Study of Some Stages of Poliovirus Morphogenesis in MiO Cells

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Five to seven minutes after addition of ^{14}C -amino acids to poliovirus-infected MiO cells, radioactivity is found only in 5S virus-specific structures. With increased time of labeling, radioactivity appears in considerable amounts also in the 14S area. In the presence of guanidine blocking the transition of 14S particles in subsequent structures in MiO cells, radioactivity is first detected in the 5S zone and then accumulates predominantly in 14S particles. These results indicate that formation of 5S particles and conversion of a part of their protein into 14S particles reflect the earliest stages of the morphogenesis of poliovirus. In contrast to poliovirus-infected HeLa cells, no 73S particles could be detected in MiO cells, nor could 73S structures be detected in poliovirus-infected MiO cells after a short incubation period (20 to 30 min) with radioactive amino acids, although formation of 150S virions was observed. Addition of guanidine to infected MiO cells leads to accumulation of 14S particles; however, 73S particles are not detected. After removal of guanidine, radioactivity increases only in the 150S area. Incubation of 14S particles isolated from the sucrose gradient of the cytoplasmic extract of infected MiO cells resulted in formation of 73S particles. The results obtained show the modification of the late stages of poliovirus morphogenesis in MiO cells.

The existence of "light" (with sedimentation constants of 10S and 5S) protein poliovirus-related particles in HeLa cells infected with poliovirus was first described by Watanabe et al. (13) and Scharff et al. (12). Penman et al. (7) showed that formation of proteins of poliovirus capsid occurred within 20 to 30 min, with the initial formation of structures with a sedimentation constant less than 20S followed by 73S and finally by 150S particles (virions). In the studies by Phillips et al. (8, 10), the light virus-related proteins were represented in the cytoplasmic extract of HeLa cells by particles with sedimentation constants of 5S and 14S, and the latter could be converted in vitro into 73S particles.

In the study of Jacobson and Baltimore (4) carried out in HeLa cells infected with poliovirus, these 73S particles, called procapsids, were the immediate protein precursors of virions.

The task of this study was to investigate some stages of the morphogenesis of poliovirus in MiO cells, a continuous line of tonsil cells of *Macaca rhesus* monkeys which are highly sensitive to poliovirus. The results indicate, firstly, that formation of 5S particles and conversion of a part of their proteins into 14S particles reflect the earliest stages of the morphogenesis of polio-

virus and, secondly, that in MiO cells the modification of the late stages of poliovirus morphogenesis takes place.

MATERIALS AND METHODS

Virus. The experiments were carried out with the MEF₁ strain of type 2 poliovirus and type 1 poliovirus, Mahoney strain.

Cells. MiO cells (continuous line of tonsil cells of *Macaca rhesus* monkeys prepared at this institute by N. E. Gulevich) were grown in monolayer in medium 199 with 10% bovine serum.

Analysis of poliovirus-related particles in the sucrose gradient. MiO cells were infected with poliovirus at a multiplicity of 100 plaque-forming units (PFU)/cell. After adsorption for 1 hr at room temperature, medium 199 containing guanidine (300 $\mu\text{g}/\text{ml}$) was added and incubation was continued for 1 hr at 36 C. Guanidine was then washed off, and incubation of the cells continued in amino acid-deficient medium 199 containing actinomycin D (5 $\mu\text{g}/\text{ml}$). The time after reversal of guanidine was taken as the beginning of infection. Conditions of labeling are described in legends of the corresponding figures. Preparation of the cytoplasmic extract, analysis of poliovirus-related particles in the sucrose gradient, and the assay of radioactivity have been described previously (2, 3, 6).

Materials. ^{14}C -L-valine, ^{14}C -L-leucine, and ^{14}C -L-phenylalanine (specific activity 100 to 150 $\mu\text{Ci}/$

mmole) were obtained from UVVVR Co., Prague, Czechoslovakia; actinomycin D was obtained from the Reanal Co., Hungary; and guanidine-hydrochloride from NBC, Ohio.

