Characterization of Some Poliovirus Temperature-Sensitive Mutants and Poliovirus-Related Particle Formation Under Nonpermissive Conditions

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Investigation of 15 poliovirus temperature-sensitive (ts) mutants by using physiological tests [formation of virus-specific antigen and ribonucleic acid (RNA) under nonpermissive conditions] permitted us to divide them into three groups. From each group, one mutant was selected (ts 2, 5, 11), and a comparative study of poliovirus-related particle (5, 10, 73, and 150S) formation under permissive (36°C) and nonpermissive (40°C) conditions was carried out. The ts 2 and ts 11 are mutants with greatly reduced RNA synthesis which at 40°C produce particles with a sedimentation constant of 5S, and the ts 5 (RNA⁺) mutant produces both 5 and 10S particles. The relationship between different temperature-sensitive defects in the mutants is discussed. The results obtained indicate a possible role of 5S protein structures in morphogenesis of poliovirus.

Conditionally lethal mutants are a convenient model for investigating the genetics and physiology of viruses. Temperature-sensitive (ts) mutants which are unable to multiply at high temperatures constitute one type of conditionally lethal mutant. A detailed analysis of a number of ts mutants of poliovirus has been done by Cooper et al. (2, 3, 12), who determined the impairment of known physiological functions of poliovirus in cells infected with ts mutants under nonpermissive conditions. At the present time, it is known that poliovirus morphogenesis, the final stage of which consists of the formation of an infectious particle with a sedimentation constant of 150S, occurs in several stages. As shown by Jacobson and Baltimore (8), the immediate precursors of mature virus are the so-called procapsids with a sedimentation constant of 73S. They contain no RNA and differ in polypeptide composition from 150S particles (9). Phillips, Summers, and Maizel (10) showed further that precursors of procapsids are protein structures with a sedimentation constant of 10S. In poliovirus-infected cells, there are also structures with a sedimentation constant of 5S which possess a complex polypeptide composition (10). The role of these structures in morphogenesis of poliovirus is unknown thus far.

The aim of the present study was to investigate by physiological tests, the temperature-sensitive defect of 15 ts mutants of poliovirus. It was intended to study the capacity of some mutants

to undergo separate stages of morphogenesis, that is, to form 5, 10, 73, and 150S structures under nonpermissive conditions. It was demonstrated that, by the capacity to synthesize ribonucleic acid (RNA) and antigens under nonpermissive conditions, the mutants could be divided into three groups. Under nonpermissive conditions, some of the mutants were found to form virus-specific structures with a sedimentation constant of 5S and one of them formed 5 and 10S structures.

MATERIALS AND METHODS

Virus. Experiments were carried out with ts mutants of poliovirus type I, strain Mahoney, and type 3, strain Saukett, incapable of multiplication at 40°C. The mutants were obtained as described previously (4, 5).

Cells. HeLa S3 cells were grown in monolayer culture in Eagle's medium with 10% bovine serum.

Virus-specific antigen determination. The HeLa cells were grown on cover slips, infected at a multiplicity of 100 plaque-forming units (PFU) per cell, and incubated at the corresponding temperature (36 or 40 ± 0.2°C) for 8 hr in a thermostated water bath. Then the cover slips were removed, fixed with acetone, and treated with specific Mahoney antisera labeled with fluorescein by the method described by Coons and Kaplan (1).

Synthesis of viral RNA. The HeLa cells were infected at a multiplicity of 100 PFU/cell and left for adsorption for 1 hr at 5°C. The unadsorbed virus was removed, and the cells were overlaid with Eagle's medium containing actinomycin D (5 µg/ml) and

incubated at 36 or 40 C for 2 hr. Then the cells were labeled for 2 hr with 1 μ Ci of 14 C-uridine per ml, and acid-precipitable radioactivity was determined. Synthesis of cellular RNA in uninfected cells in these experiments was 1 to 2% of the level of the synthesis in cells not treated with actinomycin D.

