Effect of Inactivation by Hydroxylamine on Early Functions of Poliovirus

KARIN BORGERT, KLAUS KOSCHEL, HELMUT TÄUBER, AND EBERHARD WECKER

Institut für Virologie der Universität Würzburg, D8700 Würzburg, Germany

Received for publication 29 December 1970

Evidence is presented that poliovirus particles with a single lethal hit by hydroxylamine do not induce in host cells either inhibition of cellular protein synthesis or viral ribonucleic acid (RNA) replication. The RNA of these viruses is not replicated even if the cells are simultaneously infected with both active and inactivated viruses. The damaged viral RNA seems to have lost both its template function and its function in the translation of normal viral proteins.

The replication of poliovirus is likely to depend upon the expression of all of the genetic information present in the viral ribonucleic acid (RNA). In infected cells, one can, however, observe several events that take place before virus maturation. One of them is the inhibition of cellular protein synthesis (shut-off phenomenon; references 1, 12, 17, 22, 30), and another is the replication of viral RNA. Both of these processes may be regarded as the result of viral gene expression, e.g., the function of newly synthesized virus-specific proteins. If the corresponding genes could be expressed independently of others, they might be able to do so even if some different parts of the viral genome were rendered non-functional.

We have investigated the question of whether the two early functions of poliovirus, the shut-off phenomenon and the replication of viral RNA, can be induced by viruses that are inactivated by treatment with hydroxylamine.

MATERIALS AND METHODS

Cells and virus. HeLa S3 cells were obtained from Flow Laboratories, Scotland. Cells were grown in Roux flasks; for 24 hr before use, they were suspended in Spinner medium. Poliovirus type I (Mahoney) was purified by high-speed centrifugation from lysates of infected cells, concentrated 150-fold, and stored at -70°C until use. The infectivity of each preparation was determined by plaque assay (6). In each case, 10 to 15 plates of an appropriate dilution were counted to assure sufficient accuracy.

Inactivation by hydroxylamine. Virus preparations were treated at 30°C with 0.1 M or 0.2 M hydroxylamine-hydrochloride at pH 6.8. Inactivation was stopped by a 10-fold dilution into ice-cold phosphate-buffered saline (PBS), immediately followed by dialysis against PBS at 4°C .

Determination of host-cell protein synthesis. Samples (ca. 10^6 cells) were taken from infected suspension cultures at various times and incubated for 15 min with 0.7 μCi of ^{14}C -protein hydrolysate (54 mCi/m-atom of C; Radiochemical Centre, Amersham, England). The pulse was terminated by addition of frozen PBS and immediate sedimentation of the cells in the cold. The sediment was washed three times in PBS and then precipitated by 5% trichloroacetic acid. The precipitates were collected on membrane filters (0.45 μM), washed three times with 5% trichloroacetic acid and once with 0.1 N HCl, and dried.

Determination of viral RNA synthesis. Cells infected in suspension and treated with 5 μg of actinomycin D per ml were incubated for 3 hr at 37°C . This was followed by a 30-min pulse with ^{14}C -uridine (5 μCi , specific activity, 500 $\mu\text{Ci}/\text{mmole}$; Radiochemical Centre, Amersham, England). The pulse was terminated as described above. Cytoplasmic extracts were prepared as described by Penman et al. (18), treated with 1% sodium dodecyl sulfate (SDS), and analyzed by zonal centrifugation (30) in a sucrose gradient (15 to 30%, w/w; 0.1% SDS, 0.1 M sodium phosphate buffer, pH 7.2; for 16 hr at $35,800 \times g$ at 28°C with a SW 25.1 rotor).

Fractions were collected and analyzed for optical density (OD) at 260 nm. Each fraction was precipitated by an equal volume of 10% trichloroacetic acid. The precipitates were collected on membrane filters, washed, and dried as described above.

To correct the amount of viral 35S RNA for possible variations in the total yield, the "relative amount" of 35S viral RNA is given, that is the ratio of counts per minute (35S) to OD (28S).