RESULTS

Poliovirus-related particles produced in MiO cells. Using MiO cell cultures for the study of poliovirus replication, we analyzed production of poliovirus-related particles in infected cells. The preliminary analysis demonstrated that, under the given conditions, mainly (>93%) virus-specific proteins were synthesized (14).

Figure 1 shows that, in MiO cells infected with poliovirus MEF₁ strain, virus-related particles with sedimentation constants of 5S, 14S, and 150S are formed. No peak of radioactivity is detectable in the zone of the gradient corresponding to 73S particles. Similar results were obtained when the cells were infected with Mahoney strain of poliovirus.

FIG. 1. Sedimentation analysis of cytoplasmic extracts made from poliovirus-infected MiO cells. Monolayer culture (about 2×10^7 cells) was infected as described in Materials and Methods. At the time of maximal viral RNA and protein synthesis after 3.5 hr of incubation in amino acid-deficient medium, which contained 5 μ g of actinomycin D per ml, cells were labeled for 90 min with 0.25 μ Ci each of 14 C-leucine, 14 C-valine, and 14 C-phenylalanine per ml. Then cells were chilled and disrupted by using a glass Dounce homogenizer in a hypotonic TNM buffer (0.01 M Triethanolamine, pH 7.4, 0.01 M NaCl, 0.002 M MgCl₂). The cytoplasmic extract was divided into two equal samples, one of which (A) was made 1% with respect to sodium deoxycholate. Crystalline bovine albumin was added to another sample (B). The samples were layered on a linear 5 to 20% (w/w) sucrose gradient (18 ml) in TNM buffer and centrifuged at 28,000 rev/min in a 3 by 23 ml swing-out rotor of a MSE-65 centrifuge at 12°C for 90 min (A) and for 16 hr (B). Fractions of the gradients were diluted 1:3 with distilled water, and optical density at 260 nm (A) or at 280 nm (B) was determined. Then fractions were assayed for acid-insoluble radioactivity. The arrows indicate the position of the 260-nm absorbance peak due to 74S ribosomes and the position of the 280-nm absorption peak due to bovine albumin (4.6S).

Kinetics of labeling of 5S and 14S poliovirus-related particles. To study the kinetics of labeling of 5S and 14S particles, the MiO cells were incubated with labeled amino acids for 4, 10, and 20 min at 3.5 hours after infection. The cytoplasmic extracts were then prepared and analyzed in the sucrose gradient. Analysis of the extract after a 4-min labeling failed to detect significant accumulation of the label in the fractions corresponding to 5S and 14S particles. After a 10-min incubation period with 14 C-amino acids, two peaks of radioactivity were revealed with predominant accumulation of the label in the area of 5S (Fig. 2A). With an increased incubation time of 20 min, radioactivity was approximately equally distributed between 5S and 14S particles (Fig. 2B).

Pulse-chase labeling of 5S and 14S particles. For the study of the relation between 5S and 14S particles, pulse-chase experiments were made with the morphogenesis stopped at the stage of formation of 14S particles. We have demonstrated (see below) that in poliovirus-infected MiO cells, addition of guanidine resulted in accumulation of 14S particles but not of 73S particles, as found by Jacobson and Baltimore (4) in studies of poliovirus-infected HeLa cells. Proceeding from these results, experiments were carried out in which at 3.5 hr after infection, guanidine (300 μ g/ml) was added to the cells, followed by the addition of labeled amino acids. After 5 min of incubation, the excess of unlabeled amino acids was added and the incubation con-

FIG. 2. Kinetics of labeling of 5S and 14S poliovirus-related particles. Monolayer cultures were infected as described in Materials and Methods. At the time of maximal viral RNA and protein synthesis, after 3.5 hr of incubation in amino acid-deficient medium containing 5 μ g of actinomycin D per ml, cells were pulsed with 0.25 μ Ci each of 14 C-leucine, 14 C-valine, and 14 C-phenylalanine per ml for 10 min (A) and for 20 min (B). Then cells were chilled, and cytoplasmic extracts were prepared and analyzed in sucrose gradients as described in Fig. 1(B).

