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Rescue of Temperature-sensitive Poliovirus[1]

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A temperature-sensitive strain of type 1 poliovirus, LSc, was functionally rescued when infected cells were incubated at 40 C in the presence of Mahoney, a temperature-resistant strain of type 1 poliovirus. The rescue value was 9% of the mutant yield obtained under permissive conditions. Rescued virus underwent replication, because the progeny of ^{32}P-labeled LSc were not radiosensitive. Serum inactivation studies with Mahoney specific antiserum indicated that a small amount of phenotypic mixing occurred among the rescued particles. The temperature-sensitive event occurred between 2 and 4 hr postinfection in the developmental cycle of LSc. Neither viral polymerase activity nor virus-induced ribonucleic acid could be demonstrated in infected cells between 2 and 4 hr after infection at 40 C with the temperature-sensitive mutant.

Functional rescue of conditional lethal mutants of poliovirus by homologous or heterologous enteroviruses is well known (7, 9). Mutants of poliovirus which fail to grow under nonpermissive conditions are able to replicate to some degree in the presence of helper virus. Restrictive conditions included the presence of guanidine hydrochloride during the growth of sensitive virus or the absence of drug during the growth of drug-dependent virus. The rescue values are seldom greater than 1% of the mutant yield under nonselective conditions.

Cooper (4) reported complementation between pairs of temperature-sensitive mutants of poliovirus, but was unable to demonstrate rescue of a temperature-sensitive mutant by the wild type. This paper reports the results of rescue experiments involving a temperature-sensitive strain of type 1 poliovirus, LSc, and a temperature-resistant strain of the same type, Mahoney. In addition to rescue, experimental results are presented indicating the time of occurrence of the temperature-sensitive step in the LSc growth cycle.

MATERIALS AND METHODS

Tissue cultures. The S3 strain of HeLa cells was used in serial culture. The growth medium (HLS) was Hanks balanced salt solution (BSS) which contained (per liter): 0.5 g of $NaHCO_3$, 100 ml of calf serum, 5 g of lactalbumin hydrolysate, 10 mg of streptomycin, 10^4 units of penicillin, 2.5×10^4 units of polymyxin, and 5×10^4 units of nystatin. Virus titrations were performed according to the standard agar overlay procedure of Hsiung and Melnick (8). When guanidine hydrochloride was used in the agar overlay, the medium contained 100 μg of inhibitor per ml.

Virus. The Mahoney $g^s t^r$ (guanidine-sensitive, temperature-resistant) and LSc $g^r t^s$ (guanidine-resistant, temperature-sensitive) strains of type 1 poliovirus were used. Temperature sensitivity is defined by the failure of virus to grow at 39 to 40 C.

Radiochemicals. Guanosine-5'-triphosphate-*8-^{14}C* tetralithium (26 mc/mmole) and tritiated uridine (24.7 c/mmole) were obtained from Schwarz Bio Research Inc., Orangeburg, N.Y. Carrier-free $H_3{}^{32}PO_4$ was obtained from the New England Nuclear Corp., Boston, Mass.

Preparation of Mahoney specific antiserum. A 4-ml amount of growth medium containing 7.4×10^9 plaque-forming units (PFU)/ml of Mahoney poliovirus was irradiated for 3 min in a 90-mm petri dish with ultraviolet (UV) light from a Westinghouse GI5T8 Sterilamp emitting 59.4 ergs per mm^2 per sec in the UV range. After irradiation, there was no demonstrable infectivity in the sample.

Two young rabbits were injected intramuscularly with 0.5 ml of irradiated virus fluid. An equivalent dose was administered 1 week later, and 2 weeks after the initial injection the animals were bled and serum was obtained.

