NDV_o ts mutants for embryonated eggs*[a]

Virus	No. dead/no. of eggs inoculated		
	24 h	48 h	72 h
NDV_o	1/27	27/27	
NDV_{pi} Cl 20	1/24	7/24	14/24
NDV_{pi} ts$^+$ 20R3	0/24	0/24	17/24
NDV_{pi} Cl 38	0/24	2/24	18/24
NDV_{pi} ts$^+$ 38R1	0/24	13/24	24/24
NDV_{pi} ts$^+$ 38R4	0/24	1/24	24/24
NDV_{pi} ts$^+$ 38R6	0/24	2/24	20/24
NDV_o ts 17	1/24	17/24	
NDV_o ts 29	0/24	23/24	
NDV_o ts 31	0/24	0/24	15/21
NDV_o ts 43	0/24	24/24	

[a] Each of 2 dozen eggs was inoculated into the allantoic cavity with 10^4 PFU of virus. The infected eggs were incubated at 36 C and cumulative dead eggs were recorded at 24, 48, and 72 h.

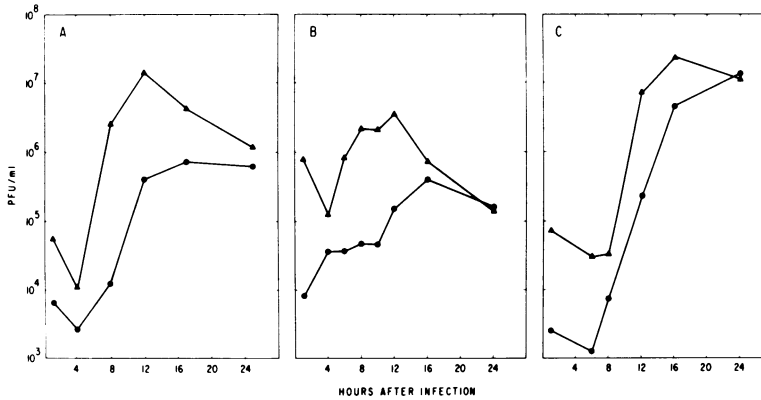

FIG. 3. *Replication of NDV_o, NDV_{pi} ts$^+$ 38R4 and NDV_{pi} clone 38 in mouse L cells. Mouse L-cell cultures were infected (MOI = 1-5 PFU/cell) and incubated at 37 C. At various times, RV (●) and CAV (▲) were determined. A, NDV_{pi} ts$^+$ 38R4; B, NDV_o; C, NDV_{pi} clone 38.*

TABLE 3. *Thermostability of virion-associated activities of NDV_{pi} ts$^+$ revertants and NDV_o ts mutants*

Experiment no.	Virus	Fraction surviving[a] infectivity after:		Fraction remaining HAU after 35 min at 55 C[b]	Fraction remaining neuraminidase after 35 min at 55 C[b]
		30 min	60 min		
1	NDV_o	0.36	0.15	<0.2	<0.1
	NDV_{pi} Cl 20	3.1×10^{-3}	3.8×10^{-4}	0.5	0.80
	NDV_{pi} ts$^+$ 20R3	4×10^{-3}	4×10^{-4}	ND[c]	ND[c]
	NDV_{pi} Cl 38	1.9×10^{-2}	4×10^{-3}	0.71	1.0
	NDV_{pi} ts$^+$ 38R1	0.022	1×10^{-3}	1.0	2.5
	NDV_{pi} ts$^+$ 38R4	0.01	1.5×10^{-3}	1.0	2.5
	NDV_{pi} ts$^+$ 38R6	8.7×10^{-2}	8.9×10^{-4}	0.7	0.95
2	NDV_o	0.75	0.35	<0.2	<0.1
	NDV_o ts 17	0.64	0.12	<0.2	0.086
	NDV_o ts 29	0.41	0.15	<0.2	0.405
	NDV_o ts 43	0.67	0.21	<0.2	0.162

[a] CE cell-grown virus diluted 1:10 in PBS and heated for 30 or 60 min at 50 C.
[b] Egg-grown virus diluted 1:50 in PBS and heated for 35 min at 55 C.
[c] Not determined; titer of egg-grown virus was not high enough to perform these tests.

production did not occur until 16 h postinfection and 60% of the accumulated CAV was released by 24 h after infection. These results suggested that the ability of NDV_{pi} to replicate in L cells may be linked to the ts defect of the virus.