tinued for 2, 10, and 35 min (Fig. 3). As shown in Fig. 3A, 7 min after addition of ^{14}C -amino acids, radioactivity accumulated predominantly in 5S particles. With increasing time of incubation, the label also appeared in a significant amount in the 14S area, and a predominant accumulation of radioactivity in 14S particles occurred (Fig. 3B-C).

Absence of 14S particle formation from 5S protein structures in vitro. Phillips et al. (8, 10) showed that conversion of 14S virus-related particles into 73S particles could be achieved in vitro by incubation of 14S particles in the presence of the cytoplasmic extracts of the infected cells. It was of interest to find out whether it was possible to convert 5S particle proteins into 14S particles by using the same method since, as shown by the above results, this process appeared to occur in vivo. No formation of 14S particles could be demonstrated upon incubation of 5S structures isolated from poliovirus-infected MiO cells with the cytoplasmic extract of the infected cells (Fig. 4).

Kinetics of labeling of 150S poliovirus. As it has been shown in the first section of this paper on poliovirus-infected MiO cells, we failed to detect poliovirus-related particles with a sedimentation constant of 73S. Taking into account an important role played by 73S particles in the morphogenesis of poliovirus (4), we decided to study the detected phenomenon in more detail.

As is known from the literature, with a short incubation of poliovirus-infected HeLa cells with radioactive amino acids (20 to 30 min), radioactivity in the area of 73S particles ac-

FIG. 4. Behavior of 5S structures upon incubation with infected cell extract. 5S structures were isolated from a sucrose gradient in an experiment with infected cells which were labeled for 60 min at 3.5 hr after infection. A 0.5-ml sample containing 5S structures (about 4,000 counts/min) was mixed with 1 ml of cytoplasmic extract made from 2×10^7 infected MiO cells at 3.5 hr after infection. After incubation at 36°C for 40 min with continuous shaking, the mixture was chilled and analyzed as described in Fig. 1(B).

cumulates to a much greater degree than in 150S virions (4, 7). Taking this evidence into consideration, we analyzed formation of 73S particles under the following conditions. Poliovirus-infected MiO cells were incubated with ^{14}C -amino acids for 10 min, after which time the excess of unlabeled amino acids was added, and the cultures were incubated for 10, 15, or 20 min (Fig. 5). 73S particles could not be detected even at the time when the radioactivity only began to appear in 150S virions (Fig. 5).

Formation of poliovirus-related particles in MiO cells in the presence of guanidine. Jacobson and Baltimore (4) showed that addition of guanidine to poliovirus-infected HeLa cells at the period of maximum synthesis of viral RNA and protein arrested the production of mature virus and led to the accumulation of 73S particles. The results of analysis of poliovirus-related particles accumulating in MiO cells in the presence of guanidine can be seen in Fig. 6. Under these conditions, no 150S and 73S particles are found (Fig. 6A), and radioactivity accumulates predominantly in 14S particles (Fig. 6B). After guanidine removal, the virions (150S) are formed; however, no significant peak of radioactivity in the area of 73S particles can be found (Fig. 6C).

Conversion of 14S particles into 73S particles in vitro. Phillips et al. (8, 10) demonstrated that incubation of 14S particles isolated from a sucrose gradient with the cytoplasmic extract

FIG. 3. Pulse-chase labeling of 5S and 14S particles in the presence of guanidine. Guanidine (to the final concentration of 300 $\mu\text{g}/\text{ml}$) was added to the infected cells 3.5 hr after infection. Five minutes later, cells were pulsed for 5 min with ^{14}C -amino acids. Then replacement by a warm label-free guanidine-containing medium with 100-fold concentration of unlabeled amino acids was carried out, and incubation was continued for 2 min (A), 10 min (B) and 25 min (C). Cells were chilled, and cytoplasmic extracts were made and analyzed in sucrose gradients as described in Fig. 1(B).

