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Short Communication

Lack of evidence for strand-specific inhibition of poliovirus RNA synthesis by 3-methylquercetin

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Summary

Buffalo Green Monkey cells were infected with poliovirus-1 in the presence of 3-methylquercetin or guanidine, and the formation of positive- and negative-strand viral RNA was monitored using single-stranded RNA probes.

Both 3-methylquercetin and guanidine prevented the formation of plus-strand as well as minus-strand viral RNA, although, due to the high multiplicity of infection used, a high number of genomic viral RNA was always present in the cells.

Poliovirus; 3-Methylquercetin; Guanidine; Plus-strand RNA; Minus-strand RNA

3-Methylquercetin (3-MQ) has been shown to have antiviral activity (Van Hoof et al., 1984; Vrijsen et al., 1984) against some enteroviruses. The drug does not prevent the shut-off of cellular protein synthesis caused by poliovirus infection, nor does it stop the formation of viral proteins if it is added at 90 min postinfection (Vrijsen et al., 1984; Castrillo et al., 1986). However, it stops the incorporation of radioactive precursors into viral RNA almost completely (Castrillo et al., 1986) if added at any time postinfection. Additionally, the drug alters the antigenic properties of polio-1 virions (Rombaut et al., 1985). In our hands 3-MQ, at 20 μg/ml, completely prevented the replication of poliovirus-1.

It has been reported that in the presence of 3-MQ some residual synthesis of RNA occurs (Castrillo and Carrasco, 1987) and that during sucrose gradient cen-

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trifugation and sepharose-2B-chromatography this RNA behaves like the replicative form of poliovirus. From these findings it has been inferred that the synthesis of minus-strand RNA might be less sensitive to 3-MQ than that of the plus-strand or even totally resistant to the drug. However, it could be argued that 3-MQ allows some kind of aberrant RNA synthesis which leads to the formation of double-stranded RNA forms.

In this paper we have reconsidered the question whether the synthesis of the minus-strand RNA is resistant to 3-MQ.

To achieve a clear-cut identification of any RNA which might be synthesized in the presence of 3-MQ we used nucleic acid hybridization with specific probes. One of the problems of identifying polio RNA using hybridization to nick-translated double-stranded cDNA probes is that the assay does not discriminate between plus- and the minus-strand RNA. It is therefore not possible to unequivocally identify the polarity of the strand synthesized (or inhibited) in the presence of 3-MQ.

In order to ascertain the polarity of the synthesized RNA we used the plasmid pHK8 (Fig. 1), which has the polio-1-genomic cDNA integrated between a T7- and