A similar experiment with the nitrous acid-induced mutants, however, indicated that the ts marker alone may not be sufficient for a productive replication cycle of NDV in L cells. The NDV_o ts mutants, like the parental NDV_o virus, also produced a covert infection in L cells using an input MOI of 1 to 5 PFU/cell (Table 4). A comparison of the underlined values for RV and CAV for each virus listed in Table 4 reveals that with both NDV_o and each of the NDV_o ts mutants, accumulation of CAV was maximal at 12 h after infection, and that less than 10% of this infectivity was released as mature virus. In all cases, CAV declined by at least 50% between 12 and 24 h after infection without a concomitant increase in released virus. The fate of the CAV in the covert infection of L cells by NDV_o, NDV_o ts mutants or NDV_{pi} ts^+ revertants remains unknown.

Properties of spontaneous ts mutants. To investigate further the relationship between the ts marker and the evolution of NDV_{pi}, 351 clones of Herts NDV were isolated and screened by the welled-tray technique for ability to replicate at 43 C. This technique determines the number of ts clones present in the virus population tested but does not measure the mutation rate of this marker. Four spontaneous ts mutant clones were obtained. The reversion frequency of the ts marker for these clones was $<3 \times 10^{-5}$ in all four cases. The replication efficiency in CE cells at 43 C for each clone and other properties of the spontaneously-occurring mutants are shown in Table 5. All of the spontaneous mutants could be classified as "NDV_o": they produced large (2-3 mm) plaques in CE cells at 37 C; the HA and neuraminidase were not stable to heating, and they produced a covert infection in mouse L cells in which a large amount of replicated CAV was never released into the medium. These data confirm that with naturally occurring clones, as well as with mutagenized clones, no relationship exists between temperature-sensitive markers and other properties associated with NDV_{pi}.

One other result is worth noting. The spontaneous ts clones were tested for the ability to synthesize virus-specific RNA in CE cells at 43 C by using methods which have been described in detail (10). All four spontaneous mutants failed to synthesize virus-specific RNA

TABLE 4. *Replication of NDV_o ts mutants in mouse L cells at 37 C*

Virus	Released virus (PFU/ml)			Cell-associated virus (PFU/ml)		
	0 h	12 h	24 h	0 h	12 h	24 h
NDV_o	1.8×10^4	1.7×10^6	$\underline{5 \times 10^6}$	6.1×10^5	$\underline{3.7 \times 10^7}$	5×10^6
NDV_o ts 17	8×10^3	8×10^4	$\underline{2 \times 10^5}$	3.3×10^5	$\underline{5 \times 10^6}$	9.7×10^5
NDV_o ts 29	4.1×10^3	1.3×10^5	$\underline{3 \times 10^5}$	2.4×10^5	$\underline{1.2 \times 10^7}$	1.6×10^3
NDV_o ts 31	1.2×10^3	1×10^3	$\underline{5 \times 10^3}$	6.1×10^4	$\underline{9.1 \times 10^5}$	9.4×10^4
NDV_o ts 43	3.7×10^3	1.5×10^5	$\underline{6.8 \times 10^5}$	1.1×10^5	$\underline{1.9 \times 10^7}$	1.6×10^6
NDV_{pi} Cl 38	2.5×10^3	2.5×10^5	$\underline{1.4 \times 10^7}$	7.5×10^4	$\underline{7.5 \times 10^6}$	1.1×10^7

TABLE 5. *Properties of spontaneously-occurring Herts NDV ts mutants*

Clone	Replication in CE cells[a] (43 C/37 C)	Plaque size at 3 days (mm)	Egg lethality[b] at 48 h (%)	Residual activity[c] after 35 min at 55 C		Replication in L cells
				HA	Neuraminidase	
NDV_o	0.40	2-3	100	<0.01	0.28	Covert
ts 107	8.1×10^{-4}	2-3	90	<0.03	0.29	Covert
ts 110	3.4×10^{-4}	2-3	100	<0.06	0.28	Covert
ts 113	2.9×10^{-5}	2-3	70	<0.03	0.30	Covert
ts 119	3.0×10^{-4}	2-3	100	<0.03	0.31	Covert
NDV_{pi} 35	2.8×10^{-4}	<1	50	1.0	1.0	Productive

[a] Infected CE cells were incubated at 37 or 43 C and virus yields after 24 h were assayed on CE cells at 37 C.

