Differential Effect of Phleomycin on the Infectivity of Poliovirus and Poliovirus-Induced Ribonucleic Acids

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The infectivity of intact poliovirus was not affected by exposure to the antibiotic phleomycin at concentrations as high as 200 $\mu\text{g/ml}$, whereas that of the single-stranded poliovirus ribonucleic acid (RNA) was inactivated to 99% by pretreatment of the RNA with phleomycin at a concentration of 2 $\mu\text{g/ml}$. The infectivity of double and multistranded RNA was 10 times less sensitive than that of single-stranded RNA to the action of this antibiotic. Preincubation of HeLa cells for 30 min with 10 to 50 μg of phleomycin reduced the sensitivity of the cells to infection by viral RNA and intact virus, indicating that phleomycin interferes with cellular functions necessary for virus replication. When phleomycin was added to cells at different times after infection with single- or double-stranded RNA, the highest inactivation of infective centers was observed immediately after infection. With time of incubation at 37 C, the infective centers became more resistant to the action of phleomycin.

Recently Hecht and Summers (5) reported that phleomycin interferes with the replication of poliovirus ribonucleic acid (RNA). Phleomycin added 30 min after infection of cells with poliovirus type 1 inhibits virus-directed incorporation of radioactive uridine to 100% at a concentration of 100 $\mu\text{g/ml}$ and to 90% at a concentration of 67 $\mu\text{g/ml}$. The authors suggested that phleomycin might act by binding to poliovirus RNA early in the infectious cycle or by preventing the synthesis of virus-specific protein(s) necessary for virus replication. The present report summarizes results on the differential effect of phleomycin: (i) on the infectivity of poliovirus-induced single-, double-, and multistranded RNA; (ii) on infective centers obtained by infection with poliovirus RNA or with poliovirus; and (iii) on the cell capacity for virus production after interaction with RNA or virus.

MATERIALS AND METHODS

Propagation of cells and virus. Suspension cultures of HeLa S₃ cells were grown in Eagle's medium supplemented with 5% horse or fetal calf serum at a concentration of 2×10^5 to 7×10^6 cells/ml. For growth of Mahoney strain type 1 poliovirus, cells were washed with serum-free medium, concentrated, and resuspended in serum-free medium at a concentration of 10^7 cells/ml. They were infected with 5 to 10 plaque-forming units (PFU) of partially purified

poliovirus and incubated at 37 C in the presence of 2 μg of actinomycin D per ml. The pH was readjusted periodically with 1 M tris(hydroxymethyl)aminomethane (Tris) to 7.4. Six hours after infection the cells were sedimented and resuspended at 5×10^7 in RSB (0.01 M Tris-hydrochloride, pH 7.4; 0.01 M NaCl; 0.0015 M MgCl_2) and frozen.

After thawing and warming to 20 C, the cells were lysed by the addition of sucrose and Triton N101 to a final concentration of 0.6 M sucrose and 0.3% Triton and kept at room temperature for 15 min. Cell debris and nuclei were sedimented for 10 min at 10,000 rev/min in a Sorvall SS34 rotor. Sodium dodecyl sulfate was added to the supernatant fluid to a final concentration of 0.25%, and the virus was sedimented at 30,000 rev/min. The virus from the pellet was further purified by equilibrium density gradient centrifugation in CsCl and by subsequent dialysis (4, 13).

Single-stranded poliovirus RNA was obtained from purified virus by phenol extraction (8). Poliovirus-induced double-stranded RNA (RF-RNA) and multistranded RNA (RI-RNA) were prepared from virus-infected cells 4 hr after infection by phenol extraction at room temperature (1), differential precipitation with 1 M LiCl, and agarose gel filtration (10).

Titration of infectious RNA. Titers of infectious RNA species and virus were determined with an agar-cell suspension-plaque assay (9). Concentrated cells at 6×10^6 were mixed 1:1 with a solution containing 20% dimethylsulfoxide (DMSO) and 320 μg of diethylaminoethyl (DEAE) dextran per ml in Eagle's medium without serum and without Ca^{2+} and incu-

bated at 37 C for 1 min (G. Koch, *in preparation*). Portions (0.2 ml) of these cells (experimental cells) were added to tubes containing 10- μ l samples of RNA (with or without prior incubation with phleomycin) or virus and further incubated at 37 C for 15 min or longer. Unless otherwise stated, the RNA was diluted in TM buffer (0.01 M Tris-hydrochloride, pH 7.4; 0.5 mM $MgCl_2$), and the virus was diluted in Eagle's medium without serum. In several experiments, phleomycin was added at different times after cell-RNA or cell-virus interaction, to a final concentration of 10 to 100 μ g/ml. A 1.5-ml amount of indicator cells at 6×10^6 /ml was added to each tube; the contents were mixed with agar and plated as described previously (9). Plaques were counted after 2 and 3 days.