Inhibition of the synthesis of RNA and protein in HeLa cells infected with poliovirus. The HeLa cells were infected at a multiplicity of 100 PFU/cell and incubated at the corresponding temperature for 4 hr. Then the cells were labeled for 1 hr with 1 μ Ci of 14 C-uridine per ml or 0.25 μ Ci of 14 C-algal protein hydrolysate per ml, and the acid-precipitable radioactivity was determined. Results are expressed as percentages of uninfected cells.

Analysis of poliovirus-related particles in sucrose gradient. The methods described by Maizel, Phillips, and Summers (9) and Phillips, Summers, and Maizel (10) were used with some modifications. HeLa cells (10^7 cells per experiment) were infected with the virus at a multiplicity of 100 to 200 PFU/cell. Adsorption was for 1 hr at 5 C, and unadsorbed virus was removed. For analysis of 73 and 150S particles, Eagle's medium lacking valine, leucine, and phenylalanine and containing 5 μ g of actinomycin D per ml was added, and the cells were incubated at 40 or 36 C for 2 hr in a water bath. For analysis of 10 and 5S structures, medium was supplemented with guanidine hydrochloride (300 μ g/ml). This medium was removed after incubation for 2 hr by washing three times with Earle's saline, and Eagle's medium lacking valine, leucine, and phenylalanine and containing 5 μ g of actinomycin D per ml was added. The cells were then incubated at 40 or 36 C for 2 hr. Afterwards the cells were labeled for 2 hr with 0.5 μ Ci/ml (L form each) of 14 C-leucine, -valine, and -phenylalanine. Cytoplasmic extracts of infected cells were prepared by homogenizing the cells in a glass Dounce homogenizer after a 5-min period of cell swelling in a hypotonic RSB buffer [0.01 M NaCl, 0.0015 M $MgCl_2$, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4]. Nuclei and heavy cell debris were removed by centrifugation at $900 \times g$ for 2 min. For analysis of 73 and 150S structures, the supernatant was treated with 1.0% deoxycholate (DOC); for analysis of 5 and 10S structures, no treatment with DOC was used. Cytoplasmic extracts were immediately layered onto a preformed 5 to 22% (w/w) sucrose-RSB gradient. For analysis of 5 and 10S structures, crystalline bovine albumin (4.6S) was added as a marker for determination of the sedimentation constants of viral structures. After appropriate centrifugation, gradient fractions were analyzed for absorbance in a spectrophotometer and for acid-precipitable radioactivity after collecting the precipitate on membrane filters (Synthesia, Praha).

Assay of radioactivity. Radioactivity was counted in a liquid scintillation spectrometer USS-1 (USSR) by using scintillation liquid of the following composition: 4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-2-(5-phenyloxazolyl) in 1 liter of toluene.

Materials. Uridine-2- 14 C (5.6 mCi/mmol) and L-phenylalanine-1- 14 C (1.14 mCi/mmol) were from "Isotop," Leningrad; 14 C-algal protein hydrolysate, 14 C-L-valine (52 mCi/mmol), and 14 C-

L-leucine (52 mCi/mmol) were from "Isotop," Moscow. Actinomycin D was from Merck Sharp and Dohme Research Laboratories, Rahway, N.J. Polio-specific antisera labeled with fluorescein were prepared at the N. F. Gamaleja Institute of Epidemiology and Immunology, Moscow.

RESULTS

Characteristics and classification of ts mutants of poliovirus. Selection of viral strains from the 30 mutants obtained from wild-type poliovirus strains by means of different mutagens was carried out by determination of efficiency of plating (EOP) under agar at 40 C as compared with that at 36 C (EOP 40/36). For wild-type viruses, this ratio was 1. For subsequent tests, 15 mutants were selected with an EOP 40/36 of from 0.09 to 0.0008 (Table 1).

Investigation of the synthesis of viral RNA upon infection with ts mutants showed that under permissive conditions (36 C) the level of this synthesis was 35 to 82% of that observed in cells infected with wild-type virus. Under non-permissive conditions (40 C), no synthesis of viral RNA could be detected with the majority of mutants (ts 2, 9, 11, 12, 16, 22, 23, 25, 26, 27, and 28).