[b] Each of 2 dozen fertile eggs was injected into the allantoic cavity with 10^4 PFU and dead and live eggs were tallied after 48 h.

[c] Stock virus preparations were diluted in PBS, heated at 55 C and residual hemagglutinin and neuraminidase activities were assayed as previously described (10).

at the nonpermissive temperature. In addition to the four spontaneous ts mutants, we have now tested three nitrous acid-induced mutants, and at least 20 ts mutants isolated from L cells persistently infected with Herts NDV and all of these ts mutants were RNA^-. A possible exception was NDV_o ts 43 which synthesized about 16% of the wild-type level of RNA at 43 C; however, the reversion frequency of this mutant was fairly high, and the level of RNA synthesis obtained may have been due to the presence of revertants.

Persistent infection with NDV_{pi} ts^+ revertants and NDV_o ts mutants. The results of the experiments with NDV_{pi} ts^+ revertants, NDV_o ts mutants generated with nitrous acid, and spontaneous Herts NDV ts clones, coupled with previous results with NDV_{pi}, strongly suggested that selection of ts mutants within the persistent infection was not random and might play a role in either establishment or maintenance of the persistently infected state. To investigate this possibility, L-cell cultures were infected with NDV_o, NDV_{pi}, NDV_{pi} ts^+, and NDV_o ts mutant clones, by using procedures similar to those used for establishment of the original L_{NDV} cell cultures in 1967 (see Materials and Methods). Seventy-two hours later, all infected cultures appeared healthy and were subcultured (P_1) into new 2-oz bottles. The infected cultures retained their normal appearance and have been subcultured at the same rate as control L cells once per week for over 30 passages.

Pertinent data on the early passages of the infected L cells are given in Table 6. Assay of infected centers in cells and culture fluids from P_0 revealed that very little, if any, virus replication had occurred during the first 72 h of infection. In most cases, interferon synthesis was minimal due to the low MOI (23). After P_0, most of the virus in the persistently infected cultures remained cell-associated and this was reflected in the value for cells per plaque. This value fluctuated widely from sample to sample and from week to week, but average values indicated that one cell in 100 or in 1,000 was usually capable of yielding virus. These values are in agreement with those previously reported for L_{NDV} cultures (23).

By P_{15}, the original virus inoculum had been diluted by cell passage appoximately 10.5 logs. Since the original virus inoculum was 0.1 PFU per cell or about 3×10^5 PFU per bottle, infectivity at P_{15} was clearly the result of virus replication. The notation $L_{(virus\ clone)}$ was adopted to denote each type of persistently infected culture, so that L_{Herts} and L_{38R1} were persistently infected with uncloned Herts virus and NDV_{pi} ts^+ 38R1, respectively.

Persistent infection, therefore, can apparently be established by all NDV clones except NDV_{pi}. NDV_{pi} clone 20 induced significant levels of interferon during P_0 and was rapidly eliminated from the cultures (Table 6); after P_3 the cultures were considered no longer infected and were discarded. Disruption of the cells by sonic oscillation prior to plating had no effect on the number of infective centers per culture. Other attempts to establish L-cell cultures persistently infected with NDV_{pi} have also been unsuccessful (Thacore and Youngner, unpub-

TABLE 6. *Early passage history of L cells persistently infected with various types of NDV*

