Early Functions of Poliovirus

III. The Effect of Guanidine on Early Functions

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(Z. Naturforsch. 26 b, 940—944 [1971]; received March 3, 1971)

Guanidine does not inhibit the translation of viral RNA into virus specific proteins but it seems to prevent the generation of functional viral polymerase from precursor material. In the presence of guanidine, "inhibitory protein(s)" responsible for the shut-off of cellular protein synthesis are still produced provided that the multiplicities of infection are high. The incoming (parental) viral RNA appears to be functional as messenger for only a limited number of transcriptional cycles.

In two previous reports the influence of hydroxylamine inactivation on two early functions of poliovirus was described: The shut-off of cellular protein synthesis and the replication of viral RNA 1, 2. It was found that a single lethal hit by hydroxylamine in the viral genome abolishes either function possibly in two ways:

1. Via a malfunction of the damaged viral RNA in translation.
2. By depriving the viral RNA of its template function in replication. This in turn, leads to a lack of viral RNA and thus of messenger function as compared to normally infected cells.

These investigations had, by necessity, to be performed with virus populations that consisted of various amounts of infectious viruses and viruses with one or several lethal hits by hydroxylamine. The respective ratios are dependent only on the degree of inactivation. In order to study if the introduction of 1 lethal hit results, either directly or indirectly, in a lack of viral messenger function, one ought to infect cells with a high multiplicity of 1 hit-particles only. This, of course, is impossible.

However, guanidine is reported to specifically inhibit viral RNA replication 3 and not to interfere with viral protein synthesis 4. In that case one should be able to study the messenger function of viral RNA in the absence of viral RNA replication. Experiments carried out along this line are described in this report.

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Materials and Methods

Cells: HeLa S 3-cells (Flow Laboratories, Irvine, Scotland) in stationary and suspension cultures were used as previously described 2.

Viruses: Poliovirus Type 1 (Mahoney) 5, guanidine sensitive clone.

Experimental procedures

a) Guanidine and virus structural proteins

Three aliquots each consisting of 1.4 x 10^8 cells were infected with 50 PFU/cell in a volume of 10 ml E a gle's spinner medium without serum. After spinning for 30 min at 35 °C, 40 ml of E a gle's spinner medium containing 5% calf serum were added and the cells further incubated for 180 minutes. The cells were sedimented and washed in spinner medium containing 1/10 of the normal amino acid concentration and resuspended in 50 ml of this medium. Guanidine (3 mm) was added to a sample, another one was treated with actidione (1 mm), a third remained as control. 10 min after addition of the inhibitors all three samples received a 30 min pulse of 33.3 μCi of H^14C-protein-hydrolysate each (54 mCi/m Atm C). The pulse was limited by the addition of a 2000 fold excess of unlabelled amino acids followed by sedimentation of the cells. The sediments were resuspended in 30 ml of prewarmed medium containing additional 25 mg of unlabeled amino acids and were incubated for a total of 22 hrs. Virus was harvested as described 5. The concentrated virus preparations were sonified and purified by passing through a Sepharose column (Sepharose 2 B, Pharmacia, Uppsala, Sweden) (Column 38 cm x 1.8 cm), equilibrated with PBS and eluted with the same buffer. The virus peak fractions (plaquetests) were concentrated to about two milliliter volumes by dialysis against 40% Ficoll-solution (Pharmacia) and


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the viruses were further purified by CsCl-gradient centrifugation. The subsequent procedures are described in the legends to the respective figures.

b) Guanidine and the replication of viral RNA

Five aliquots of HeLa S 3-cells (5.3 × 10^7 cells each) from a spinner culture were suspended in 10 ml of GBI-spinner medium without serum containing actinomycin D (5 μg/ml). They were infected with 40 PFU of poliovirus. After 40 min each aliquot received 90 ml of Eagle’s spinner medium containing 5% calf serum, actinomycin D (5 μg/ml) and ^14C-uridine (20 μCi; 53 mCi/m mole, Radiochemical Center, Amersham, England). 5 ml samples were taken for each point in Fig. 2; they were processed as described below. At the indicated time after infection (Fig. 2) two of the aliquots were made 1 mM with respect to actidione, and two of them 3 mM with respect to guanidine. One aliquot was used as control. Further samples were taken as shown in the Figure. At each time point the samples were put into cold PBS (5 ml) and centrifuged and washed twice in 10 ml PBS in the cold. There washed cell pellets were precipitated with 2 ml of 5% TCA and collected on membrane filters and washed again with 10 ml of 5% TCA. The filters were dried and the radioactivity measured in 10 ml POPOP/PPO/toluene solution in a liquid scintillation counter (Packard, Tricarb).