Serum neutralization experiments. Mahoney specific antiserum was diluted 1:100 in BSS, and 1-ml samples were added to tubes and incubated at 35 C. Various prewarmed samples of virus were added to different tubes as follows: (i) 1 ml of LSc (4.5×10^5 PFU/ml, final dilution), (ii) 1 ml of Mahoney (1.5×10^6

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PFU/ml, final dilution), (iii) 1 ml of a virus mixture (1.4×10^5 PFU/ml of LSc, 1.2×10^6 PFU/ml of Mahoney, final dilution) which was designated the artificial mixture, and (iv) 1 ml of experimental fluid from a rescue experiment, diluted 1:70 in BSS (5.0×10^5 PFU/ml of LSc, final dilution). The samples were thoroughly mixed, and 0.2-ml portions were removed at intervals, diluted 10-fold in cold BSS, and titrated. Mixed samples containing two viruses were titrated in the presence of guanidine; single samples were titrated in the absence of the drug. Hence, in all mixed samples only LSc was titrated.

Preparation of ^{32}P-labeled LSc poliovirus. Bottles (2 oz) containing confluent monolayers (2×10^6 cells) were drained and incubated at 36 C for 1 hr with 5 ml of phosphate-free BSS containing 20% dialyzed calf serum. The monolayers were then washed with phosphate-free medium and incubated an additional 2 hr in the phosphate-free medium.

Cultures in triplicate were then incubated for 1 hr with 0.8 ml of phosphate-free medium containing 1.5 mc of carrier-free ^{32}P phosphoric acid. The ^{32}P medium was removed, and 0.2 ml (1.7×10^7 PFU) of dialyzed LSc fluid was added to each monolayer. After 40 min of incubation, the cells were washed twice with 5-ml portions of phosphate-free medium and inoculated with 1.2 ml of ^{32}P medium (2.0 mc of ^{32}P). After 6 hr at 36 C, the cells were washed twice with 5-ml portions of phosphate-free medium, 1 ml of HLS was added to each bottle, the bottles were frozen and thawed three times, and the contents were pooled. The fluid was centrifuged at $1,000 \times g$ for 20 min; the supernatant fluid was diluted 1:100 in HLS and frozen at −20 C.

Preparation of virus-induced polymerase. The enzyme extraction (water homogenization) and assay procedures were those of Baltimore and Franklin (2). Enzyme was obtained from 10^8 HeLa cells 4.5 hr after infection at a ratio of 100 PFU/cell. The enzyme assay mixture contained, in a final volume of 0.65 ml: 1.5 to 2.5 mg of protein extract; cytidine triphosphate, 51.3 mμmoles; uridine triphosphate, 46.6 mμmoles; adenosine triphosphate, 49.4 mμmoles; guanosine triphosphate-8-^{14}C tetralithium (9,896 counts per min per mμmole), 21.0 mμmoles; tris-(hydroxymethyl)aminomethane (Tris)-chloride buffer, *p*H 8.8, 30.0 mμmoles; magnesium acetate, 5.0 μmoles; actinomycin D, 4.36 μg; creatine phosphate, 10.0 μg; and creatine phosphokinase, 40.0 μg. Triphosphates and the energy-generating mixture were obtained from Schwarz Bio Research Inc. Actinomycin D was a gift of the Merck, Sharp and Dohme Research Laboratories, Rahway, N.J.

Samples were dissolved in 1 ml of NCS solubilizer (Nuclear-Chicago Corp., Des Plaines, Ill.) and were counted in 20 ml of dioxan scintillator (5) in a Packard Tri-Carb Model 2002 scintillation spectrometer. Efficiency of counting ^{14}C was 70%.

Methylated albumin chromatography. Ribonucleic acid (RNA) was obtained from about 3×10^8 HeLa cells that were labeled 2 to 4 hr after infection with 1 mc of tritiated uridine. Cells were infected at a ratio of 350 PFU/cell in 40 ml of medium containing 7% dialyzed calf serum and 4 μg of actinomycin D per ml.

