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Action of 3-Methylquercetin on Poliovirus RNA Replication

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3-Methylquercetin is a natural flavone that powerfully blocks poliovirus replication. This compound inhibits selectively poliovirus RNA synthesis both in infected cells and in cell-free systems. Poliovirus double-stranded RNA (replicative forms) is still made in the presence of this inhibitor, whereas the synthesis of single-stranded RNA and the formation of replicative intermediates are drastically blocked.

Poliovirus contains a 7,500-nucleotide RNA genome of positive polarity (12, 17). This genomic RNA carries a 22-amino-acid protein (VPg, also called viral protein 3B) covalently bound to its 5′ end (10, 13). The 3′ end contains a poly(A) tail with an average length of 75 nucleotides (21). Upon infection the parental RNA first must be translated to generate, among other proteins, an RNA-dependent RNA polymerase (viral protein 3D) (4, 9, 14). This polymerase utilizes the genomic RNA as template and makes a copy of minus-strand RNA in vitro when oligo(U) is added as a primer (2, 11, 18). In addition to this viral subunit, other less-well-characterized proteins participate in the replication of viral RNA (5, 14, 23). The poly(A) tail of virion RNA can be copied to oligo(U) by a host factor that possesses uridylyltransferase activity, forming a hairpin structure that can be used in vitro as a primer for the virus-coded replicase (2, 11, 22). Thus, in the presence of host factor, no priming by oligo(U) is required in vitro for the viral polymerase to copy the genomic RNA. Transcription of parental viral RNA yields a negative RNA copy that in turn serves as a template to make more copies of genomic RNA (2, 18). Detailed knowledge of the viral and cellular proteins that participate in these processes is still lacking.

The availability of inhibitors that selectively block viral transcription is of great help in characterizing the different steps that take place in this process. Thus, guanidine, an inhibitor of poliovirus growth, primarily inhibits the synthesis of viral RNA, probably at the initiation step (7, 20). Strikingly, about 75% of the guanidine-resistant mutants analyzed contain a mutated 2C protein (1, 16). Sequencing analyses of these mutants show that they contain one or two amino acid changes within the 2C region. These results suggest that protein 2C might be involved in viral RNA synthesis also. Recently, we described a more powerful and selective inhibitor of poliovirus replication than guanidine: 3-methylquercetin (3-MQ) (8). The addition of this compound from the beginning of poliovirus infection prevented the appearance of viral proteins, although the shutoff of host translation still took place (8). Addition of 3-MQ late in infection did not inhibit viral protein synthesis, whereas viral RNA synthesis was drastically inhibited, suggesting that this process was the target of 3-MQ action (8).