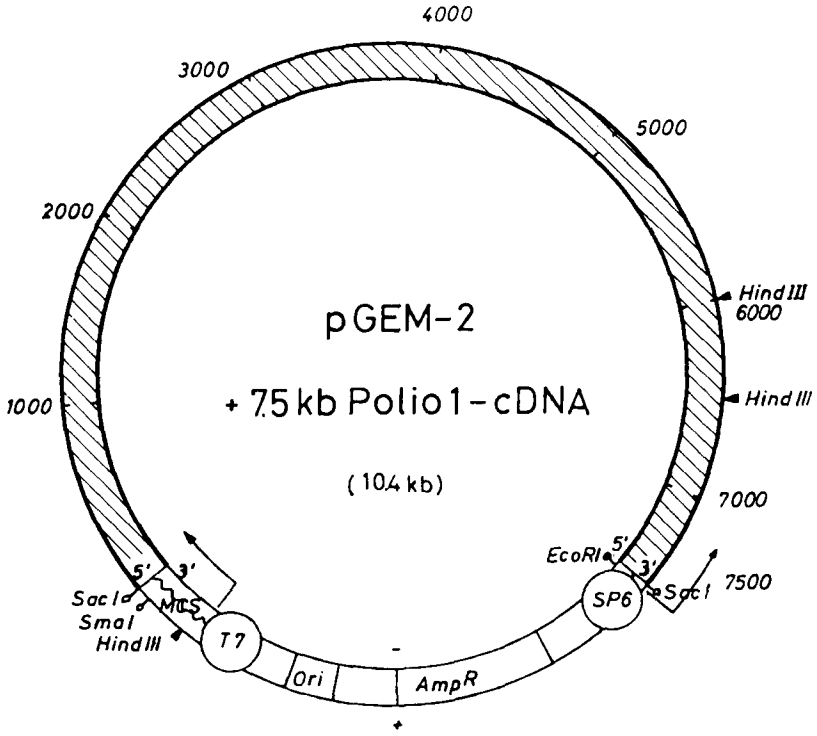

Fig. 1. Plasmid pHK8 used as template for the synthesis of ^{32}P-labeled polio RNA of positive (using T_7-polymerase) or negative (using SP6-polymerase) polarity. The map is approximately to scale. The plasmid was linearized prior to transcription with *Eco*RI or *Sma*I. The cloning vector pGEM-2 was purchased from Promega Biotec, Madison, U.S.A.

an SP6-promoter. For cloning procedures see Cova et al. (1988) and Kopecka et al. (1988). Using the proper RNA polymerases, labeled RNA of plus or minus polarity can be synthesized and used as a probe. This approach specifically allows the detection of less than 1 pg polio RNA of either polarity.

Petri dishes (9 cm D) were seeded with Buffalo Green Monkey (BGM) cells at 3×10^7 cells/dish and the cultures grown at 36°C for 24 h under 5% CO_2 in 10 ml Eagle's MEM supplemented with 10% FCS. The cultures were then treated with 3-MQ at 20 μl/ml and were incubated with the drug for another 60 minutes. The medium was removed and the cells exposed during 30 min to poliovirus-1 (Lsc 2ab) at an MOI of 50 PFU/cell in 0.9 ml MEM without serum and in the presence of 3-MQ at 20 μg/ml. After 30 minutes, excess medium was removed and the cultures were further incubated with 10 ml MEM plus 2% FCS and 3-MQ at 20 μg/ml. At 1.5, 3.0, 4, 5, 6 and 7 h postinfection, one Petri dish was taken out, the medium discarded, the monolayer rinsed twice with 0.5 ml ice-cold PBS. The total RNA was extracted according to the procedure described by Kirkegaard and Baltimore (1986), and resuspended in a final volume of 20 μl distilled water. One additional Petri dish was extracted at zero time after infection.

Control poliovirus- or mock-infected cultures run in parallel were either treated with 3-MQ or not treated. Another set of cultures received 2 mM guanidine instead of 3-MQ. The control and guanidine cultures were treated identically as the 3-MQ cultures.

Two 5-μl aliquots of the final extract were run by electrophoresis on 1% agarose (Bio-Rad Minicell.) at 30 mA and pH 8.2 for 5 h in the following solution: H_3BO_3, 50 mM; $Na_2B_4O_7$, 5 mM; Na_2SO_4, 10 mM and EDTA, 1 mM. After staining with ethidium bromide the gel was incubated in 250 ml electrophoresis buffer containing 5 mM methylmercury(II)hydroxide for at least two hours. We carried out the gel denaturation not before but after electrophoresis for the following reasons: if electrophoresis was carried out under denaturing conditions, minus-strand and plus-strand RNA moved to the same position. Since genomic plus-strand RNA is much more abundant than its minus-stranded counterpart, it will, even after its fixation to the gel, compete for minus-strands with the (also plus-stranded) radioactive probe used for detection, and by doing so, diminish the sensitivity of the method. If the separation was carried out under non-denaturing conditions, the minus-strand RNA (which is in a double- or multi-stranded state) moved slower than, and became separated from, the bulk of single-stranded genomic RNA. Detection then became more sensitive because the single-stranded genomic RNA did not interfere with hybridization.