TABLE 1. *Rescue of temperature-sensitive poliovirus by temperature-resistant virus*

Virus	Temp of infection	PFU/ml	
		No guanidine in overlay	Guanidine (100 μg/ml) in overlay
	C		
LSc $g^r t^s$	36	1.5×10^8	2.0×10^8
LSc $g^r t^s$	40	1.0×10^6	2.0×10^6
Mahoney $g^s t^r$	36	2.6×10^9	7.0×10^4
Mahoney $g^s t^r$	40	8.0×10^8	7.0×10^4
LSc $g^r t^s$ plus Mahoney $g^s t^r$	40	2.8×10^8	2.0×10^7

The cells were homogenized in RSB (10^{-2} M Tris, *p*H 7.0, 10^{-2} M KCl, 1.5×10^{-3} M $MgCl_2$), and RNA was extracted from the cytoplasmic fractions with phenol and 0.5% sodium dodecyl sulfate at 60 C.

RNA was chromatographed on methylated albumin-Kieselguhr (MAK) columns containing the middle layer described by Mandell and Hershey (11). Stepwise elution was performed at room temperature with different concentrations of NaCl in 0.05 M phosphate buffer, *p*H 6.8. Five 4.5-ml fractions were collected from columns with bed volumes of 4 ml. The nucleic acids were precipitated with trichloroacetic acid (6% final concentration) and collected on 0.45-μ filters (Millipore Corp., Bedford, Mass.); radioactivity was determined in 3 ml of dioxan scintillator (5). Recovery of nucleic acids from the columns was always 90 to 100% when up to 1 mg of RNA per column was used.

RESULTS

The following experiments were designed to determine the degree of rescue of temperature-sensitive poliovirus by temperature-resistant virus. Once rescue was established, experiments were conducted to determine the time of occurrence and the nature of the temperature-sensitive step.

Rescue of temperature-sensitive LSc poliovirus. Prescription bottles (2 oz) containing about 2×10^6 HeLa cells in confluent monolayers were drained, rinsed with 5 ml of HLS, refed with the same volume of HLS, and incubated at either 35 or 40 C for 0.5 hr. After preincubation, cultures were infected with either Mahoney or LSc virus at a ratio of 25 PFU per cell. Five cultures of each were incubated at 35 C and another five cultures were held at 40 C. Another set of cultures was inoculated with the same multiplicities of both viruses and incubated at 40 C. After adsorption for 1 hr, each monolayer was washed twice with 5-ml portions of prewarmed HLS and refed with a similar volume of growth medium. At 9 hr after infection, all bottles were frozen and thawed three times, and replicate samples

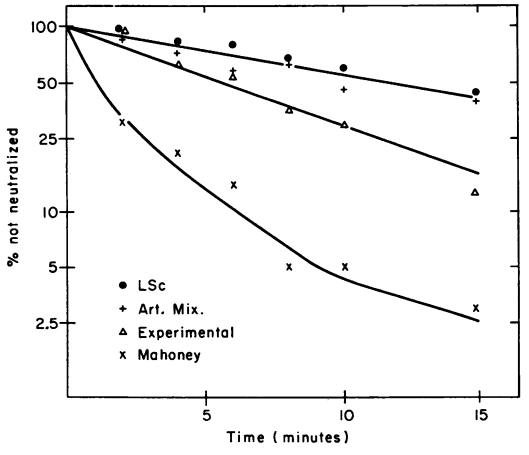

FIG. 1. *Neutralization of LSc and Mahoney polioviruses by Mahoney specific antiserum;* (●) *LSc virus;* (+) *LSc in an artificial mixture of LSc and Mahoney;* (△) *LSc virus from a rescue experiment;* (×) *Mahoney virus.*

were pooled and titrated in the presence and absence of guanidine. In the presence of guanidine, only LSc is titrated, since the compound inhibits Mahoney virus but not LSc.

The results of a representative experiment are presented in Table 1. The inhibition of LSc development at 40 C was 99% (1.5×10^8 versus 1.0×10^6 PFU/ml) and inhibition in the presence of Mahoney was about 90% (1.5×10^8 versus 2.0×10^7 PFU/ml). This 10-fold increase in LSc titer represents 9% of the control LSc titer and is termed rescue. In several experiments, the degree of rescue was as high as 50%. However, about 9% was obtained most consistently.