Determination of infectious RNA. The cytoplasmic extracts (see above) were extracted by phenol as described by Wecker et al. (27). The RNA in the aqueous phases was coprecipitated by the addition of 0.2 M phosphate buffer (pH 7.2) and 2 volumes of ethanol at -20°C . After centrifugation (10 min, $1,500 \times g$), the precipitates were washed twice as

described (27) and dissolved in 0.001 M ethylenediaminetetraacetic acid (EDTA; pH 7.2). The solutions were then incubated for 10 min at 37°C and assayed for infectious RNA as described (27). Samples were analyzed by zonal centrifugation, and the OD at 260 nm and the radioactivity of the fractions were determined as described above.

Measurement of radioactivity. Acid-precipitable material was collected on membrane filters (Sartorius, Göttingen). The filters were dried and counted in a Packard liquid scintillation spectrometer after the addition of toluene-2,5-diphenyloxazole-1,4-bis-2-(5-phenyloxazolyl)benzene scintillation fluid.

RESULTS

Virus-induced inhibition of cellular protein synthesis requires de novo synthesis of virus-specific proteins. First it was shown that de novo protein synthesis is essential for the shut-off phenomenon to occur (Fig. 1). Cycloheximide present during the first 2 hr after infection completely prevented its development. When the inhibitor was removed 2 hr postinfection, it took, as usual, 3 hr before the protein synthesis in the poliovirus-infected cells became maximally reduced. Indeed, the shut-off phenomenon evolved exactly as if the cells had only been infected at the time of reversal of the inhibition by cycloheximide.

This result substantiates the finding that appropriate concentrations of *p*-fluorophenylalanine and puromycin, if added early in the infectious cycle, prevent the shut-off phenomenon caused by picornaviruses (3, 7, 19, 26).

Holland and Peterson (14) have shown that actinomycin D does not interfere with the virus-induced inhibition of host cell protein synthesis. This was fully confirmed by our own experiments. Therefore, the notion seems justified that de novo synthesis of viral proteins is necessary for the specific shut-off of cellular protein synthesis.

Inactivated viruses cannot provoke the shut-off phenomenon. Having obtained evidence that viral gene expression is probably necessary for the development of the shut-off phenomenon, we now examined whether inactivated viruses still can provoke it.

Penman and Summers (19) have found that heavily ultraviolet (UV)-inactivated polioviruses (99.9%) no longer induce the phenomenon. In that case, however, most viruses had received multiple lethal hits. Therefore, the question remained of whether a virus with a single lethal hit in its genome might still provoke an inhibitory effect upon host cell protein synthesis.

This problem can be investigated provided that there is a demonstrable correlation between

FIG. 1. Influence of cycloheximide (Acti-Dione) on the development of virus-induced inhibition of cellular protein synthesis. Ten milliliters each of three identical cell cultures in suspension were treated as follows: sets 1 and 2 were uninfected and set 3 was inoculated with an MOI of 100. After 30 min, all three samples received 90 ml of Spinner medium containing 5% calf serum each. Set 1 did not contain cycloheximide; sets 2 and 3 contained cycloheximide (1.07 mM). At the times indicated, 5-ml samples were withdrawn, incubated with ^{14}C -amino acids for 15 min, and further processed as described. The concentration of cycloheximide was kept at 1.07 mM during the pulses of sets 2 and 3. Two hours after infection, all three sets were harvested, washed twice with 100 ml of medium without serum, and adjusted to the original cell concentration with 80 ml of Spinner medium containing 5% calf serum. Further samples were taken, but from all three sets they were pulsed with ^{14}C -amino acids without cycloheximide. Symbols: $\circ$, uninfected, no cycloheximide (set 1); $\bullet$, uninfected, plus cycloheximide (set 2); $\circ$, infected, plus cycloheximide (set 3). (Sets treated with cycloheximide do not reach the values of the control, probably due to incomplete removal of cycloheximide.)

the multiplicity of infection (MOI) and the degree of the shut-off phenomenon. As shown in Fig. 2, there is such a correlation up to an input MOI of between 10 and 50. Higher MOI values do not enhance either the degree or the kinetics of the shut-off phenomenon beyond a certain plateau.