Chemicals. Phleomycin (lot A-9331-616) was a gift from K. E. Price of Bristol Laboratories, Syracuse, N.Y.

DEAE dextran was purchased from Pharmacia, Uppsala, actinomycin D was from Merck & Co., Inc., Rahway, N.J.; and DMSO was from Eastman Organic Chemicals, Rochester, N.Y. Eagle's medium (Joklik modified) and serum were obtained from Grand Island Biological Co., Grand Island, N.Y.

RESULTS

Differential sensitivity to phleomycin of the infectivity of poliovirus and of poliovirus-induced RNA species. The experimental results reported by Hecht and Summers (5) indicated that phleomycin inhibits poliovirus growth in HeLa cells by interference with viral RNA replication, either by binding to the RNA template or to the viral RNA polymerase. The first of these alternatives can be readily tested by incubation of viral RNA with phleomycin.

Single-stranded poliovirus RNA, poliovirus-induced RF-RNA and RI-RNA, and intact poliovirus were exposed to different concentrations of phleomycin for 5 min at room temperature and subsequently assayed for infectivity by the agar-cell suspension-plaque technique. The experimental results are summarized in Fig. 1.

The infectivity of poliovirus was not affected by exposure to phleomycin in concentrations from 1 to 100 μ g/ml. In contrast, the infectivity of single-stranded RNA was inactivated to 93% by 0.5 μ g/ml, to 99% by 2 μ g/ml, and to 99.9% by 10 μ g of phleomycin per ml.

A comparable inactivation of the infectivity of RF-RNA required 10 times more phleomycin. The differential sensitivity could be due to a faster or tighter binding of phleomycin to single-stranded than to double-stranded RNA. It was of interest, therefore, to study the sensitivity of the RI-RNA to phleomycin. The dose-response curve obtained with RI shows that RI infectivity was affected like RF-RNA at low phleomycin concentrations. A better binding of phleomycin

to single-stranded RNA than to RI is best explained by the assumption that phleomycin binds preferentially to a single-stranded 3' end of poliovirus RNA, which is either hydrogen-bound to the minus strand or not present on the uncompleted plus strands of RI.

The reaction between phleomycin and RNA was not significantly affected by the time and temperature of the incubation and was not dependent on the salt composition during the incubation (Table 1). A preincubation of phleomycin at 37 C for 10 min did not alter its RNA-inactivating ability. The absence of a time and temperature effect on the interaction with RNA is, therefore, not due to an instability of the antibiotic. The interaction of viral RNA with polycations was augmented by DMSO in concentrations of 2 to 20%. DMSO breaks hydrogen bonds (6) and unfolds single-stranded RNA and thereby

FIG. 1. Dose-dependent inactivation of poliovirus-specific RNA species by phleomycin. Phleomycin was added to samples of viral RNA, RF-RNA, and RI-RNA or poliovirus in TM buffer at the indicated final concentrations and incubated for 10 to 15 min at 23 C. The RNA and virus samples (20 μ l) were diluted 1:10 in 0.2 ml of 2×10^6 to 3×10^6 HeLa cells/ml and preexposed for 1 min at 37 C to Eagle's medium without serum containing 10% DMSO and 160 μ g of DEAE dextran per ml. Infective centers were assayed as described in Materials and Methods. Symbols: ■, RF-RNA; ○, RI-RNA; ●, viral RNA; □, intact virus.