Antigenic character of rescued poliovirus. Since the assays of virus yields from mixed infections are done in the presence of guanidine, the viruses capable of replicating in the presence of this compound have the genetic character g^r and are genotypically similar to LSc. Under the conditions of mixed infection employed in this study, the possibility arises that phenotypic mixing occurs and that some of the rescued LSc particles have coat protein similar to the rescuing virus. In other words, some of the input LSc RNA could have been encapsulated by protein from helper virus. The antigenic character of the coat protein of the rescued particles was ascertained by comparing their rate of neutralization by Mahoney specific antiserum to the rates for LSc and Mahoney.

The results of serum neutralization (Fig. 1) indicate similarity in neutralization rates of rescued LSc virus and LSc in an artificial mixture of LSc and Mahoney. Mahoney virus was inactivated at a faster rate by its homologous antiserum. Rescued LSc was neutralized faster than the LSc control, but not as fast as Mahoney. The intermediate neutralization of rescued LSc indicates that the coat protein of the rescued particles in the experimental sample has a mixed antigenic character, suggesting a small amount of phenotypic mixing. The results of the neutralization of LSc in the artificially mixed sample indicates that the presence of Mahoney in the sample had little effect upon the neutralization of LSc.

Rescue of ^{32}P-labeled LSc. The rescue of LSc virus by Mahoney could be complicated by encapsulation of input LSc genomes with protein subunits of helper virus. The serum neutralization experiments indicated that this occurred to a small degree, if at all. To document this, ^{32}P-labeled LSc was used in a rescue experiment in nonradioactive medium, the rationale being that if replication of LSc actually occurred the progeny viruses would be unlabeled and no loss of infectivity should occur with time due to ^{32}P disintegration. A rescue experiment with ^{32}P-labeled LSc was performed. In this particular study, the yield of LSc virus in doubly infected cultures incubated at 40 C was about 7% of that in the control culture of LSc incubated at 35 C. Figure 2 indicates that the infectivity of the rescued LSc virus remained constant over a period of 42 days, whereas the infectivity of the labeled parent virus decreased about 2 $\log_{10}$ in the same period of time. The small shoulder on the suicide curve is not reproducible. These data strongly suggest

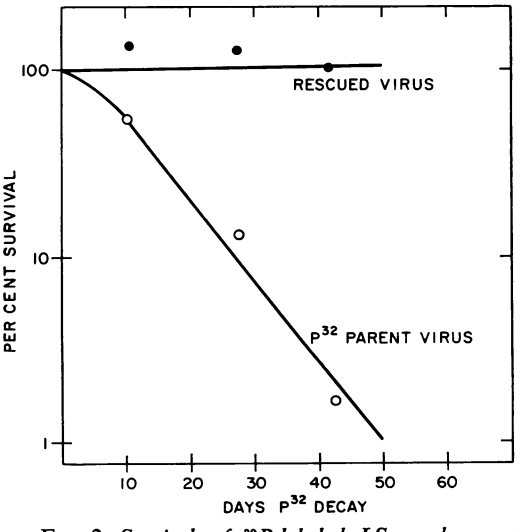

FIG. 2. *Survival of ^{32}P-labeled LSc and rescued LSc. Semilog plot of the survival of ^{32}P parent virus* (○) *and rescued virus* (●).

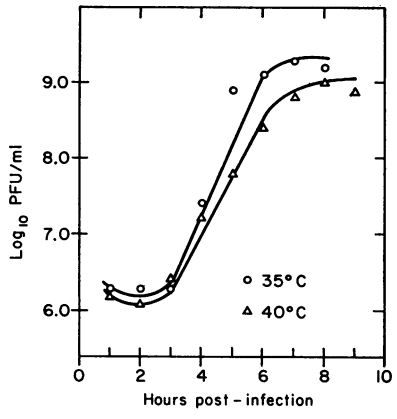

FIG. 3. *Step-down growth curve of LSc poliovirus. Flasks containing 2.5 × 10⁷ HeLa cells in 6 ml of medium were infected with LSc at a ratio 25 PFU/cell. One flask was held at 35 C throughout the study* (○) *and the other was incubated at 40 C during the first 2 hr of infection and then placed at 35 C* (△).

that rescued LSc actually underwent replication under selective conditions in the presence of Mahoney virus.