As shown for other RNA viruses (8, 21, 23), UV irradiation and treatment with hydroxyla-

FIG. 2. Inhibition of cellular protein synthesis and multiplicity of infection.

mine also inactivate poliovirus following first-order kinetics. Thus, by applying the Poisson distribution (11), one can calculate the relative amount of particles with 1, 2, 3...n lethal hits within a given virus population for any degree of inactivation as determined by plaque assay.

For the subsequent experiment, a virus preparation was inactivated by 98.8%. The composition of the resulting population with regard to particles containing 0, 1, 2,...n lethal hits (P_0, P_1, P_2 , etc.) is indicated in the legend to Fig. 3, which also gives the respective multiplicities (M_0, M_1, M_2 etc.) as actually employed in the experiment. The results obtained with this virus preparation and the appropriate controls are shown in Fig. 3.

It is apparent that the partially inactivated virus population with a residual MOI of 2.6 was even less effective in reducing host cell protein synthesis than was the corresponding control adjusted to the same MOI. This is due to a competition between inactivated and infectious viruses. The competition becomes only apparent if the ratio of active to inactivated viruses is significantly smaller than 1:10 (Koschel, unpublished data). In the present experiment the ratio ($P_0:P_n$) was $2.6:218 = 1:84$. If the virus with one lethal hit (P_1) could also contribute to the shut-off phenomenon, the ratio ($P_0 + P_1:P_{>1}$) would be $18:202 = 1:11$. This ratio is outside

the range of significant competition and thus the respective multiplicity ($M_0 + M_1$) should amount to an actual MOI of about 18. This, however, is clearly not borne out by the experimental results. They instead suggest that even viruses with a single lethal hit no longer participate in inhibition of host cell protein synthesis.

RNA of inactivated viruses is not replicable. One of the possible explanations for the findings described above is that a viral RNA molecule containing one or more lethal hits cannot be replicated. This question was studied by applying basically the same approach as for the experiments mentioned above. Consequently, a correlation between MOI and viral RNA synthesis was first established. The amount of labeled 35S material served as a measure for the amount of viral RNA synthesized between 3 and 3.5 hr postinfection (see above).

The results displayed in Fig. 4 and 5 indicate that a correlation between MOI and viral RNA synthesis exists up to a multiplicity of about 16,

FIG. 3. Inhibition of cellular protein synthesis by a partially (98.8%) inactivated poliovirus preparation. Symbols: $\circ$, uninfected control; $\blacktriangle$, infected by inactivated virus (composition in per cent: $P_0 = 1.2$; $P_1 = 7$; $P_2 = 17$; $P_{>2} = 74.8$; for multiplicities, see insert of figure); $\triangle$, infected by untreated virus ($M_0 = 2$); $\blacksquare$, infected by untreated virus ($M_0 = 17$); $\square$, infected by untreated virus ($M_0 = 220$).

FIG. 4. Incorporation of ^{14}C -uridine into 35S sedimenting viral RNA from sucrose-sodium dodecyl sulfate gradients, depending on multiplicity of infection. III: alternate dots and dashes, $M = 0.5$; solid line, $M = 1$; dashed line, $M = 2$. II: solid line, $M = 4$; dotted line, $M = 8$. I: solid line, $M = 16$; dashed line, $M = 32$.

when saturation is reached. The data in Table 1 represent the composition of three virus preparations partially inactivated by hydroxylamine and the corresponding controls. They also indicate the relative amounts of viral RNA obtained after infection with these virus preparations.

Without exception, the amount of viral RNA obtained after infection with partially inactivated virus preparations was less than that produced by the corresponding controls. Thus, viruses with one or more lethal hits do not seem to induce viral RNA replication. In fact, they exert an inhibitory effect upon the RNA replication of infectious viruses.

There remains the question of whether they

FIG. 5. Relationship of synthesized 35S RNA (relative amounts) and multiplicity of infection.

simply cannot give rise to functional RNA polymerase or whether their RNA has lost its template function, or both. We therefore examined whether inactivated viral RNA is replicated within a cell that is simultaneously infected with infectious virus.