TABLE 1. *Influence of salt environment and time of incubation on the interaction of phleomycin with single-stranded viral RNA^a*

Determination	Infective centers as per cent of control		
	Control	With phleomycin at 0 C	With phleomycin for 7 min at 37 C
TM buffer.....	(2,400) ^b	15 (362)	11 (264)
Eagle's medium..	(2,460)	13 (329)	12 (305)
Eagle's medium + 10% DMSO....	(2,610)	17 (409)	13 (329)
Tris-EDTA.....	(1,920)	19 (367)	13 (252)
Tris-EDTA + 1% DMSO.....	(1,870)	16 (309)	14 (254)
Tris-EDTA + 10% DMSO....	(1,890)	19 (378)	13 (249)

^a Viral RNA and phleomycin were diluted and mixed in TM buffer in Eagle's medium with and without DMSO and in 0.2 M Tris buffer containing 10⁻³ M EDTA with different concentrations of DMSO. The final concentration of RNA was 0.06 µg/ml and that of phleomycin was 0.4 µg/ml. The mixtures were either kept at 0 C and 10-µliter samples were withdrawn and immediately assayed for infectivity, or the RNA-phleomycin mixtures were incubated for 7 min at 37 C and duplicate 10-µliter samples were withdrawn and assayed. The numbers in parentheses are the average number of plaques from four plates.

^b The RNA from control tubes without phleomycin were diluted 1:10 before assay.

exposes phosphate groups which can then interact more readily with polycations. Mg²⁺ counteracts the DMSO effect (D. Hauliza and G. Koch, *in preparation*). Both DMSO and Mg²⁺ did not affect the interaction of phleomycin with RNA. Since the inactivation of RNA by phleomycin is not dependent on the addition of salts and DMSO and on time and temperature of incubation, this indicates that phleomycin binds to part(s) of the RNA which is (are) readily available in any configuration of the RNA.

Partial reaction of phleomycin-inactivated viral RNA. Is the interaction between RNA and phleomycin dependent on the ratio of phleomycin to RNA and how tightly is the antibiotic bound to the RNA? Both questions can be studied by incubating different concentrations of RNA with identical amounts of phleomycin and assaying the remaining biological activity of the RNA before and after dilution. The results of such an experiment are given in Table 2. The degree of inactivation was of the same order whether 0.1, 1, or 10 µg of RNA/ml was exposed to as much as 4 µg of phleomycin/ml, provided

the RNA assay was carried out immediately (Table 2). A 100-fold variation in the concentration of RNA did not change the degree of inactivation. Cellular RNA at a concentration as high as 20 µg/ml did not interfere with the inactivation of poliovirus RNA by phleomycin. However, a different degree of RNA inactivation by phleomycin was obtained by the following procedure. The interaction between RNA and phleomycin was stopped by a 1:10 or 1:100 dilution; the RNA was not immediately transferred to HeLa cells but kept in the diluted state for 5 min at room temperature (Table 2). The partial restoration of the biological activity from 0.6 or 1% to 6% at a 1:10 dilution or 18 to 22% at a 1:100 dilution indicates a loose and reversible bonding of the antibiotic to RNA.

Phleomycin-mediated inactivation of infective centers induced by poliovirus and poliovirus-specific RNA species. Hecht and Summers (5) suggested that phleomycin might act inside of the cell by binding to the RNA of the virus. As soon as the RNA from infecting virus is uncoated, it should become sensitive to the action of phleomycin. To confirm this finding with our test system, phleomycin was added to HeLa cells at different times after infection with intact virus. HeLa cells did not deplete the medium of the antibiotic activity within a short time. This was shown in an experiment in which 3 × 10⁶ HeLa cells were incubated with 5 or 20 µg of phleomy-

TABLE 2. *Phleomycin inhibition of single-stranded RNA infectivity at different RNA concentrations^a*

Phleomycin (µg/ml)	RNA (0.1 µg/ml) 0 min before plating	RNA (1 µg/ml) 0 min before plating	RNA (10 µg/ml) diluted 1:10 0 min before plating	RNA (1 µg/ml) diluted 1:10 at 5 min before plating	RNA (10 µg/ml) diluted 1:100 at 5 min before plating
0.1	43 ^b			46	
0.5	12	4.5		43	33
1.0	3	2.5		39	61
2.0	3	1.1	1.0	6	22
4.0	7	0.6	0.4	6	18

^a RNA in TM buffer at concentrations ranging from 0.1 to 10 µg/ml was incubated for 10 min at room temperature with 0.1 to 4.0 µg of phleomycin per ml. Samples (20 µliters) of the reaction mixture and of 1:10 dilutions were added to 0.2 ml of HeLa cells, and the yield of infective centers was determined. In addition, samples of the reaction mixture were withdrawn, diluted 1:10 and 1:100, kept at room temperature for 5 min, and assayed for biological activity.

