Virus-Induced Interference in Heterologously Infected HeLa Cells

M. DOYLE AND J. J. HOLLAND

Department of Biology, University of California, San Diego, La Jolla, California 92037

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In doubly infected HeLa cells, poliovirus type 1 rapidly and completely dominates vesicular stomatitis virus (VSV) plaque-forming unit production. Poliovirus type 1 shuts off incorporation of amino acids into VSV-specific proteins within 2 hr after superinfection of cells already committed to massive synthesis of VSV proteins. However, poliovirus type 1 appears to have little, if any, direct effect upon incorporation of uridine into VSV-directed ribonucleic acid (RNA) synthesis. Poliovirus apparently interferes with VSV virion production only at the level of translation of viral messenger RNA, although it interferes with host cell macromolecular syntheses at the levels of translation and transcription.

Poliovirus type 1 and vesicular stomatitis virus (VSV) are both single-stranded ribonucleic acid (RNA) viruses capable of producing marked cytopathic effects in sensitive cells in tissue culture. In addition to inhibiting host RNA and protein production, these viruses substitute synthesis of their own specific proteins and RNA species to varying and distinctly different extents (1, 15, 16, 18). The experiments reported here utilized these two viruses to analyze the nature of virus-virus interactions in doubly infected cells. This analysis of poliovirus type 1 and VSV might then be useful in an attempt to understand the nature of poliovirus-cell interactions, specifically the inhibition of host macromolecular syntheses.

A number of investigators have examined the process of interference between poliovirus and various other viruses. McCormick and Penman (12) found no interference between nonreplicating poliovirus and mengovirus. They concluded that the "inhibiting protein" of one virus did not affect the other virus and therefore the two viruses possess initiators of translation which differ from those of host cells. Choppin and Holmes (3) reported that monkey kidney cells infected with the paramyxovirus SV5 and superinfected with poliovirus showed no inhibition of production of SV5 infective virus. The poliovirus was, however, still active in inhibiting both host cell RNA and protein synthesis.

Ledinko and Melnick (11) and Ledinko (10) showed that poliovirus type 1 can completely exclude the growth of superinfecting poliovirus type 2. Virus multiplicity of infection (MOI) and the time between the interfering and challenging infections were both factors affecting the interference. Higher MOI of interfering virus resulted in an earlier appearance of complete interference.

Cords and Holland (5) studied homologous poliovirus interference, as well as interference between poliovirus and coxsackievirus. Neither virus had a marked advantage in these infections, and the results of double infection depended upon which virus could replicate the greater number of genomes first. Interference seemed to be due to a simple competition for some limiting cell component(s).

In contrast, the present study shows that poliovirus type 1 established complete dominance over VSV in doubly infected cells regardless of relative MOI values or times of infection. Possible mechanisms for interference between poliovirus and VSV are discussed.

MATERIALS AND METHODS

Virus stocks. The poliovirus type 1 was a very rapidly lethal, clinical isolate kindly typed for us by Edwin Lennette. With the exception of one experiment with guanidine-dependent virus, reported below, the virus strain used was guanidine-sensitive. VSV was an Ogden strain kindly supplied by F. Schaffer.

Cell culture. HeLa and L cells were obtained from Flow Laboratories and cultured as monolayers in screw-cap glass bottles under 5% CO₂. Cells were grown in MEM containing 5% calf serum.

Virus assay. Virus titers were obtained by a 0.4% agarose layer plaque technique as described by Holland and McLaren (7). To assay VSV in the presence of poliovirus, samples were plated on L cells, to which poliovirus will not attach. To assay polio-
virus in the presence of VSV, a sample of the original pool was treated with an equal volume of ether for 5 min with occasional rotary mixing. The ether was evaporated and appropriate dilutions were plated on HeLa cells. This treatment destroyed all VSV infectivity present.

Amino acid labeling. The 1H-labeled amino acids obtained from Schwarz BioResearch Inc. were L-phenylalanine (40 Ci/m mole), L-tyrosine (41.5 Ci/m mole), and L-valine (25.4 Ci/m mole). The 14C-labeled amino acids from the same source were L-phenylalanine (300 mCi/m mole), L-tyrosine (455 mCi/m mole), and L-valine (260 mCi/m mole). Labeling media were prepared by adding the 1H- or 14C-amino acids to MEM minus the same unlabeled amino acids. Incorporation of label was stopped by pouring off the radioactive media, washing the monolayers three times with cold 0.15 M NaCl and adding 1 ml of 1× sample buffer (see below). The samples were dialyzed overnight against 1× sample buffer and specific activities (counts per minute per microgram of protein) were determined.

Uridine labeling. The [5-3H]uridine obtained from Schwarz BioResearch Inc. had a specific activity of 26.1 Ci/m mole. Labeling medium was standard MEM containing 3H-uridine.