A "trans" function between certain poliovirus mutants with regard to the replication of their RNA (4, 28) has already been described. The results of such an experiment are shown in Table 2. The amount of 35S viral RNA (counts per minute) and the infectivity contained in that material [plaque-forming units (PFU)] were determined, and the ratio of counts per minute to PFU, that is the specific infectivity, was calculated. The specific infectivity was taken as a measure of the type of viral RNA which was replicated, e.g., only infectious or infectious plus inactivated RNA.

The data in Table 2 reveal that the specific infectivities of both the control and the sample obtained with a partially inactivated virus preparation were identical within the limits of error. This strongly suggests that noninfectious viral RNA was excepted from replication even in the presence of functional RNA polymerase.

DISCUSSION

The experiments presented here and previous findings by others (12, 14, 19, 29) indicate that it is very likely that virus-specific protein is directly or indirectly involved in the shut-off of host cell protein synthesis by polioviruses. There is no doubt that a viral gene product, the polymerase, is essential for viral RNA replication (2, 10). Of course, neither of these two virus-specific

TABLE 1. *Experiments with hydroxylamine-inactivated poliovirus and control multiplicities and the relative amount of viral RNA synthesized*

Expt no.	Set ^a	Degree of inactivation (%)	Multiplicities				Relative amount of 35S RNA
			M ₀	M ₁	M ₀ + M ₁	M _{total}	
1	Inactivated	94.4	0.5	1.5	2.0	9.0	3.3
	Control	0	0.5	0	0	0.5	9.4
	Control	0	2.0	0	0	2.0	17.8
2	Inactivated	66.5	0.5	0.54	1.0	1.5	9.4
	Control	0	0.5	0	0	0.5	19.5
3	Inactivated	96.8	0.5	1.8	2.3	15.8	1.7
	Inactivated	95.8	0.5	1.7	2.2	12.1	2.6
	Control	0	0.5	0	0	0.5	19.1

^a See figures.TABLE 2. *Determination of specific infectivity of virus RNA from cells infected with untreated and hydroxylamine-treated virus*

Expt	Multiplicity of infection for P ₀ , P ₁ ... P _n particles				Amt of virus RNA ^a	Infectivity ^b	Specific infectivity ^c	Relative specific infectivity (control = 1)	Theoretical relative specific infectivity for replication of RNA from			
	M ₀	M ₁	M ₂	M _{res}					P ₀	P ₀ + P ₁	P ₀ + P ₁ + P ₂	P ₀ + P _n
Control (untreated virus)	18				5.33	9.2 ± 5%	0.6 ± 5%	1	1			
Inactivated virus	3.3	5.3	4.8	4.6	0.448	0.96 ± 4.5%	0.5 ± 4.5%	0.8	1	2.6	4.1	5.5

^a Expressed as ϵ counts per minute (35S) $\times 10^4$.^b Expressed as PFU per milliliter $\times 10^4$.^c Expressed as counts per minute per PFU per milliliter.

functions will require all of the genetic information contained in the viral genome.

We have presented evidence that the shut-off phenomenon and viral RNA replication are abolished by the introduction into viral RNA of a single lethal hit by hydroxylamine. Thus, it seems as if the "target size" for either of the respective gene functions was identical with that of the sum of all gene functions, e.g., the infectivity. This is readily explained by our finding that a single lethal alteration of viral RNA renders it nonfunctional as template. Since RNA of poliovirus has to be regarded as an autoreplicative messenger, a lack in replication implies a lack in messenger function as well.

We have shown that lethally damaged viral RNA is unable to be replicated. In addition, it exerts a striking inhibition upon the replication of perfectly normal poliovirus RNA within the same cell. This can be accounted for in two ways. (i) The damaged and nonreplicable viral RNA still associates with RNA polymerase

originating from the infectious virus. By doing so it deprives the normal RNA of its polymerase. Yet, this is only possible if the damaged RNA does not give rise to functional polymerase on its own account. (ii) The damaged viral RNA leads to the production of faulty, nonfunctional polymerase. This occupies and thus blocks some of the normal viral RNA.