^b Values represent yield of infective centers as per cent of control.

cin for 1 to 10 min at 37 C in the presence or absence of 10% DMSO and 160 μ g of DEAE dextran. The cells were sedimented, and the supernatant fluids were assayed for the inactivating effect on viral RNA and RF-RNA. No decrease in phleomycin activity was detectable in the supernatant fluids at 1, 5, and 10 min at 37 C.

A 20-min exposure to 25 μ g of phleomycin per ml from 20 to 40 min after infection prevented virus replication in 35 to 40% of the infective centers. Phleomycin added at 40 min after infection for 1 min reduced the infective center yield by 35%, and phleomycin present from 40 to 60 min postinfection reduced the infective center yield by 43%. After uncoating, the viral RNA appeared to become sensitive to phleomycin for at least a short period of time.

After exposure of cells to RNA, one can differentiate between RNA which still adheres to the cell surface and RNA which has entered the cell by treatment of the cell-RNA mixture with ribonuclease (2, 7). Measured in this way, 60% of the adsorbed double- and 75% of single-stranded RNA penetrated into HeLa cells within 5 min of interaction. Thereby the sensitivity of RNA inside of cells to the action of phleomycin can be analyzed (Fig. 2 and 3).

At 1 min postinfection, an appreciable amount of RNA was adsorbed, and most of the adsorbed potentially infectious RNA was taken up by the cells. The RNA inside the cell was still very sensitive to phleomycin inactivation. One can conclude, therefore, that viral RNA at 1 and 5 min postinfection is not yet protected to a measurable extent against the action of phleomycin. At 15 and 20 min postinfection, the degree of inactivation of infective centers by phleomycin markedly decreased. It is apparent that the viral RNA is now protected in some way against the action of phleomycin.

RF-RNA bound and penetrated into HeLa cells was most sensitive to 25 μ g of phleomycin between 1 and 5 min postinfection. This can be explained by the observed conversion of RF-RNA from ribonuclease-resistant to a partially ribonuclease-sensitive form after penetration into HeLa cells (K. Wieggers and G. Koch, Abstr., 6th FEBS Mtg., Madrid).

At 10 and 20 min postinfection, infective centers induced by single- and by double-stranded RNA showed almost the same sensitivity against phleomycin and became more resistant to the action of phleomycin with time of incubation either by protection of the RNA inside of the cell or by the accomplishment of early functions in the viral replication cycle before the antibiotic reached the RNA.

FIG. 2. Phleomycin inhibition of infective centers induced by single-stranded RNA at different times after infection. Infection of cells with single-stranded viral RNA was carried out as described in *Materials and Methods*. Thirty to 40 tubes containing equal amounts of HeLa cells (6×10^6) and viral RNA (10^{-4} μ g) in 0.2 ml were incubated at 37 C. At different times after infection, phleomycin was added to a final concentration of 25 μ g/ml and then further incubated for various time intervals before 1.5 ml of the indicator cells and 1 ml of the agar were added and the mixture was plated (phleomycin at 1 min postinfection, ▲; 5 min postinfection, ■; 10 min postinfection, □; 15 min postinfection, ●, and 20 min postinfection, ▽). The interaction of RNA and cells was stopped in control samples without phleomycin by adding 1.5 ml of indicator cells (□) or 1.5 ml of indicator cells containing 20 μ g of ribonuclease per ml (△) to 0.2 ml of experimental cells and RNA. All experimental points were calculated from at least two duplicate experiments.