HeLa cells grown as monolayers were infected or mock infected with poliovirus at a multiplicity of infection of 20 PFU per cell. After a 0.5-h absorption, the excess virus was removed, and culture medium containing 2% calf serum plus 10 μg of actinomycin D per ml was added. After 3 h of incubation at 37°C in a 7% CO_2 atmosphere, different concentrations of 3-MQ were added. After 15 min of further incubation the incorporation of [^{3}H]uridine into trichloroacetic acid-precipitable material was measured. 3-MQ had no effect on RNA synthesis in control (uninfected) HeLa cells, whereas 2 μg of 3-MQ per ml blocked by about 50% RNA synthesis resistant to actinomycin D in poliovirus-infected cells (Fig. 1). These results support the conclusion that 3-MQ is a potent and selective inhibitor of RNA synthesis in poliovirus-infected cells. To determine whether this inhibition of RNA synthesis was due to a direct effect of 3-MQ on poliovirus RNA synthesis or whether it was a consequence of indirect inhibition, such as nucleotide metabolism, we prepared a membrane fraction from infected cells by previously described procedures (6, 19). This endogenous membrane-bound RNA replication complex predominantly synthesizes plus-strand RNA (6, 18, 19). HeLa cells were infected with poliovirus type 1 (Moloney strain) at a multiplicity of infection of 50 PFU per cell. Infected HeLa cells were harvested at 4.5 h postinfection, suspended in 10 mM Tris hydrochloride buffer (pH 7.5) containing 10 mM NaCl and 1.5 mM magnesium acetate, and homogenized in 20 strokes in a Dounce homogenizer. The cell lysate was centrifuged at 700 × *g* for 10 min. The supernatant was subsequently centrifuged at 25,000 × *g* for 30 min. The resulting pellet was suspended in 10 mM Tris hydrochloride buffer (pH 8.0) containing 50 mM KCl and 1.5 mM magnesium acetate and used as the membrane fraction as previously described (19). Each RNA synthesis assay was done in a total volume of 50 μl, which contained 10 μl of membrane fraction, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.0), 3 mM $MgCl_2$, 10 mM dithiothreitol, 10 μg of actinomycin D per ml, 1 mM each of ATP, CTP, and UTP, and 10 μM GTP–0.2 mCi of [^{3}H]GTP per ml (Amersham Corp.). Incorporation of [^{3}H]GTP into trichloroacetic acid-precipitable material by this fraction was blocked by 3-MQ (Fig. 2). This reaction was almost totally inhibited by 20 μg of 3-MQ per ml, although some incorporation was still apparent even with these high 3-MQ concentrations. This may represent the synthesis of some form of poliovirus RNA resistant to 3-MQ inhibition. Initiation of plus-strand RNA synthesis might require viral protein VPg, whereas the initiation of minus-strand RNA synthesis requires in vitro an oligo(U) primer (2, 5, 14, 18, 19, 23). Therefore, it is possible that 3-MQ selectively inhibits the synthesis of one or another

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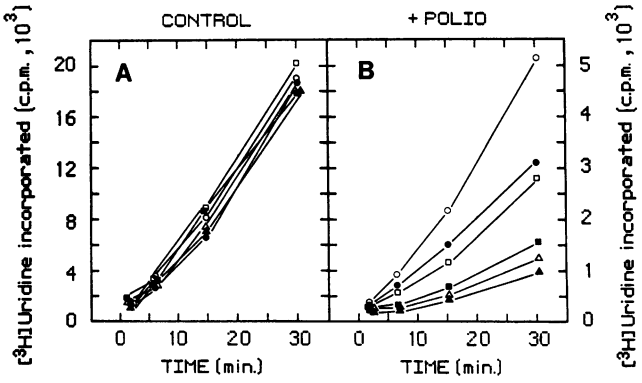

FIG. 1. Kinetics of [^{3}H]uridine incorporation in HeLa cells infected with poliovirus at different concentrations of 3-MQ. (A) RNA synthesis in uninfected HeLa cells. (B) RNA synthesis in poliovirus-infected cells (plus 5 μg of actinomycin D per ml). The concentration of 3-MQ (in micrograms per milliliter) was as follows: 1 (●), 2 (□), 5 (■), 10 (△), or 20 (▲). ○, Untreated cells.

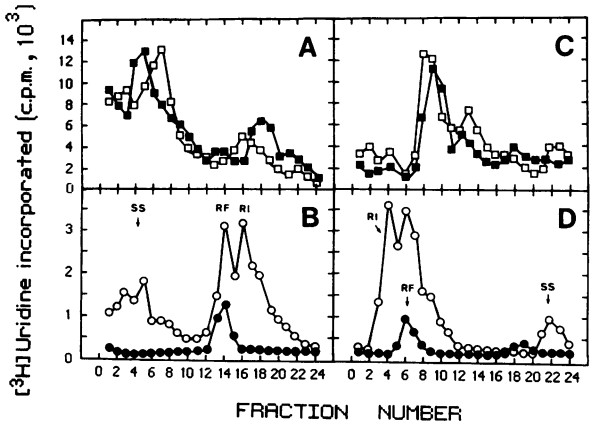

FIG. 3. Sucrose gradients (A and B) and Sepharose-2B chromatography (C and D) of the extracted RNA. (A and C) Uninfected-HeLa cell RNAs. (B and D) Poliovirus RNAs. Symbols: □ and ○, cells not treated with 3-MQ; ■ and ●, cells treated with 3-MQ. Arrows indicate the positions of replicative intermediate RNA (RI), replicative form (RF), and single-stranded RNA (SS).