However, we must underline the sensitivity of the detection of new viral RNA permitted us by

TABLE 1. *Some properties of poliovirus ts mutants*

Virus	EOP 40/36	Synthesis of viral RNA at 40 C ^a	Formation of virus-specific antigen at 40 C ^b	Synthesis of macromolecules in cells infected by poliovirus ts mutants	
				Protein	RNA
				%	%
Mahoney	1	100	100	78	86
ts 2	1.5×10^{-2}	<10	100	84	98
ts 5	3.5×10^{-3}	38	100	93	98
ts 6	8.0×10^{-4}	58	50	40	65
ts 7	7.0×10^{-3}	55	100	77	90
ts 9	9.0×10^{-4}	<10	100	72	96
ts 11	3.8×10^{-3}	<10	0	30	46
ts 12	5.3×10^{-3}	<10	100	28	64
ts 16	9.0×10^{-3}	<10	100	67	87
ts 20	1.5×10^{-3}	30	100	85	92
Saukett	1	100	100	90	98
ts 22	4.6×10^{-2}	<10	80	84	97
ts 23	4.6×10^{-3}	<10	0	73	90
ts 25	9.0×10^{-2}	<10	80	79	97
ts 26	4.6×10^{-2}	<10	0	82	94
ts 27	5.2×10^{-2}	<10	0	76	93
ts 28	6.1×10^{-2}	<10	0	75	90

^a Expressed as per cent of wild-type virus.

^b Per cent of cells with immunofluorescence.

saying only that the synthesis induced by these mutants (RNA⁻) was reduced by 90 to 95%. With the remaining mutants (ts 5, 6, 7, and 20), RNA synthesis at 40 C was from 30 to 58% of that of the wild-type virus (Table 1).

Determination of poliovirus antigen production capable of reacting with specific fluorescent anti-serum showed no cytoplasmic immunofluorescence in cells infected with ts 11, 23, 26, 27, and 28 mutants and incubated for 8 hr at 40 C, whereas in experiments with the remaining mutants (ts 2, 5, 6, 7, 9, 12, 16, 20, 22, and 25) 80 to 100% of the cells incubated under similar conditions contained the virus-specific antigen (Table 1). In cells infected with the mutants and incubated at 36 C, poliovirus antigen could be detected in all instances by the 8th hr postinfection.

In studies of inhibition of synthesis of cellular macromolecules (RNA and protein), it was found that all of the mutants as well as wild-type viruses at 36 C inhibited synthesis of cellular proteins and RNA by the 5th hr after inoculation by 50 to 70% and 65 to 90%, respectively. At 40 C, inhibition of proteins by wild-type viruses and most of the mutants was 67 to 93% and inhibition of RNA was 86 to 98%. Some mutants, however, namely, ts 6, 11, and 12 under nonpermissive conditions, inhibited synthesis of cellular macromolecules less effectively; proteins were inhibited by 28 to 40% and RNA was inhibited by 46 to 65% (Table 1).

Table 2 summarizes properties of the ts mutants of poliovirus studied in this work. Since not a single mutant with a complete loss of the capacity to inhibit synthesis of cellular macromolecules was found, division of the ts mutants into three groups was done on the basis of only two tests: synthesis of RNA under nonpermissive conditions and antigen formation capacity. Mutants of group I showed no impairment of these two functions. Mutants of group II exhibited no synthesis of viral RNA and virus-specific antigen. Group III included mutants which at greatly reduced viral RNA replication synthesized antigen reacting with specific antiserum. Mutants with reduced ability to inhibit synthesis of cellular macromolecules (ts 6, 11, and 12) belong to three different groups.

For further elucidation of the specific physiological defect preventing formation of complete virus under nonpermissive conditions, one mutant was selected from each group (ts 2, 5, 11), and a comparative study of the formation, under permissive and nonpermissive conditions, of poliovirus-related particles with sedimentation constants of 5, 10, 73, and 150S, the presence of which in the cell characterizes poliovirus infection (9, 10), was carried out.