The same technique was applied to determine the viral RNA synthesis at high multiplicities (500 PFU/cell) with and without guanidine.

c) Guanidine and development of the shut-off phenomenon

All respective procedures in the experiments were exactly as described previously. The concentration of guanidine was 3 mM.

Results

Caliguiri and Tamm concluded from their experiments that guanidine inhibits the RNA synthesis of poliovirus without impairing the synthesis of viral proteins. This is completely born out by the following experiments.

1. Guanidine and the synthesis of viral structural proteins

Aliquots of infected cells were treated with guanidine or actidione as described in Materials and Methods a (see legend of Fig. 1 also).

The specific radioactivity (cpm/PFU) of the purified viruses produced was determined. As shown in Figs. 1 a, b and c, guanidine did not influence the specific radioactivity of the viruses as compared to the controls. In contrast, actidione reduced it significantly as expected, to about 10% of the control values. Thus, the synthesis of viral proteins which subsequently become part of mature poliovirus

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Fig. 1. Influence of actidione and guanidine upon the synthesis of viral proteins. Experimental procedures as described in Materials and Methods. The figure shows the CsCl density gradient analysis of virions formed under the various conditions (centrifugation: 18 hrs., 35000 rpm, 4 °C, rotor SW 39, after mixing 2.6 ml virus preparation with 1.4 ml saturated (4 °C) CsCl solution). a) Control, no inhibitor present during pulse, b) with actidione, present during pulse, c) with guanidine, present during pulse. •—• Radioactivity (cpm per fraction; 0.04 ml), o——-o Infectivity (PFU per ml). The radioactivity in the fractions was determined after addition of 0.46 ml of water and 10 ml of Br a’s solution. The specific radioactivities (cpm/PFU) calculated for the virus peak fractions are: a) (1.61 ± 0.18) × 10^-5, b) (0.12 ± 0.02) × 10^-5, c) (1.85 ± 0.36) × 10^-5. (The error represents the mean variation of the arithmetic mean.)

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is not affected by guanidine in any demonstrable fashion.

2. Guanidine and the replication of viral RNA

Each of five aliquots of cells received $^{14}$C-uridine 45 min after infection with poliovirus in the presence of actinomycin D. Then 5 ml samples of the respective sets were taken for each time point in Fig. 2. This reflects the accumulative incorporation of $^{14}$C-uridine into viral RNA.

At two different times (140 min and 185 min, respectively) guanidine was added to four aliquots. One remained as control.

Samples were further collected after the addition of the inhibitors in order to study their effect upon viral RNA synthesis. This is also shown in Fig. 2. In the early phase when guanidine or actidione were added, viral RNA synthesis was immediately arrested (sets 1 and 2). When guanidine was added during the linearly increasing phase of viral RNA synthesis the inhibitor reduced the RNA synthesis immediately and brought it to a complete halt within 30 - 35 min (set 4).

On the other hand, with actidione viral RNA synthesis continued normally for approximately 18 min and was arrested only during the subsequent 20 min (set 3).

From these results it may be concluded that guanidine affects viral RNA synthesis in a rather direct way and quite promptly. The marked difference between the effects of guanidine and actidione also strongly suggests that guanidine does not act via inhibition of viral protein synthesis.

3. Guanidine and the shut-off of cellular protein synthesis by poliovirus

According to the results mentioned above the use of guanidine appears suitable to study the messenger function of the infecting (incoming) viral RNA in the absence of viral RNA replication. Here, the shut-off of cellular protein synthesis was taken as a measure for the production of an early functional viral protein in the presence of guanidine. Similar investigations have already been published by others with variable results. HOLLAND found an inhibitory effect of guanidine on the shut-off of cellular protein synthesis at low multiplicities of infection ($m = 10$). The inhibition, however, was overcome at $m = 10,000$.

BABLANIAN et al. described that the virus-induced depression in the rate of protein synthesis is delayed but not prevented by guanidine in HEL-cells (at $m = 100$ of polio typ 2 and 1.11 mM guanidine).

PENMAN et al. reported the absence of any respective inhibition of the shut-off by guanidine both at low or high multiplicities. The question was therefore reinvestigated in some detail.

The results of our own experiments are shown in Fig. 3. At $m = 10$, guanidine effectively prevented the virus induced shut-off phenomenon. At $m = 500$, however, the onset and the initial development of the inhibitory process were quite comparable to the corresponding control. A continued reduction of cellular protein synthesis was only observed in the absence of guanidine, however.