FIG. 5. Kinetics of labeling of 150S poliovirus in infected MiO cells. At 3.5 hr after guanidine reversal, the infected cells were pulsed with ^{14}C -amino acids for 10 min. Then replacement by a warm label-free medium with a 100-fold concentration of unlabeled amino acids was carried out, and incubation was continued for 10 min (A), 15 min (B), and 20 min (C). Cells were chilled and cytoplasmic extracts were prepared and analyzed as described in the legend of Fig. 1(A).

FIG. 6. Poliovirus-related particle formation in MiO cells in the presence of guanidine and after its removal. Guanidine (to the final concentration of 300 $\mu\text{g}/\text{ml}$) was added to the infected cells 3.5 hr after infection. Ten minutes later, cells were pulsed with ^{14}C -amino acids for 15 min in the presence of guanidine. Then replacement by a warm label-free guanidine-containing medium with a 100-fold concentration of unlabeled amino acids was carried out for (A) and (B). The cells of the third sample (C) were washed free of guanidine, whereupon the same replacement was carried out except that guanidine was omitted from the medium. Incubation was continued for 1 hr. Cytoplasmic extracts were prepared and analyzed as described in the legend of Fig. 1.

of poliovirus-infected HeLa cells led to the formation of 73S particles. This suggests the presence in HeLa cells of a factor responsible for conversion of 14S particles into 73S particles. It was of interest to discover whether the cytoplasmic extract of infected MiO cells contains a similar activity. As shown in Fig. 7, the cytoplasmic extract of infected MiO cells is capable of promoting conversion of 14S particles into 73S particles. The cytoplasmic extracts of uninfected cells showed no such activity.

Detection of 73S particles in poliovirus-infected MiO cells under certain experimental conditions. In some experiments, monolayer cultures of MiO cells were infected with poliovirus strains Mahoney or MEF₁. At 3.5 hr, the cells were taken off from the glass with ethylenediamine-tetraacetic acid, suspended in fresh medium, and

incubated in suspension for 15 min. Labeled amino acids were then added for 30 min, whereupon the cytoplasmic extract was prepared and analyzed in the sucrose gradient. (In all previous experiments, the cells were not taken off the glass and were incubated with ^{14}C -amino acids in monolayers.) Under these conditions, a peak of radioactivity in the area of 73S particles could be determined in the case of Mahoney strain (Fig. 8A), whereas, in the case of MEF₁ strain, 73S particles were barely detectable (Fig. 8B.)

DISCUSSION

There have been no experimental data on definite precursors of 14S virus-related particles formed in cells infected with poliovirus reported in literature. Rueckert et al. (11) assumed, on the

FIG. 7. Conversion of 14S particles to 73S particles upon incubation with cytoplasmic extracts made from poliovirus-infected MiO cells. 14S particles were isolated from a sucrose gradient in an experiment such as the one described in Fig. 1(B). A 0.5-ml sample containing 14S particles (about 5,000 counts/min) was mixed with 1 ml of cytoplasmic extract (made from 3×10^7 infected MiO cells at 3.5 hr after infection). After incubation at 36°C for 40 min with continuous shaking, the mixture was chilled, made 1% with sodium deoxycholate, and analyzed in a sucrose gradient as described in Fig. 1(A).