Preexposure of cells to phleomycin and its effect on the establishment of infective centers by viral RNA species or virus. HeLa cells exposed to actinomycin D show an enhanced competence for infection by single-stranded RNA and a reduced competence for infection by double-stranded RNA (10). Phleomycin is taken up by HeLa cells and interferes with their DNA metabolism (12). It was of interest to determine whether phleomycin has a similar effect as actinomycin on the cell competence for infection by viral RNA species. HeLa cells were incubated in the presence of 10 to 20 μ g of phleomycin per ml for 30 or 60 min at 37 C. In the experiments concerned with RNA infection, unadsorbed phleomycin was removed from the cells by centrifugation before infection with RNA. Phleomycin reduced the cell competence for infection

FIG. 3. *Phleomycin inhibition of infective centers induced by RF-RNA at different times after infection. For details see Fig. 2. Double-stranded RNA is taken up by HeLa cells instantaneously after adsorption (7), and the same number of infective centers is obtained when indicator cells are added without and with 20 μ g of ribonuclease per ml. All tubes contained 10^{-4} μ g of RF-RNA. Symbols: Δ , phleomycin added at 1 min postinfection; $\blacktriangle$, 5 min postinfection; $\circ$, 10 min postinfection; $\bullet$, 20 min postinfection.*

by single- and double-stranded RNA (Tables 3 and 4).

The yield of infective centers obtained by single-stranded RNA after a preexposure of the cells to 20 μ g of phleomycin for 30 min at 37 C is only 5% of the control. A prolongation of exposure to 60 min did not lead to further inhibition of infection. The inhibition was of the same order as that obtained by adding phleomycin to infectious centers 1 min after exposure to RNA. This might indicate that penetrating RNA is inactivated by phleomycin which is bound by the host cell. This view is supported by the finding that a subsequent incubation of the cells in the absence of phleomycin restores 50% of the competence of the cells for infection by viral RNA. The reversibility is due to a reduction in the amount of phleomycin present or available for RNA interaction intracellularly or to a restoration of the cell capacity to synthesize poliovirus or to both.

When HeLa cells were exposed to 10 or 20 μ g of phleomycin per ml for 30 to 60 min, the yield of infectious centers induced by RF-RNA was only 3 to 10% of the control (Table 4). This is the same order of reduction in the cell competence as seen in the experiment with single-stranded RNA. However, a subsequent incubation of cells

in the absence of phleomycin did not restore significantly the competence for infection with RF-RNA.

Preincubation of HeLa cells with 50 μ g of phleomycin per ml for 30 to 40 min before infection with intact virus resulted in a 45 to 60% lower yield of PFU than in the control with cells incubated for the same time in the absence of phleomycin. This would indicate that phleomycin interferes with cellular functions which are needed for virus growth. Hecht and Summers (5) reported that phleomycin in concentrations as high as 100 μ g/ml gave less than 20% inhibition of HeLa DNA, RNA, or protein synthesis. The mode of action of phleomycin on the cell capacity for poliovirus growth remains to be elucidated.

DISCUSSION

The results reported here confirm and extend the findings of Hecht and Summers (5) on the inhibition of poliovirus replication by phleomy-

TABLE 3. *Reversible inhibition by phleomycin of the cell competence for infection by viral RNA^a*

Time of preincubation	No. of plaques as per cent of control
With phleomycin (20 μ g/ml)	
0-30 min.....	5
0-60 min.....	6
Without phleomycin	
31-60 min ^b	47
0-60 min.....	100
61-120 min ^c	56
0-120 min.....	100
With phleomycin (10 μ g/ml)	
0-60 min.....	10
Without phleomycin	
61-120 min ^c	53
0-120 min.....	100

^a HeLa cells from Spinner cultures were sedimented, washed, and resuspended at 6×10^6 cells/ml in Eagle's medium without serum and incubated at 37 C in closed tubes in a shaking-water bath with and without addition of phleomycin. After 30 or 60 min, cells from both tubes were centrifuged (1,000 rev/min, 2 min), resuspended at 5×10^6 cells/ml in medium, and further incubated at 37 C as described above. Samples from both cell cultures were mixed 1:1 with medium containing 20% DMSO and 320 μ g of DEAE dextran per ml, incubated for 1 min at 37 C, and then added to duplicate tubes containing 10^{-4} μ g of viral RNA. The experiment was carried out three times, and the average number of plaques obtained was used to obtain the per cent yields given in the table.

^b With phleomycin 0-30 min.

^c With phleomycin, 0-60 min.

TABLE 4. *Irreversible inactivation by phleomycin of the cell competence for infection by double-stranded RNA^a*

Time of preincubation	No. of plaques as per cent of control
With phleomycin (20 μ g/ml)	
0-30 min.....	10
0-60 min.....	3
Without phleomycin	
31-60 min ^b	12
0-60 min.....	100
61-120 min ^c	9
0-120 min.....	100
With phleomycin (10 μ g/ml)	
0-60 min.....	5
Without phleomycin	
61-120 min ^c	4
0-120 min.....	100

^a See Table 3 for details. Experiments with single- and double-stranded RNA were run in parallel.