Polyacrylamide gel electrophoresis. Radioactively labeled protein samples were dialyzed overnight against 1× sample buffer [0.1% sodium dodecyl sulfate (SDS), 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.001% ethylenediaminetetraacetic acid (EDTA), 0.005 M sodium acetate, pH 9.0] and then subjected to electrophoresis overnight at 70 V on 5% polyacrylamide gels as described by Kiehn and Holland (9). The electrophoretic buffer also contained 0.1% mercaptoethanol and 0.02% thioglycollate. Radioactively labeled RNA samples were run on 2.5% acrylamide, 0.5% agarose gels, using the system of gels and buffers described by Bishop et al. (2). The gels were fractionated and counted by the procedure described by Kiehn and Holland (9).

RESULTS

Double infection with poliovirus type 1 and VSV. Inhibition of VSV PFU production by poliovirus type 1 is shown in Table 1. All cells were infected with VSV for a total of 8 hr. At various times before completion of VSV infection, the cells were superinfected with poliovirus and incubated until 8 hr post VSV infection. The monolayers were then assayed for VSV and poliovirus PFU production. Addition of poliovirus at any time before completion of VSV macromolecular synthesis led to marked inhibition of VSV replication. On the other hand, the replication of poliovirus type 1 was apparently unaffected by the presence of VSV. Cells simultaneously infected with VSV and poliovirus type 1 in the presence of 2 mM guanidine yielded no replication of poliovirus, but did yield a 50-fold reduction of VSV replication compared with VSV PFU formation alone in presence of guanidine. When cells were simultaneously infected with VSV and a poliovirus type 1 mutant which requires guanidine for replication, there was a 10-fold reduction in VSV titer in the absence of guanidine; and in the presence of guanidine there was a 100-fold reduction. Evidently replication of poliovirus RNA is not necessary for interference with VSV PFU formation.

The kinetics of amino acid incorporation into VSV, poliovirus, or doubly infected cells are

Table 1. Plaque-forming unit production in HeLa cells preinfected with vesicular stomatitis virus (VSV) superinfected with poliovirus type 1

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<th>Total time of infection (hr)</th>
<th>Total time of VSV infection (hr)</th>
<th>Total time of poliovirus infection (hr)</th>
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HeLa monolayers were infected with VSV at multiplicity of infection (MOI) 10. At various times postinfection, the cells were superinfected with poliovirus type 1 at MOI 50. At 8 hr post VSV infection, the cells were frozen until assayed. Results of the plaque assays are expressed as percentage of the control level 8 hr postinfection with VSV alone or poliovirus alone.
shown in Fig. 1A. Poliovirus type 1 superinfection of cells preinfected with VSV caused a dramatic decrease in the level of amino acid incorporation. Poliovirus inhibited host cell amino acid incorporation at the same rate. There is a peak of poliovirus-specific synthesis at 3 hr post poliovirus infection despite VSV preinfection. Both VSV and poliovirus showed a stimulation of amino acid incorporation during the first hour postinfection. The meaning of this stimulation is unclear, but it is not due to refeeding effects because it did not occur in the mock-infected controls. Figure 1B shows kinetics of amino acid incorporation into infected cells in the presence of guanidine. Again poliovirus type 1 inhibited host cell and VSV amino acid incorporation. These kinetic experiments were quantitatively controlled and repeated many times with results identical to those shown here. Poliovirus in the presence or absence of guanidine consistently lowered the rates of amino acid incorporation into both host cell and VSV-specific proteins.

Figure 2A through D shows a series of polyacrylamide gel electrophoresis patterns of selected time points from Fig. 1. These gels were run in order to analyze qualitatively the types of proteins present in mixedly infected cells where quantitative analysis showed poliovirus-specific kinetics of amino acid incorporation. Figure 2A shows the polyacrylamide gel pattern of proteins present in cells infected with poliovirus type 1 and pulse-labeled with amino acids for 15 min at 4 hr postinfection. Figure 2B shows the pattern in VSV-infected HeLa cells 4 hr after infection. The pattern of VSV polypeptides is similar to that published by Wagner et al. (16) except that polypeptide L is absent. The pattern is also similar to those published by Mudd and Summers (13). However, throughout these studies very little label was incorporated into the 40,000 molecular-weight VP-4 component of VSV. Therefore, distinguishing this peak from the large VP-3 peak is difficult. When 2 mM guanidine is present in cells infected with VSV for 2 hr and superinfected with poliovirus type 1 for an additional 2 hr, the gel pattern of the proteins which were synthesized at a reduced rate is typical of VSV, although it is clear that VP-2 and VP-5 have been more strongly inhibited by poliovirus superinfection than has VP-3. (Compare Fig. 2B and 2C.) Figure 2D is the pattern in cells treated exactly as in Fig. 2C except that guanidine was not present to inhibit poliovirus. VSV proteins VP-2, VP-3, and VP-5 are still present, but the majority of proteins are poliovirus specific.