Step-down growth curve of LSc. To determine the time of occurrence of the temperature-sensitive block, cultures were infected with LSc at a ratio of 25 PFU/cell. One culture was held at 35 C throughout the study, and a second culture was incubated at 40 C for the first 2 hr of infection and then transferred to a 35 C water bath. At intervals, samples were frozen, thawed, and titrated.

The rate of virus production and the time of appearance of new virus in the step-down culture were similar to the control (Fig. 3). The final yield of virus was slightly lower in the step-down cycle and could be a reflection of deleterious effects of high temperature of incubation on HeLa cells. Comparison of the two curves indicates that the thermosensitive event occurs some time after 2 hr, since there was no lag when the culture was held at 40 C during the first 2 hr of infection.

Effect of elevated temperature on LSc polymerase production. Since poliovirus polymerase is first detectable in infected cells about 2 hr after infection, studies were performed to determine whether at 40 C there is a blockage of formation of active enzyme. The conditions of infection, isolation, and assay of this enzyme were as described in Materials and Methods.

The results presented in Table 2 indicate a reduction of approximately 95% in the polymerase activity produced in LSc-infected cells incubated at 40 C, as compared to 35 C.

TABLE 2. *Production of LSc gᵗᵗˢ-induced polymerase at 40 C*

Experimental conditions	Acid-insoluble RNA in vitro (counts per min per 1.3 × 10⁸ cells)
Uninfected cells	141
Infected, 40 C	287
Infected, 35 C	3,608

Methylated albumin chromatography of RNA from LSc-infected cells. In an effort to determine the type of RNA formed under selective conditions, the profile of RNA from LSc-infected HeLa cells incubated at 35 and 40 C was determined by chromatography on MAK columns. The results reported in Fig. 4 are representative of several experiments. Note that RNA eluted in the last fraction of the lower salt concentration, because 4.5-ml elution volumes were used on columns with bed volumes of 4.0 ml.

Labeled RNA from cells infected with LSc and incubated at 35 C showed a large peak of radioactivity eluting at 0.9 M NaCl and a smaller peak eluting at 0.8 M NaCl. The data are in agreement with MAK profiles obtained with poliovirus by Bishop and Koch (3). The peaks are somewhat different, but this can be attributed to the preparation of methylated albumin and the conditions of elution. Treatment of the different fractions with 1.5 μg of ribonuclease per ml (five times crystallized; Sigma Chemical Co., St. Louis, Mo.) in 0.6 M NaCl, 0.05 M phosphate buffer, *p*H 6.8, for 15 min at 36 C shows that the 0.8 M fraction was resistant to enzymatic degradation and was probably double-stranded RNA (*see* insert, Fig. 4). The 0.9 M fraction was completely degraded by the enzyme and was presumed to be single-stranded RNA (3). Samples from cells infected with LSc and held at 40 C had no demonstrable RNA profile, indicating that no virus-induced RNA was made under the selective conditions. The optical density trace is due to background cellular RNA and deoxyribonucleic acid (DNA). Philipson (12) showed that S3 HeLa cell ribosomal RNA eluted at 0.86 M NaCl and 0.90 M NaCl when chromatographed on MAK columns, whereas soluble RNA and DNA eluted at 0.5 M NaCl and 0.71 M, respectively.