FIG. 8. Poliovirus-related particle production in MiO cells incubated in suspension. MiO cells were infected with Mahoney (A) and MEF₁ (B) strains of poliovirus as described in Materials and Methods. At 3.5 hr after infection, cells were taken off the glass by ethylenediaminetetraacetic acid and were suspended in a warm amino acid-deficient medium (about 2×10^6 cells/ml). After 15 min, cells were pulsed with ^{14}C -amino acids for 30 min with continuous shaking. Cells were chilled, and cytoplasmic extracts were made and analyzed in sucrose gradients as described in Fig. 1(A).

basis of polypeptide composition of 14S and 73S structures and total molecular weight of the polypeptides, that the smallest structural precursors of poliovirus capsid must be the structures with a 5S sedimentation constant.

The results presented above show that, 7 to 10 min after addition of ^{14}C -amino acids, only the protein structures with the sedimentation constant of 5S are labeled. Pulse-chase experiments disclosed that proteins of 5S particles were precursors of 14S particles. The results obtained appear to permit the conclusion that formation of 5S particles and conversion of a part of their proteins into 14S particles reflect the earliest stages of poliovirus morphogenesis.

A 5S $\rightarrow$ 14S conversion is accompanied by change in the antigenic specificity since, as shown by previous studies (3), the antigenic specificity of 5S and 14S particles is different.

Investigation into conversion of proteins of 5S particles into 14S particles *in vitro* failed to give positive results. At the same time, according to the results of Phillips (8) and our own studies, conversion of 14S particles into 73S particles does occur under the analogous experimental conditions. It seems that the conditions of conversion of proteins of 5S particles into 14S particles differ from those of the 14S $\rightarrow$ 73S reaction.

In the second part of our investigation, we demonstrated that fractionation in a sucrose gradient of the cytoplasmic extracts of poliovirus-infected MiO cells failed to detect 73S particles despite the formation of 150S virions. At the same time, in our experiments (2, 3, 6) as well in the studies by Maizel et al. (5) and Jacobson and Baltimore (4) in HeLa cells, 73S particles are produced in considerable amounts. The amount of radioactivity at a peak of 73S particles in HeLa cells, according to these data, constitutes 50 to 70% of that in 150S virions. In MiO cells (Fig. 1A), radioactivity in the corresponding zone is only about 15% and shows no peak. Therefore, it follows that, in MiO cells, production of 73S particles is greatly reduced or they are not formed at all.

If the infected MiO cells are incubated with labeled amino acids in the presence of guanidine, radioactivity is observed in 5S and particularly in 14S particles. In contrast to the data of Jacobson and Baltimore (4) obtained in studies of poliovirus-infected HeLa S3 cells, we failed to detect 73S particles in MiO cells under similar experimental conditions. Washing off guanidine resulted only in increase of radioactivity in the 150S peak.

The results obtained in our studies of the late stages of poliovirus morphogenesis in MiO cells indicate that formation (or accumulation) of 73S particles may be controlled by the host cell. This may explain the fact that a change in the conditions of incubation of the infected cells

(Fig. 8A) may result in considerable production of 73S particles.

It seems significant that in vitro experiments have demonstrated that cytoplasmic extracts of infected MiO cells are capable of promoting conversion of 14S particles into 73S particles. This evidence appears to indicate that, in infected MiO cells, there is a factor conducive to in vitro conversion of 14S particles into 73S particles as has been reported for HeLa cells (8, 10).

The total information obtained indicates that, in the described system, there occurs considerable modification of the late stages of poliovirus morphogenesis due to the host cell, as a result of which 73S particles prove undetectable.

There are several possible explanations for the obtained data. (i) Morphogenesis under the conditions described runs a course of $14S \xrightarrow{\text{RNA}} 150S$ and omits the stage of 73S particles formation. In this case, 73S structures are not the precursors of 150S virions. (ii) 73S particles are precursors (i.e., procapsids) of poliovirus virions in MiO cells similar to what has been described for HeLa cells (4), but they do not reach appreciable amounts. A lack of accumulation of procapsids in MiO cells may be the consequence of a different ratio of the reacting components (RNA/procapsid, the amount of 5S and 14S structures, etc.) as well as of the features of the membrane structures participating in the morphogenesis of poliovirus (1, 7-9).

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