L cells infected with:	P_0			P_4		P_{15}	
	PFU[a]/ml $\times 10^2$	Cells[b]/ plaque	Interferon titer[c]	PFU/ml $\times 10$	Cells/ plaque	PFU/ml $\times 10^2$	Cells/ plaque
Herts	2.6	3.0×10^2	<32	<1	1.3×10^4	0.2	3.6×10^4
NDV_o	2.5	3.0×10^2	<32	<1	5.0×10^3	3.3	4.0×10^2
NDV_o ts 17	4.5	1.0×10^2	<32	<1	5.0×10^3	0.2	3.7×10^2
NDV_o ts 29	4.0	7.5×10^1	<32	<1	6.0×10^3	2.6	3.6×10^3
NDV_o ts 43	2.1	3.0×10^2	<32	<1	3.0×10^3	10	3.0×10^2
NDV_{pi} Cl. 20	1.0	7.0	120	<1[d]	>10^6[d]		
NDV_{pi} ts^+ 20R3	0.4	5.0×10^2	<32	10	5×10^2	8.0	5.6×10^2
NDV_{pi} ts^+ 38R1	41	7.5	<32	100	40	5.8	7.8×10^2
NDV_{pi} ts^+ 38R4	110	6.0	64	64	2.5×10^2	290	2.4×10^2
NDV_{pi} ts^+ 38R6	90	8.0	<32	1700	4.5	490	2.1×10^2

[a] Infective virus in culture fluid (7 ml) assayed in CE cell monolayers.

[b] Infected cells were scraped from the glass into 2 ml of MEM, counted in a hemocytometer, and plated for infective centers on CE cell monolayers.

[c] Interferon in culture fluids at selected passages was assayed by the plaque reducton technique; values are U/3 ml.

[d] Cultures concluded to be no longer infected and were discarded after P_3.

lished data). Although attempts to establish persistent infection with NDV_{pi} clones 25 to 38 were not included in these experiments, one can assume that they would have behaved similarly to clone 20. Subtle differences between these clones and clone 20 exist (10) but the replication patterns of all NDV_{pi} clones in L cells are very similar.

As a result of persistent infection with NDV, L_{NDV} cultures have been shown to become resistant to superinfection with unrelated cytolytic viruses, such as VSV (3, 7, 14, 23). The putative L_{NDV} cultures and uninfected control L-cell cultures were challenged at P_8 with VSV, NDV_o, or NDV_{pi} clone 35, as described in Materials and Methods. Plaques of NDV challenge virus were produced 1 to 2 days earlier than NDV plaques caused by the endogenous, carried virus in the L_{NDV} cultures.

The yield of challenge virus from each persistently infected culture is shown in Table 7. Clearly, VSV replicated only in the uninfected L cells; the yield of VSV from any of the L_{NDV} cultures was at least 4 logs lower than the yield of VSV from the control L-cell cultures. Similarly, no virus replication was observed when L_{NDV} cultures were challenged with either NDV_o or NDV_{pi} clone 35. The interferon titers in all culture fluids harvested from P_8 at the time of superinfection were less than 16 U per 3 ml.

The data given in Tables 6 and 7 clearly demonstrate that the L-cell cultures were persistently infected with NDV. It was also observed during the course of the cell passages described above that the time required for development of NDV plaques in infective center assays in CE cell cultures increased from 3 to 4 days to 6 days; this suggested that selection of slower growing variants or mutants had occurred. To test this possibility, and to determine the temperature-sensitivity of virus present, clones of virus were reisolated from each persistently infected culture and screened for ability to replicate at 37 and 43 C by using the "welded-tray" technique described in Materials and Methods. At 37 C, all infected monolayers showed extensive cytopathology by 48 h. In contrast, in one experiment, only 6 out of 65 monolayers infected with virus clones from L_{NDV} cultures showed minor cytopathic changes at 43 C, although all NDV_o-infected monolayers at this temperature were completely destroyed. Culture fluids from about 40% of the wells at 43 C were assayed for infectivity and significant virus replication was detected only in fluids from wells with degenerative changes in the cells.