In either case, a lethal hit anywhere in the RNA of poliovirus not only abolishes its template function but also seems to affect its messenger function, at least with respect to the production of active polymerase and, perhaps, a "shut-off protein." Indeed, the model of a polycistronic messenger function and the post-translational cleavage of a primary viral protein (13, 15) are quite easily reconciled with our results. Whether a faulty polymerase or none at all results from a damaged viral RNA cannot be decided as yet.

From the experiments described here, it becomes quite clear that a gradual inactivation of

polioviruses by hydroxylamine is not suited for localizing individual genes within the viral genome. It remains to be seen if this is a unique feature of picornaviruses. Perhaps it applies to other RNA viruses as well. The successive inactivation by UV irradiation (16) or treatment with an ethylene-iminoquinone (24, 25) or hydroxylamine (24) of various functions of influenza virus does not necessarily preclude such a possibility. Actually, the genome of influenza viruses has been shown to consist of five individual RNA molecules (5, 20). Each of them, if lethally hit, could be subject to mechanisms similar to those discussed here for the RNA of poliovirus, leaving the functions coded for by the unchanged molecules still intact. In that case, the formation of viral hemagglutinins, on the one hand, and that of viral RNA and s-antigen, on the other, should be coded for by different viral RNA molecules, because they possess quite distinct rates of inactivation (24). In contrast, Newcastle disease virus contains only one molecule of RNA, and inactivation by UV abrogates virus infectivity together with viral RNA replication (9). Similarly, treatment by ethylene-iminoquinone fails to inactivate individual functions of mouse encephalomyelitis virus selectively and successively (Scholtissek and Rott, *personal communication*). Again, the genome of mouse encephalomyelitis virus consists of one RNA molecule.

To probe the notion that our findings with polioviruses may not be unique for picornaviruses, analogous studies ought to be extended to other RNA-containing animal viruses. A more detailed account of the results presented here will be published elsewhere.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft and the Stiftung Volkswagenwerk.

We thank Angelika Kreisel for technical assistance.