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6 J. J. HOLLAND, J. molecular Biol. 8, 574 [1964].
7 R. BABLANIAN, H. J. EGGERS, and I. TAMM, Virology 26, 100 [1965].
8 S. PENMAN and D. F. SUMMERS, Virology 27, 614 [1965].
In order to investigate the possibility that at multiplicity of 500 viral RNA was replicated in spite of the presence of guanidine, the following experiment was performed. The accumulative incorporation of $^{14}$C-uridine in actinomycin D treated cells was determined in controls and after infection with $m = 500$ in the presence and absence of guanidine. As Fig. 4 shows, the viral RNA synthesis is completely inhibited by guanidine even after infection at $m = 500$. The results of the experiments presented in Figs. 3 and 4 lead to the following conclusions.

1. If viral RNA replication is made impossible in the presence of guanidine at low multiplicities of infection, the viral messenger function does not express itself in a measurable way.

2. By sufficiently supplying incoming viral RNA owing to high multiplicities of infection, one can observe viral messenger function even in the absence of viral RNA replication.

**Discussion**

The experiments clearly suggest that the primary messenger function of polio RNA is not affected by guanidine: amino acids are still incorporated into polypeptides which subsequently can become part of the structural protein moiety of virions. Similarly, a seemingly virus coded "inhibitory protein (s)" which is responsible for the shut-off of cellular protein synthesis is formed in the presence of guanidine.

By comparing the effect of guanidine on the one hand and actidione on the other upon viral RNA synthesis it is evident that guanidine inhibits more quickly. The inhibitory effect of actidione is demonstrable only after a lag period although the drug arrests protein synthesis by more than 98% within 1 min (l.c. 9, KOSCHEL unpublished). The kinetics of the inhibition of viral RNA synthesis by actidione is therefore likely to reflect two events which continue after the inhibition of protein synthesis:

1. The production of an active polymerase complex from a pool of inactive precursor material and the time required to exhaust this pool.
2. The loss of active polymerase by subsequent events, e.g. rate of turnover.

Both, the generation of active polymerase from precursor proteins and the disappearance of active polymerase are compatible with the model of JACOBSON and BALTIMORE 10. These authors suggested that the viral genome is read in one piece. The growing peptide chain is cleaved into large pre-
cursor proteins which are split into structural and functional viral proteins. Polymerase proteins and others are bound to the membrane structures of the replicative complex, the site of synthesis of viral RNA. Guanidine, unlike actidione, leads to an immediate drop in the rate of viral RNA synthesis. This may suggest the existence of a pool of inactive polymerase precursors which, in the presence of guanidine, can no longer be utilized.

In vitro studies have failed to indicate any direct effect of guanidine upon existing polymerase and its function on the replicative intermediate complex of poliovirus. Thus, guanidine is more likely to inhibit the generation of the active polymerase complex from inactive polypeptide precursors present in a pool. Preliminary results obtained by acrylamide-gel analysis of the viral protein associated with the replicative complex in the absence and presence of guanidine support this notion (Koschel, in preparation).

Another functional viral protein, the "inhibitory protein (s)" directly or indirectly responsible for the shut-off of cellular protein synthesis can, however, still be formed in guanidine inhibited cells. This provided a tool to study its formation in the absence of viral RNA replication.

In that case, viral proteins should be synthesized solely under the control of the parental or incoming viral RNA. At low multiplicities of infection ($m = 10$) the virus induced shut-off of cellular protein synthesis could still be prevented by guanidine in our system. At $m = 500$ in the presence of guanidine, however, the onset and the initial development of the phenomenon were very similar to those found in infected control cells, although there was actually no viral RNA synthesis.

This can be interpreted by assuming that a certain critical amount of "inhibitory protein (s)" has to be accumulated before the shut-off phenomenon becomes demonstrable. If viral RNA replication is made impossible by guanidine, the rate of production of "inhibitory protein (s)" depends solely on the number of incoming viral RNA and their function as m-RNA in translation. Thus, only a rather high multiplicity of infection provides for a sufficient viral messenger function.

It is noteworthy, however, that even then the shut-off phenomenon did not reach the extent regularly found in the controls either at high or at low multiplicities. This suggests that the incoming viral RNA may serve as a messenger only for a rather limited number of translational cycles.

We thank Mrs. A. Kreisel for her technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft and the Stiftung Volkswagenwerk.

9 D. Baltimore, M. Girard, and J. E. Darnell, Virology 29, 179 [1966].