After denaturation the gel was put between two sheets of Whatman paper and dried under vacuum at room temperature. The completely dry gel was humidified with sterile water to allow the Whatman papers to be peeled off. The gel was then cut over its length into two equal halves, each containing a set of separated extracts. The halved gels were then incubated overnight at 42°C with 5 ml of an hybridization buffer containing 5 to 10×10^5 cpm of ^{32}P-labeled RNA of plus or minus polarity. The probes had been synthesized from the plasmid pHK8, and the transcription carried out according to Krieg and Melton (1984). The gels were then

washed three times for 30 min at 65°C with 250 ml of 2×, 1× and 0.5 × SSC, respectively, which contained 0.1% SDS. The gels were placed on a sheet of Whatman paper, covered with thin plastic foil, dried under vacuum in a gel-drier at room temperature and incubated for 1 day with X-Ray-films at −70°C using intensifying screens. The electrophoresis of the extracts of the cultures treated with 3-MQ or guanidine was carried out in two separate gels from the beginning. These gels were incubated for 4 days with the X-ray films.

Fig. 2 shows the results of the control cultures which had been infected with poliovirus, but not treated with 3-MQ. Panels B and C show runs of the extracts collected at times ranging from 0 to 7 h postinfection. In all tracks 18S and 28S ribosomal RNA can be seen. *Hin*dIII-cut lambda DNA was run as a standard between panels B and C. Starting at 5 h postinfection, a band corresponding to the molecular weight of poliovirus became apparent. Panel D of Fig. 2 depicts the autoradiography of the gel in panel C, after hybridization with plus-strand probe. At approximately 3 h postinfection, minus RNA appeared as a high-molecular-weight band which became somewhat smaller with time. Panel A of Fig. 2 shows the autoradiography of the gel in panel B after hybridization with minus-strand probe. Plus-strand RNA (which in control experiments had the same size as the authentic

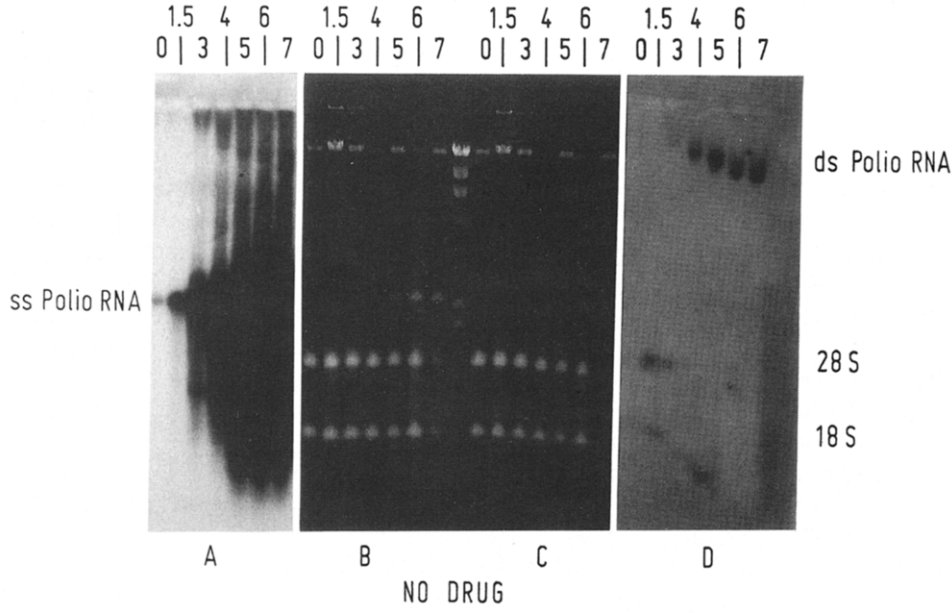

Fig. 2. Kinetics of polio RNA synthesis in BGM cells. Upon infection, lysates of the cultures were made at the times indicated at the top and duplicate samples of the lysates were separated in 1% agarose (panels B and C). The gel was denatured in methylmercury(II)hydroxide, dried, and cut along the standard track into two identical halves. Each half was then hybridized with either minus- or plus-strand RNA. Panel A shows the autoradiography of the gel hybridized with minus-strand RNA, panel D that of the gel hybridized with positive-strand RNA. The extract of the mock-infected cultures, not shown here, but shown in Figs. 3 and 4, did not elicit any radioactive signal.