DISCUSSION

The data presented in Table 1 indicate that a conditional lethal mutant of type 1 poliovirus can be functionally rescued by simultaneous infection of HeLa cells with a heat-resistant strain of poliovirus. The yield of LSc at 40 C in the presence of Mahoney virus was 10-fold higher

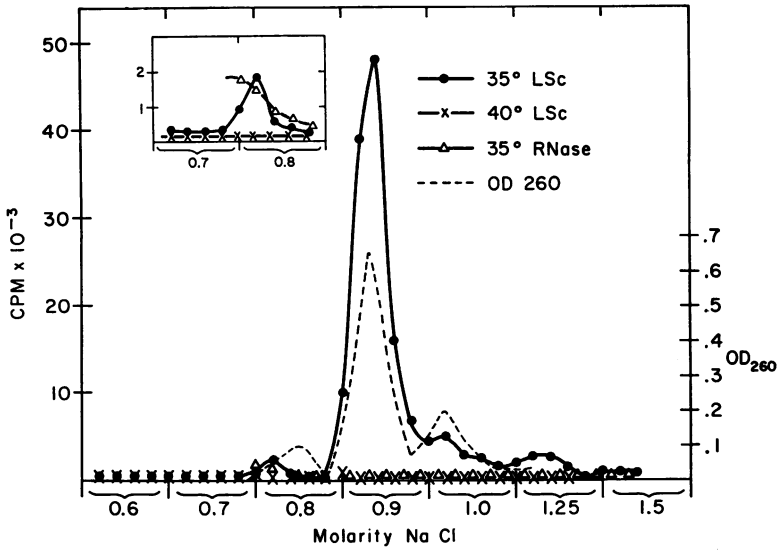

FIG. 4. *MAK chromatography of LSc-induced RNA. Infection at 35 C (●), infection at 40 C (×), infection at 35 C plus 1.5 μg/ml of ribonuclease before chromatography (△). All cultures were infected in the presence of actinomycin D. The column was loaded with 170 μg of RNA from each sample.*

than the yield of LSc alone at 40 C. The observation coincides with reports from several laboratories (7, 9) dealing with rescue of guanidine-dependent poliovirus in drug-free medium and the rescue of guanidine-sensitive virus in medium containing the inhibitor. The rescue value reported here is higher than those reported in the drug studies, since the maximal value of rescue attainable in those reports was about 1%.

Studies with heat-defective mutants of type 1 poliovirus failed to show rescue between mutant and wild type viruses (4). These experiments were performed with multiplicities of infection of mutant and helper virus which were lower than those used in these experiments. Perhaps this accounts in part for the discrepancy in results. Also, the heat-defective mutants employed by Cooper were induced by 5-fluorouracil, whereas the mutant used in this study arose spontaneously.

Genetic recombination has not been excluded experimentally in this study as a contributing factor in the rescue experiments However, since recombination with poliovirus is very small (6), the rescue value reported here is not likely to be due to a significant level of recombination.

That the rescued viruses actually underwent replication is shown in Fig. 2. The rescued progeny of ^{32}P-labeled LSc poliovirus were not inactivated when rescued in a nonradioactive medium. In addition, the small amount of phenotypic mixing among the rescued particles (Fig. 1) and the interference by the mutant with the growth of helper virus (Table 1) are points of evidence which suggest that the rescued particles actually replicated.

A number of experiments pinpoint the thermosensitive event somewhere after 2 hr following infection. First of all, infected cells incubated for 2 hr at 40 C and then transferred to 35 C showed no lag in virus development (Fig. 3). Secondly, at 40 C cells infected with LSc had no active RNA polymerase and also failed to induce virus RNA between 2 and 4 hr of the growth cycle (Table 2, Fig. 4). Taken together, these results suggest that either the production or activity of polymerase was temperature-sensitive or that its turnover rate was extremely high. Lwoff (10) has studied a temperature-sensitive mutant of poliovirus and has suggested that the temperature-sensitive event is the polymerization of enzyme subunits. If the production or the activity of the viral polymerase is, in fact, the temperature-sensitive step, then it follows that functional rescue of temperature-sensitive LSc under selective conditions might involve polymerase molecules induced by the helper virus.

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