Formation of poliovirus-related particles with

TABLE 2. *Classification of poliovirus ts mutants*

Group	ts mutants	Antigen formation capacity at 40 C	Synthesis of viral RNA at 40 C
I	5, 6, 7, 20	+	+
II	11, 23, 26, 27, 28	—	—
III	2, 9, 12, 16, 22, 25	+	—

sedimentation constants of 150 and 73S. Figure 1A presents the results of an analysis of a cytoplasmic extract of HeLa cells infected with the ts 11 mutant under permissive conditions of cultivation. Three peaks were distinguished by radioactivity: the fraction of 150S, 73S, and the third light component at the top of the gradient. Similar results were obtained with the other two mutants under study, ts 2 and ts 5. This pattern corresponds to the reported data concerning wild-type polioviruses (9).

Analysis of cytoplasmic extracts of HeLa cells infected with ts 2, 5, and 11 mutants at 40 C revealed no formation of radioactive proteins with sedimentation constants of 150 and 73S. Considerable radioactivity was observed only in the upper part of the gradient (Fig. 1B).

Formation of poliovirus-related particles with sedimentation constants 10 and 5S. As can be seen in Fig. 2A, analysis of a cytoplasmic extract of HeLa cells infected with the ts 2 mutant at 36 C revealed two peaks of radioactivity corresponding to 10 and 5S. Identical data were obtained with ts 5 and ts 11 mutants. Addition of guanidine simultaneously with infection of the cells, without subsequent washing of the culture completely, prevented formation of the above-mentioned structures.

Analysis of cytoplasmic extracts of HeLa cells infected with the mutants at 40 C showed that, in the cells infected with ts 2 and ts 11 mutants, only protein structures with a sedimentation constant of 5S were formed, whereas after infection with the ts 5 mutant there were both 5 and 10S structures (Fig. 2B).

Thermostability of ts 2, 5, and 11 mutants. Since thermostability usually reflects characteristics of proteins of the virus capsid and there are certain differences in the synthesis of protein structures at 40 C in cells infected with ts 2, 5, and 11 mutants, we compared the thermostability of these mutants and wild-type viruses. Heating of wild Mahoney virus at 50 C for 15 and 30 min resulted in a reduction of infectivity by 3 and 4 logs, respectively (Fig. 3). A similar reduction was observed after heating the ts 2 mutant. At the same time, ts 5 and ts 11 mutants proved to be much more thermolabile and their infectivity

FIG. 1. Sucrose gradient centrifugation of cytoplasmic extracts prepared from poliovirus-infected HeLa cells (analysis of 150 and 73S particles). HeLa cells were infected with *ts* mutants and incubated in the presence of 5 μ g of actinomycin D per ml at 40 or 36 C. After 2 hr, the cells were labeled for 2 hr with 0.5 μ Ci/ml each of 14 C-leucine, -valine, and -phenylalanine. The cytoplasmic extracts were prepared, made 1.0% with respect to deoxycholate, and analyzed by sucrose gradient zone sedimentation (5 to 22% sucrose-RSB, 39,000 rev/min, 40 min, 4 C, SW39 rotor). (A) Cells infected with *ts* 2 mutant at 36 C. (B) Cells infected with *ts* mutants at 40 C: *ts* 2 ($\bullet$), *ts* 5 ($\blacktriangle$), *ts* 11 ($\blacksquare$). The arrows indicate the position of the 260-nm absorbance peak due to 74S ribosomes and the peak of infectivity due to virions (150S).

FIG. 2. Sucrose gradient centrifugation of cytoplasmic extracts prepared from poliovirus-infected HeLa cells (analysis of 10 and 5S particles). HeLa cells were infected with *ts* mutants in the presence of guanidine. After 2 hr of incubation, guanidine was removed, and medium containing 5 μ g of actinomycin D per ml was added. At 2 hr after guanidine reversal, cells were labeled with 14 C-amino acids for 2 hr. Cytoplasmic extracts were prepared and analyzed by sucrose gradient zone sedimentation (5 to 22% sucrose-RSB, 39,000 rev/min, 9.5 hr, 4 C, SW39 rotor). (A) Cells infected with *ts* 2 at 36 C ($\bullet$); the same experiment but without removing guanidine ($\blacktriangle$). (B) Cells infected with *ts* 2 ($\bullet$), *ts* 5 ($\blacktriangle$), and *ts* 11 at 40 C ($\blacksquare$). The arrow indicates the position of the 280-nm absorption peak due to bovine albumine (4.6S).