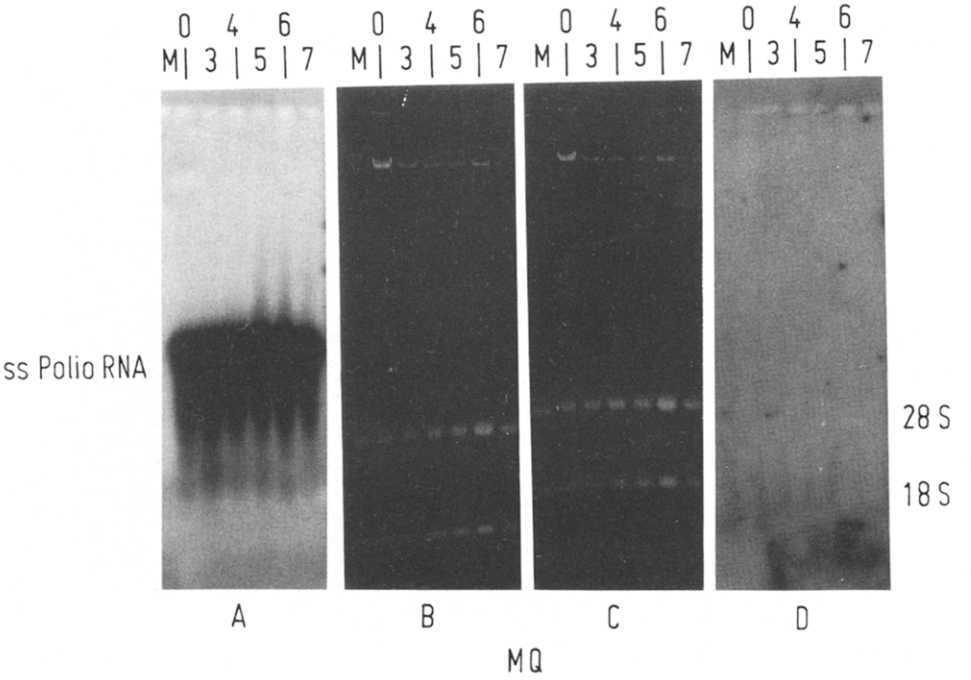

Fig. 3. The same experiment as in Fig. 2 but with cultures containing 3-MQ at 20 μg/ml. M represents the mock-infected culture.

polio genome – data not shown) was always detectable, due to the high inoculum, even at time 0. With increasing time after infection, this genomic size band became stronger, reflecting the ongoing accumulation of viral genomic RNA. At approximately 3 h postinfection a second band became detectable at the same height as in panel D. This material, detectable with the minus- as well as plus-stranded probe, most probably represents the double-stranded replicative intermediate and replicative form of poliovirus. Note that the exposure of the gels to methylmercury(II)hydroxide occurred not during, but after, electrophoresis; if electrophoresis was performed in the presence of methylmercury(II)hydroxide the high-molecular-weight material was not detectable (data not shown).

No minus-strand RNA was detected in the cultures treated with 3-MQ (Fig. 3, panel D), although, due to the high MOI, plus-strand RNA was present and detectable from the very beginning of the infection (Fig. 3, panel A), and remained constant during the experiment. Identical results are shown in Fig. 4 for cultures treated with guanidine. In the mock-infected cultures (track M in Figs. 3 and 4; not shown in Fig. 2) no radioactive signal showed up.

The constant amounts of positive-strand RNA detected in the drug-treated cultures during the experiment are in agreement with the high stability of poliovirus-RNA found in the infected cultures (unpublished results of J.M.L.P.).