In two experiments, 78 plaque-purified clones from the various persistently infected cultures were screened for temperature-sensitivity and virus progeny harvested from welled-trays at 37 C was also tested for plaque size at 4 days in CE cells at 37 C, to determine if the virus reisolated at P_8 was of a different plaque morphology from the virus used to infect P_0. By the eighth cell passage, virus carried in the persistently infected L cells was ts no matter which type of virus was used for infection (Table 8). Also, plaque size of all reisolated virus clones was decreased regardless of whether large or small plaque virus was used to initiate the persistent infection.

TABLE 7. *Yield of challenge virus from persistently infected L cells (P_8) superinfected with VSV, NDV_o, or NDV_{pi} clone 35*

L cells	Yield of challenge virus (PFU/ml × 10^3)		
	VSV	NDV_o	NDV_{pi} clone 35
Control[a]	5.0×10^4	51	170
L_{Herts}	0.7	2.7	1.0
L_{NDV_o}	1.1	1.8	3.0
$L_{ts\ 17}$	1.0	2.3	1.3
$L_{ts\ 29}$	3.9	2.6	3.2
$L_{ts\ 43}$	1.6	1.3	3.1
$L_{ts^+\ 38R1}$	0.4	4.2	2.6
$L_{ts^+\ 38R4}$	<0.1	4.6	2.7
$L_{ts^+\ 20R3}$	0.5	3.2	4.8

[a] Uninfected control L cells with the same passage history as the persistently infected cultures.

TABLE 8. *Temperature-sensitivity and plaque size of virus isolated from L cell cultures persistently infected with NDV_o, NDV_o ts mutants, or NDV_{pi} ts^+ revertants*

Plaques isolated from 8th passage of:	Plaques tested	No. ts	Plaque size and range at 4 days (mm)[a]
L_{Herts}	8	8	1.2 (1.0–1.5)
L_{NDV_o}	8	8	1.0 (0.5–1.5)
$L_{ts\ 17}$	6	6	1.0 (0.5–1.5)
$L_{ts\ 29}$	8	8	1.5 (<1–2.0)
$L_{ts\ 43}$	5	5	1.0 (0.5–1.5)
$L_{ts^+\ 38R1}$	11	8	<0.5
$L_{ts^+\ 38R4}$	13	11	<0.5
$L_{ts^+\ 38R6}$	12	11	<0.5
$L_{ts^+\ 20R3}$	8	7	<0.5

[a] NDV_o control plaques were 2 to 3 mm at 3 days, whereas NDV_{pi} clone 35 control plaques were 1 to 2 mm at 4 days.

Persistent infection of L cells with other NDV strains. To test whether the selection of ts virus was peculiar to the Herts NDV-L-cell system, two other strains of NDV were used to initiate persistent infection. L-cell monolayers were infected with the Texas-GB (Tex) or the

Kansas-Man (Kan) strains of NDV, by using conditions for establishing persistent infection (see Materials and Methods). Wild-type clones (Tex_o and Kan_o) and clones from persistently infected cultures (Tex_{pi} and Kan_{pi}) were isolated and characterized.

The properties of Tex and Kan clones are summarized in Table 9. The plaque size in CE cells of both Tex_{pi} and Kan_{pi} clones was smaller than that of the respective wild-types. The frequency of stable ts Tex_{pi} clones was much higher than the frequency of stable ts Kan_{pi} clones. This pattern was true whether clones were isolated early (after 4–19 cell passages) or late (after 37–46 cell passages) after establishment of the persistent infections and may reflect differences between the Tex and Kan strains. Many Kan_{pi} clones proved to be unstable after egg passage (Table 9) and consequently high-titered stocks of those clones were not available for use in RNA synthesis experiments. All stable ts clones tested, however, were RNA^- at the nonpermissive temperature. All clones isolated from persistently infected cultures also replicated more efficiently in L cells than did the wild-type Tex or Kan clones. In contrast to results with Herts NDV_{pi}, however, Tex_{pi} and Kan_{pi} virus clones isolated from persistently infected L cells were no different from wild-type Tex_o and Kan_o clones in the thermal inactivation profiles of infectivity, hemagglutinin, and neuraminidase.