^b With phleomycin, 0-30 min.

^c With phleomycin, 0-60 min.

cin. The different effects of phleomycin in various stages of the interaction of poliovirus and HeLa cells should be carefully enumerated. By exposure to phleomycin before infection, the capacity of the host cell for virus replication seems to be impaired most severely if the infection is induced by RF-RNA but is measurable also for infection by single-stranded RNA and even by the intact virus. We could assume that phleomycin remains in the cytoplasm of HeLa cells for some time and is able to react with viral RNA even when the infection is carried out at an interval after the exposure to the antibiotic. On the other hand, the different inactivating effect of phleomycin seen on the sensitivity for infection of HeLa cells, which is considerably more pronounced for infection by RF-RNA than for infection by viral RNA or intact virus, might indicate that more cellular functions are needed for a productive infection with RF-RNA. This view is supported by earlier studies with actinomycin (10). Pretreatment of HeLa cells with actinomycin reduces the cell competence for infection by RF-RNA to 10% and increases the competence for infection by single-stranded RNA threefold.

Poliovirus viability is not affected by phleomycin, but single-stranded viral RNA is highly sensitive to the action of phleomycin. Cellular RNA at 20 μ g/ml does not interfere with the inactivation of single-stranded viral RNA. More than 50% of the viral RNA infectivity is lost by a brief exposure to the antibiotic at 0.1 μ g/ml and 99% at 2 μ g/ml (Table 2 and Fig. 1). A compara-

ble inactivation of RF-RNA requires 10 times more phleomycin. Of special interest is the finding that RI-RNA behaves in this respect more like RF-RNA than like single-stranded RNA. It is suggested that phleomycin binds preferentially to the 3' end of the viral RNA which is protected by hydrogen bonding to the minus strand in RF- as well as in RI-RNA. Poliovirus RNA is inactivated by venom phosphodiesterase and by *Escherichia coli* ribonuclease II (G. Koch, *unpublished data*). The latter enzyme attacks only single-stranded RNA (M. Singer, *personal communication*). This indicates that the 3' end of poliovirus RNA is not protected by intramolecular hydrogen bonds. Provided that phleomycin inactivates single-stranded RNA by binding to the 3' end, the independence of phleomycin viral RNA interaction on the presence of salt or DMSO is well explained.

Infective centers initiated by virus and single- and double-stranded viral RNA show different sensitivities for the inactivation by phleomycin. Again, cells infected by intact virus appear to be most resistant to the antibiotic. Between 20 and 40 min postinfection, about 40% of the virus-induced infective centers are inactivated even by 1 min of exposure to 25 μ g of phleomycin per ml. Thus far we know little about the mechanism of the uncoating of poliovirus RNA. Furthermore, the necessary time interval between uncoating and messenger RNA action is unknown; the viral RNA might bind instantaneously, after uncoating, with ribosomes. It might be assumed, therefore, that the viral RNA is protected against phleomycin first inside the viral protein envelope and soon after uncoating by binding to ribosomes. Further studies with phleomycin might yield information on these early events in virus replication. Immediately after penetration of viral RNA into cells, the infective centers appear as sensitive as free RNA (Fig. 2). They become more resistant to the action of phleomycin with time after infection. Infective centers induced by RF-RNA are almost as sensitive to phleomycin as those obtained by single-stranded RNA (Fig. 3). This is not surprising since we have observed that RF-RNA is partially converted into a single-stranded form after infection of HeLa cells (K. Wiegiers and G. Koch, Abstr., 6th FEBS Mtg., Madrid, 1969). The different sensitivity to phleomycin of single-stranded RNA and double-stranded RNA is lost after infection. Beginning at 15 min postinfection, infective centers induced by single- and double-stranded RNA show a nearly identical low sensitivity for inactivation by phleomycin which decreases further with time of incubation.

The rapid gain in resistance of RNA-induced

infective centers against phleomycin may indicate that early function(s) of the infecting RNA is completed. The increase in phleomycin resistance might work as a tool for further studies on the early events following the penetration of viral RNA into cells.

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