FIG. 3. Thermal inactivation of wild-type poliovirus and *ts* mutants. Symbols: ●, wild-type poliovirus, strain Mahoney; ○, *ts* 2 mutant; ▲, *ts* 11 mutant; ■, *ts* 5 mutant.

during the same time of treatment was reduced by 4.5 to 5.5 logs.

DISCUSSION

Using physiological tests (synthesis of viral RNA and production of antigen reacting with specific antiserum), we divided 15 *ts* mutants of poliovirus into 3 groups (Table 2). Eleven of the *ts* mutants were classified as RNA⁻ phenotype.

It should be noted that we could not determine whether there was a complete absence of viral RNA replication or if it was only greatly reduced under nonpermissive conditions. Therefore, the definition of the phenotype of some *ts* mutants as RNA⁻ is not absolute. Sucrose gradient sedimentation analyses of hot phenol-extracted RNA indicated that *ts* 2 and *ts* 11 mutants induce detectable RNA synthesis at 40 C at about 10% the level found at 36 C or in the wild type. (These results will be published in separate paper.)

From each group, one mutant was taken (*ts* 2, 5, and 11) and formation of poliovirus-related

particles in cells infected with these mutants at 36 and 40 C was studied. At 36 C, each of these mutants induced formation of all poliovirus-related particles with sedimentation constants of 5, 10, 73, and 150S. In cells infected at 40 C, none of the mutants under study induced synthesis of 150 or 73S particles. Analysis of protein fractions in cells infected with the *ts* 5 mutant, which at 40 C induced synthesis of RNA and antigen (Table 1), revealed formation of protein structures with sedimentation constants of 10 and 5S. The *ts* 2 and *ts* 11 mutants, which at 40 C have greatly reduced RNA synthesis, were found capable at this temperature of inducing formation of protein structures with a sedimentation constant of 5S alone. Ghendon et al. (Virology, *in press*), in experiments with a temperature-sensitive (RNA⁻) mutant of type II poliovirus, demonstrated formation, under nonpermissive conditions, of virus-specific structures with sedimentation constants of 5 and 10S.

The properties of *ts* 2, *ts* 5, and *ts* 11 mutants are presented in Table 3. As can be seen in this table, *ts* 2 and *ts* 11 mutants which induce formation of 5S structures at 40 C alone differ in the capacity to induce in cells formation of the antigen reacting with specific fluorescent antiserum. The *ts* 2 mutant induces formation of such an antigen, whereas the *ts* 11 mutant does not. These data show that the proteins induced by these mutants under nonpermissive conditions are not identical and differ at least immunologically. It should also be remembered here that the thermostability of virions of *ts* 2 and *ts* 11 viruses formed under permissive conditions was found to be different, which usually indicates certain differences in proteins of the virus capsid.

The finding of mutants which at 40 C form 5S structures suggests that these structures take part in one of the early stages of poliovirus morphogenesis and are used under permissive conditions for formation of subsequent structures. The polypeptide composition of these particles is known (10) and is not contradictory to this suggestion.

Cessation of morphogenesis at the stage of 5

TABLE 3. Some functions of poliovirus *ts* mutants at nonpermissive conditions

Mutants	Antigen formation capacity	Thermostability	RNA phenotype	Poliovirus-related particle forming capacity			
				5S	10S	73S	150S
<i>ts</i> 5	+	-	+	+	+	-	-
<i>ts</i> 2	+	+	-	+	-	-	-
<i>ts</i> 11	-	-	-	+	-	-	-

or 10S in ts mutants under nonpermissive conditions may be associated with distortion of the native structure of these particles or with impairment of the processes of post-translational cleavage of primary products of translation which, according to some authors (6, 7, 11), play an important role in the process of poliovirus maturation. Analysis of these structures by electrophoresis in acrylamide gels may be useful for the definition of some defects in the particles. It may therefore give us an opportunity to study poliovirus morphogenesis.

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