DISCUSSION

The objectives of this investigation were to determine the relationship between the ts marker and the other characteristics of NDV_{pi}, and to assess whether selection of such ts mutants played a role in either establishment or maintenance of persistent infection of L cells with NDV. The experiments with NDV_{pi} ts^+ revertants, NDV_o ts mutants induced with HNO_2, and spontaneous ts mutants indicate clearly that the ts marker can be either lost or acquired without necessarily altering the physical properties or the growth rate of the virus. NDV_{pi} ts^+ clones retained the small plaque size and thermal stability profile of NDV_{pi}, while the NDV_o ts mutants remained "NDV_o" except for the ability to replicate at 43 C. Also, with spontaneous ts mutants of the population of uncloned Herts NDV, no correlation existed between the ts marker and any of the properties characteristic of NDV_{pi}; all four clones displayed the physical and biological properties of NDV_o. However, in L cells persistently infected with NDV, temperature-sensitivity, small plaque size, and, at least with the Herts strain of virus, altered thermal stability were coselected.

Other experiments showed that the ability to replicate efficiently in L cells appeared to depend on the combination of the ts marker with the other properties of NDV_{pi}; both NDV_{pi} ts^+ revertants and NDV_o ts mutants produced a covert infection in L cells. In addition, NDV_{pi}, the only NDV type able to replicate productively in L cells, was unable to establish persistent infection, whereas uncloned Herts virus, NDV_o, NDV_{pi} ts^+ revertants, and NDV_o ts mutants were all able to establish and maintain carrier states in L cells. Virus reisolated at P_8 from any of these persistent infections was ts and produced small plaques in CE cells at 37 C. These results strongly suggest that selection of virus ts mutants is not random and may play a role in maintenance of the persistent infection.

Temperature-sensitive mutants of many other enveloped RNA viruses, including spontaneous ts mutants (4), have been studied, and

TABLE 9. *Properties of wild-type Texas-GB and Kansas-Man NDV clones and of clones isolated from L cells persistently infected with these strains*

Clones	Passage number in L cells	Plaque[a] size (mm)	No. clones ts[b]/no. clones tested	RNA synthesis at 43 C	Replication in L cells
Tex_o	None	4–5	0/4	+	Covert
Kan_o	None	4–5	0/4	+	Covert
Tex_{pi}	4–19	2–4	14/18 (14/14)[c]	–[d]	Productive
Tex_{pi}	43–46	2–4	20/24 (9/10)	–	Productive
Kan_{pi}	8	2–4	12/12 (4/12)	–	Productive
Kan_{pi}	37	2–4	12/12 (4/12)	NT[e]	Productive

[a] Plaque size in mm in CE cells after 7 days at 37 C.

[b] Yield of original clone in CE cells at 43 C ÷ yield at 37 C $<10^{-2}$ or plaque titer of original clone at 43 C ÷ plaque titer at 37 C $< 10^{-3}$.

[c] Number of ts clones stable during egg passage per number of ts clones tested in eggs; eggs still alive 48 h after inoculation were harvested.

[d] RNA phenotype of stable ts mutants grown in eggs.

[e] NT, not tested.

in each case, characterization of the mutants with regard to functional defect has revealed both RNA^+ and RNA^- mutants (1, 12, 22). Although other workers (2, 8) reported isolation of ts mutants of NDV, no biochemical analysis of the mutants has been described. Results presented here suggest that ts mutants of NDV, whether spontaneously occurring, isolated after mutagen treatment, or selected by persistent infection, are always RNA^- (10). Several dozen ts mutants of NDV have been tested in this laboratory and all of the mutants consistently failed to synthesize any detectable virus-specific RNA at the nonpermissive temperature under conditions where wild-type virus produced large amounts of RNA. The functional defect in NDV_{pi} ts mutants appears to be associated with RNA polymerase (11). The other ts mutants discussed in this report have not yet been analyzed in as much depth. The significance of finding only RNA^- ts mutants of NDV is difficult to explain. The reason may lie in unusual genetic properties associated with NDV or in complicated virion protein interactions that allow only certain limited variations in structure that are not lethal.