From the above results we conclude that 3-MQ inhibits the formation of minus-

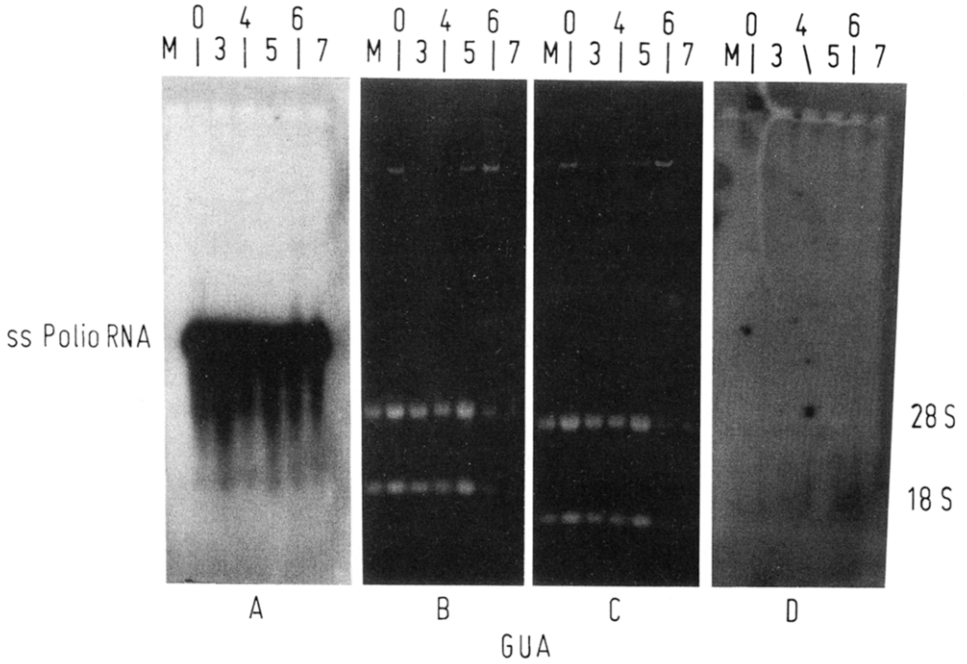

Fig. 4. The same experiment as in Fig. 2 but with cultures containing 2 mM guanidine. M represents the mock-infected culture.

strand RNA if present from the beginning of the infection. Our results contradict the findings of Castrillo and Carrasco (1987), who had observed a substantial residual synthesis of double-stranded viral RNA in cells treated with 3-MQ. While they used HeLa cells for their investigations, we carried out our work with BGM cells. It is conceivable that the cell lines used for carrying out the experiments exert some influence on the action of the drug. If host factors participate in the synthesis of viral RNA, 3-MQ, perhaps by interacting differently with these factors, might display a somewhat distinct mode of action in different cell lines. In this work we used a 60 min pretreatment of the cells with 3-MQ prior to infection. No such pretreatment was carried out by Castrillo and Carrasco. As 3-MQ is very hydrophobic, an adequate pretreatment period might be crucial for achieving optimal inhibition of RNA synthesis. Further studies must be undertaken to answer these questions.

From the evidence gathered by others (Castrillo et al., 1986) and from the present results we conclude that 3-MQ does not exert a strand-specific inhibition but inhibits the synthesis of both minus- and plus-strand RNA.

As shown in this work and as mentioned by others (Kirkegaard and Baltimore, 1986), guanidine also inhibits the formation of minus-strand RNA in vivo. The target of guanidine has been shown to be the polypeptide 2C (Pincus et al., 1986a,b) which is believed to be involved in mediating the binding of the poliovirus repli-

cative complex to the vesicular membranes where poliovirus replication takes place (Bienz, K., D. Bienz-Egger and L. Pasamontes. Abstr. Fifth Meeting of the European Group of Molecular Biology of Picornaviruses, P1.12, Mallorca, Spain, 1987). Apparently the arrangement involving protein-membrane interaction is required not only for the synthesis of plus-strand RNA but also for that of minus-strand RNA. 3-MQ may also interfere with the formation of the membrane-protein complex necessary for RNA synthesis. Alternatively, it may interact with the viral replicates or with one or several host factors participating in viral RNA synthesis. Since the molar quantities required for the action of 3-MQ are considerably less than for guanidine, one is tempted to postulate a rather specific mode of action. However, more detailed knowledge is required, especially regarding the question of whether initiation, elongation, or termination of the RNA chains is affected by 3-MQ.

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