The kinetics of uridine incorporation into VSV-infected, poliovirus-infected, or doubly infected cells are shown in Fig. 3A. Actinomycin D was present in the media during the 20-min pulse label in order to shut off host RNA synthesis.
There was a massive synthesis of poliovirus RNA at 3 to 4 hr post poliovirus infection which overwhelmed the contribution of VSV-specific uridine incorporation. An identical experiment done with guanidine (Fig. 3B) showed no poliovirus incorporation of uridine. VSV incorporation was unaffected by the presence of poliovirus in doubly infected cells. The decrease in VSV RNA synthesis late in infection in the presence of poliovirus is probably a secondary effect of the earlier inhibition of VSV synthesis.

Polyacrylamide gel electrophoresis of the labeled RNA from these experiments was used to characterize the viral RNA species being produced. Figure 4A shows VSV-specific RNA peaks 6 hr post VSV infection in the presence of guanidine. Figure 4B shows these same VSV peaks even though poliovirus and guanidine were present for 4 hr before the labeling. VSV RNA was evident even in cells superinfected with poliovirus type 1 in the absence of guanidine (Fig. 4C). The large peak of 35S poliovirus RNA overwhelmed the VSV peaks at 40 and 32S. However, the breadth of the base around the poliovirus 35S peak and the peaks around 18, 17, and 12S all indicate the presence of VSV RNA. These findings contrast strikingly with the rapid shut off of VSV proteins by poliovirus.

The results presented here showing the dominance of poliovirus over VSV in doubly infected
cells have been repeated with coxsackievirus B1 and VSV. The coxsackievirus B1 also produced a marked inhibition of VSV PFU and protein synthesis, even in the presence of guanidine, without affecting VSV RNA synthesis.

DISCUSSION

The present study shows that, in HeLa cells infected with poliovirus type 1 and VSV, the former virus predominates, interfering strongly with VSV replication and VSV protein synthesis.

Inhibition by poliovirus of host protein synthesis and VSV-specific protein synthesis may operate by the same mechanism. The similarities in kinetics of inhibition of amino acid incorporation and of changes in protein patterns in polyacrylamide gels indicate the action of similar mechanisms in both cases. Inhibition of protein synthesis evidently occurs even in the absence of detectable poliovirus RNA replication. Poliovirus inhibition of translation is evidently quite specific; it can obviously distinguish between enterovirus messenger RNA (mRNA) and host cell mRNA. Perhaps mengovirus, coxsackievirus, and SV5 mRNA species (3), but not VSV or host mRNA species, possess the same distinguishing characteristics as poliovirus messenger, thus enabling them to continue translation in the presence of poliovirus inhibitors.

Hunt and Ehrenfeld (8) reported that double-stranded RNA from poliovirus-infected cells inhibited translation in a cell-free hemoglobin-synthesizing system. Double-stranded viral RNA was reported by Cordell-Stuart and Taylor (4) to be cytotoxic to a number of uninfected cell lines.

Repeated determinations of amino acid incorporation rates into cells infected with VSV and poliovirus in the presence of guanidine have shown that poliovirus rapidly reduces the rate of VSV-directed protein synthesis. Qualitative electrophoretic gel analysis of the types of proteins made at these reduced rates demonstrates that the remaining polypeptide synthesis is VSV-specific (Fig. 2C). We have also repeatedly observed that as a result of poliovirus superinfection there is a proportionally greater decrease in the height of VP-5 and VP-2 peaks relative to the core protein peak (VP-3). In the absence of guanidine, poliovirus both lowers the rate of VSV-specific protein synthesis and replaces this VSV synthesis with poliovirus-specific protein synthesis. This results in a polypeptide gel pattern dominated by poliovirus infection (Fig. 2D), but some VSV polypeptides still remain. A difference in the location of synthesis for the core (VP-3) and membrane (VP-2, VP-5) proteins of VSV might explain the differences in the rates of inhibition by poliovirus.

Petric and Prevec (14) found that the cytoplasm of pulse-labeled VSV-infected cells contained almost exclusively core protein molecules compared
with the protein pattern in whole cells. Wagner et al. (16) have also suggested different sites for synthesis of VSV proteins. They suggest that the core protein is produced on the endoplasmic reticulum whereas the membrane-associated proteins are produced near the plasma membrane.

It is interesting that poliovirus, under the conditions used in these experiments, did not interfere with VSV RNA synthesis although it strongly inhibited host cell RNA synthesis (unpublished data). Willems and Penman (17) have suggested that the inhibition of host RNA synthesis occurs by a mechanism different from the one inhibiting host protein synthesis. Ghendon et al. (6), using temperature-sensitive mutants of poliovirus, reported that cells infected at nonpermissive temperatures produce 10S, 5S structures which react with viral antisera and that the cytoplasm of these cells contains a molecule(s) which inhibits nuclear deoxyribonucleic acid-dependent RNA polymerase.

Because limited transcription and translation of the poliovirus input RNA are both conceivable under the conditions used here, it is impossible at present to define the nature of the inhibitory molecules. The poliovirus input RNA molecule might be transcribed even in the presence of guanidine, yielding double-stranded viral RNA, or the input RNA molecule might be translated on host ribosomes to produce inhibitory proteins.

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**LITERATURE CITED**