The mechanisms for the establishment and perpetuation of L-cell cultures persistently infected with NDV are largely unknown. The experiments described above explore the possibility that selection of ts virus mutants, which also differ from wild-type virus in other properties, may be involved in maintenance of the carrier state. Other workers have also noted that virus recovered from persistently infected cell cultures may be different in properties, e.g., plaque size and virulence, from the wild-type virus used to initiate the infection (5, 17, 18, 21, 26). In several instances reports have also appeared describing ts behavior of virus associated with persistent infections. An attenuated mutant isolated from Frukto mouse sarcoma cells persistently infected with Western equine encephalitis (WEE) virus (18) also had a decreased growth potential at 42 C, whereas the wild-type virus replicated normally at this temperature (19). When the attenuated strain was passaged at 41 C and clones were selected for growth at that temperature, the resulting population retained its attenuated characteristic. In contrast, when virulent revertants were selected, the reversion to virulence had no effect on the ts marker (19). These studies, therefore, were similar to the studies with NDV_{pi} ts^+ revertants and NDV_o ts mutants reported here in which the ts and plaque type markers of NDV_{pi} were found to be independent mutations even though they were co-selected in the persistent infection. The studies with the WEE mutant support the hypothesis that mutations selected during persistent infection are not random. In another study, Nagata and coworkers (9) reported the establishment of BHK-21 cells persistently infected with Sendai virus. The viral maturation in these cultures was normal at 31 C, but ts at 37 C.

One study has also linked carrier infections in vivo with selection of ts mutants (20). Plaque production by strains of foot and mouth disease virus isolated from carrier cattle was inhibited at 40 to 41 C, whereas plaque production by vesicular isolates was not affected by the increased temperature. In addition, plaque size on BHK monolayers was smaller with carrier isolates than with vesicular isolates.

Results from several different virus-cell systems, therefore, suggest that a property of persistent infection is selection of mutants or variants with impaired ability to replicate at high temperatures. The demonstration of a small population of spontaneous ts mutants in Herts NDV preparations suggests that perhaps these virus clones are selected during early stages of the persistent infection of L cells with NDV and only later do the structural alterations implied by the altered thermal stability of NDV_{pi} evolve. It is possible that a low level of this type of virus is present even in cloned populations of virus, such as NDV_o or the NDV_{pi} ts^+ revertants since passage through eggs is known to affect the stability of NDV populations (23; Preble and Youngner, unpublished data).

We are currently investigating the generality of the phenomena observed in the NDV-L-cell system; we are testing virus-carrier systems involving other paramyxoviruses, as well as viruses unrelated to NDV, in L cells and other cell lines to determine if the outcome of persistent infection is dependent on the particular virus or host cell used.

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LITERATURE CITED

1. Burge, B. W., and E. R. Pfefferkorn. 1966. Isolation and characterization of conditional-lethal mutants of Sindbis virus. Virol. **30**:204–213.