LITERATURE CITED

- Bablanian, R., H. J. Eggers, and I. Tamm. 1965. Studies on the mechanism of poliovirus-induced cell damage. I. The relation between poliovirus-induced metabolic and morphological alterations in cultured cells. *Virology* 26:100-113.
- Baltimore, D., H. J. Eggers, R. M. Franklin, and I. Tamm. 1963. Poliovirus-induced RNA polymerase and the effects of virus-specific inhibitors on its production. *Proc. Nat. Acad. Sci. U.S.A.* 49:843-849.
- Baltimore, D., R. J. Franklin, and J. Callender. 1963. Mengovirus-induced inhibition of host ribonucleic acid and protein synthesis. *Biochim. Biophys. Acta* 76:425-430.
- Cords, C. E., and J. J. Holland. 1964. Replication of poliovirus RNA induced by heterologous virus. *Proc. Nat. Acad. Sci. U.S.A.* 51:1080-1082.
- Duesberg, R. 1952. The RNA of influenza virus. *Proc. Nat. Acad. Sci. U.S.A.* 59:930-937.
- Dulbecco, R. 1952. Production of plaques in monolayer tissue cultures by single particles of an animal virus. *Proc. Nat. Acad. Sci. U.S.A.* 38:747-752.
- Franklin, R. M., and D. Baltimore. 1962. Patterns of macromolecular synthesis in normal and virus-infected mammalian cells. *Cold Spring Harbor Symp. Quant. Biol.* 27:175-198.
- Franklin, R. M., and E. Wecker. 1959. Inactivation of some animal viruses by hydroxylamine and the structure of ribonucleic acid. *Nature (London)* 184:343-345.
- Gandhi, S. S., D. C. Burke, and C. Scholtissek. 1970. Virus-RNA-synthesis by ultraviolet-irradiated Newcastle disease virus and interferon production. *J. Gen. Virol.* 9:97-99.
- Girard, M., D. Baltimore, and J. E. Darnell. 1967. The poliovirus replication complex: site for synthesis of poliovirus RNA. *J. Mol. Biol.* 24:50-74.
- Hiatt, C. W. 1964. Kinetics of the inactivation of viruses. *Bacteriol. Rev.* 28:150-163.
- Holland, J. J. 1964. Inhibition of host cell macromolecular synthesis by high multiplicities of poliovirus under conditions preventing virus synthesis. *J. Mol. Biol.* 8:574-581.
- Holland, J. J., and D. F. Kiehn. 1968. Specific cleavage of viral proteins as steps in the synthesis and maturation of enteroviruses. *Proc. Nat. Acad. Sci. U.S.A.* 60:1015-1022.
- Holland, J. J., and J. A. Peterson. 1964. Nucleic acid and protein synthesis during poliovirus infection of human cells. *J. Mol. Biol.* 8:556-573.
- Jacobson, M. F., and D. Baltimore. 1968. Polypeptide cleavages in the formation of poliovirus proteins. *Proc. Nat. Acad. Sci. U.S.A.* 61:77-84.
- Long, W. F., and D. C. Burke. 1970. The effect of infection with fowl plaque virus on protein synthesis in chick embryo cells. *J. Gen. Virol.* 6:1-14.
- Martin, E. M., and I. M. Kerr. 1968. Virus induced changes in host-cell macromolecular synthesis, p. 15-46. *In* L. V. Crawford and M. G. P. Stoker (ed.), *The molecular biology of viruses*. 18th Symp. Soc. Gen. Microbiol., Cambridge.
- Penman, S., K. Scherrer, Y. Becker, and J. E. Darnell. 1963. Polyribosomes in normal and poliovirus-infected HeLa cells and their relationship to messenger RNA. *Proc. Nat. Acad. Sci. U.S.A.* 49:654-662.
- Penman, S., and D. F. Summers. 1965. Effects on host cell metabolism following synchronous infection with poliovirus. *Virology* 27:614-620.
- Pons, M. W., and G. K. Hirst. 1968. Polyacrylamide gel electrophoresis of influenza virus RNA. *Virology* 34:385-388.
- Rott, R., and W. Schäfer. 1963. Hydroxylamin-Empfindlichkeit des Newcastle Disease-Virus (NDV). *Z. Naturforsch.* 17 b:861-862.
- Salzman, N. P., R. Z. Lockart, and E. D. Sebring. 1959. Alteration in HeLa cell metabolism resulting from poliovirus infection. *Virology* 9:244-259.
- Schäfer, W., and R. Rott. 1962. Herstellung von Virusvacinen mit Hydroxylamin. *Z. Hyg.* 148:256-268.
- Scholtissek, G., and R. Rott. 1963. Synthesis of viral ribonucleic acid by a chemically inactivated influenza virus. *Nature (London)* 199:200-201.
- Scholtissek, C., and R. Rott. 1964. Behavior of virus specific activities in tissue cultures infected with myxoviruses after chemical changes of the viral ribonucleic acid. *Virology* 22:169-176.
- Verwoerd, D. W., and P. Hausen. 1963. Studies on the multiplication of a member of the Columbia SK group (ME virus) in L-cells. IV. Role of "early proteins" in virus induced metabolic changes. *Virology* 21:625-635.
- Wecker, E., K. Hummeler, and O. Goetz. 1962. Relationship between viral RNA and viral protein synthesis. *Virology* 17:110-117.
- Wecker, E., and G. Lederhilger. 1964. Genomic masking produced by double infection of HeLa cells with heterotypic polioviruses. *Proc. Nat. Acad. Sci. U.S.A.* 52:705-709.
- Willems, M., and S. Penman. 1966. The mechanism of host cell protein synthesis inhibition by poliovirus. *Virology* 30:355-367.
- Zimmerman, E., M. Heeter, and J. E. Darnell. 1963. RNA synthesis in poliovirus infected cells. *Virology* 19:400-408.