2. Dahlberg, J., and E. H. Simon. 1969. Recombination in Newcastle disease virus (NDV): the problem of complementing heterozygotes. Virol. **38**:490-493.
3. Deinhardt, F., V. Bergs, G. Henle, and W. Henle. 1958. Studies on persistent infections of tissue cultures. III. Some quantitative aspects of host cell-virus interactions. J. Exp. Med. **103**:573-589.
4. Flamand, A. 1970. Etude génétique du virus de la stomatite vésiculaire: classement de mutants thermosensible spontanés en groupes de complémentation. J. Gen. Virol. **8**:187-195.
5. Gavrilov, V. I., D. M. Asher, S. D. Vyalushkina, L. S. Ratushkina, R. G. Zmieva, and B. G. Tumyan. 1972. Persistent infection of continuous line of pig kidney cells with a variant of the WSN strain of influenza A_0 virus. Proc. Soc. Exp. Biol. Med. **140**:109-117.
6. Granoff, A. 1961. Induction of Newcastle disease virus mutants with nitrous acid. Virology **13**:402-408.
7. Henle, W., G. Henle, F. Deinhardt, and V. Bergs. 1959. Studies on persistent infections of tissue cultures. IV. Evidence for the production of an interferon in MCN cells by myxoviruses. J. Exp. Med. **110**:525-541.
8. Kirvaitis, J., and E. H. Simon. 1965. A radiobiological study of the development of Newcastle disease virus. Virology **26**:545-553.
9. Nagata, I., Y. Kimura, Y. Ito, and T. Tanaka. 1972. Temperature-sensitive phenomenon of viral maturation observed in BHK cells persistently infected with HVJ. Virology **49**:453-461.
10. Preble, O. T., and J. S. Youngner. 1972. Temperature-sensitive mutants isolated from L cells persistently infected with Newcastle disease virus. J. Virol. **9**:200-206.
11. Preble, O. T., and J. S. Youngner. 1973. Temperature-sensitive defect of mutants isolated from L cells persistently infected with Newcastle disease virus. J. Virol. **12**:472-480.
12. Pringle, C. R., and I. B. Duncan. 1971. Preliminary physiological characterization of temperature-sensitive mutants of vesicular stomatitis virus. J. Virol. **8**:56-61.
13. Reeve, P., and G. Poste. 1971. Studies on the cytopathogenicity of Newcastle disease virus: Relation between virulence, polykaryocytosis and plaque size. J. Gen. Virol. **11**:17-24.
14. Rodriguez, J. E., and W. Henle. 1965. Studies on persistent infections of tissue cultures. V. Initial stages of infection of L(MCN) cells by Newcastle disease virus. J. Exp. Med. **119**:895-921.
15. Rodriguez, J. E., V. terMeulen, and W. Henle. 1967. Studies on persistent infections of tissue cultures. VI. Reversible changes in Newcastle disease virus populations as a result of passage in L cells or chick embryos. J. Virol. **1**:1-9.
16. Schloer, G. M., and R. P. Hanson. 1968. Relationship of plaque size and virulence for chickens of 14 representative Newcastle disease virus strains. J. Virol. **2**:40-47.
17. Schwobel, W., and R. Ahl. 1972. Persistence of Sindbis virus in BHK-21 cell cultures. Arch. Gesamte Virusforsch. **38**:1-10.
18. Simizu, B., and N. Takayama. 1969. Isolation of two plaque mutants of Western equine encephalitis virus differing in virulence for mice. J. Virol. **4**:799-800.
19. Simizu, B., and N. Takayama. 1971. Relationship between neurovirulence and temperature sensitivity of an attenuated Western equine encephalitis virus. Arch. Gesamte Virusforsch. **35**:242-250.
20. Straver, P. J., and J. G. van Bekkum. 1972. Plaque production by carrier strains of foot-and-mouth disease virus in BHK-monolayers incubated at different temperatures. Arch. Gesamte Virusforsch. **37**:12-18.
21. Takemoto, K. K., and K. Habel. 1959. Virus-cell relationship in a carrier culture of HeLa cells and Coxsackie A9 virus. Virology **7**:28-44.
22. Tan, K. B., J. F. Sambrook, and A. J. D. Bellet. 1969. Semliki Forest virus temperature-sensitive mutants: isolation and characterization. Virology **38**:427-439.
23. Thacore, H. R., and J. S. Youngner. 1969. Cells persistently infected with Newcastle disease virus. I. Properties of mutants isolated from persistently infected L cells. J. Virol. **4**:244-251.
24. Thacore, H. R., and J. S. Youngner. 1970. Cells persistently infected with Newcastle disease virus. II. Ribonucleic acid and protein synthesis in cells infected with mutants isolated from persistently infected L cells. J. Virol. **6**:42-48.
25. Thacore, H. R., and J. S. Youngner. 1971. Cells persistently infected with Newcastle disease virus. III. Thermal stability of hemagglutinin and neuraminidase of a mutant isolated from persistently infected L cells. J. Virol. **7**:53-58.
26. Walker, D. L. 1964. The viral carrier state in animal cell cultures. Progr. Med. Virol. **6**:111-148.
27. Youngner, J. S., A. W. Scott, J. V. Hallum, and W. R. Stinebring. 1966. Interferon production by inactivated Newcastle disease virus in cell cultures and in mice. J. Bacteriol. **92**:862-868.