kind of viral RNA. To better characterize the 3-MQ-resistant fraction, the RNA labeled in mock-infected or poliovirus-infected HeLa cells in the absence or in the presence of 3-MQ was extracted and analyzed by sucrose gradient centrifugation or by Sepharose-2B chromatography (Fig. 3). HeLa cells (two p100 plates) were infected at a multiplicity of infection of 50 PFU per cell in medium containing actinomycin D (5 μg/ml). 3-MQ was added at zero time (20 μg/ml). At 30 min postinfection the culture was incubated with [^{3}H]uridine (50 μCi/ml). The medium was removed 5 h after poliovirus infection, and the cell monolayers were washed with phosphate-saline solution and chilled in Tris-saline; 2 ml of Tris-saline was added per dish. The cells were scraped off with a rubber policeman. After extraction with phenol, the RNAs were collected by centrifugation at 10,000 × *g* for 30 min at 0°C as previously described (15). Sucrose gradient fractionation (Fig. 3A and B) was done on 5 to 25% sucrose–NETS (100 mM NaCl, 2 mM EDTA, 10 mM Tris [pH 7.4], 0.2% sodium dodecyl sulfate). The gradients were centrifuged for 2 h at 40,000 rpm in a Beckman SW50 rotor. Gradient tubes, mixing chambers, and collection apparatus were pretreated with 0.01% diethyl pyrocarbonate to inactivate nucleases. All solutions were treated with 0.01% diethyl pyrocarbonate and autoclaved. Agarose chromatography fractionation was performed (Fig. 3C and D). Sepharose-2B was packed into a 1.3- by 24-cm column at room temperature. The column was equilibrated and eluted as previously described (3). In agreement with our previous results, 3-MQ had no effect on RNA synthesis in uninfected cells. However, this compound blocked the synthesis of poliovirus single-stranded RNA and of replicative intermediates. A peak of radioactivity was clearly apparent in the presence of 3-MQ in the region corresponding to the replicative forms. Treatment of these fractions with 20 μg of RNase A per ml indicated that this peak of radioactivity, tentatively characterized as replicative forms, was resistant to degradation. These results suggest that 3-MQ allows some synthesis of double-stranded RNA, perhaps because synthesis of minus strands can proceed in the presence of 3-MQ.

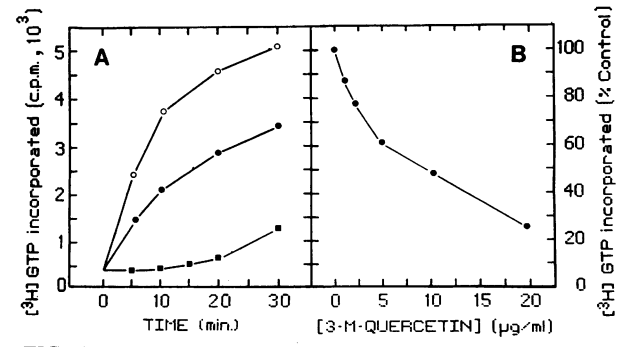

FIG. 2. RNA synthesis in membrane fractions from poliovirus-infected HeLa cells. (A) Kinetics of incorporation of [^{3}H]GTP at 30°C in the membrane fraction. 3-MQ was added at zero time at 2 μg/ml (●) or 20 μg/ml (■). ○, Control without inhibitor. (B) RNA synthesis in membrane fractions at different concentrations of 3-MQ. The samples were incubated at 30°C for 30 min.

The mode of action of 3-MQ on the poliovirus replication cycle can be envisaged as follows. After the parental poliovirus RNA is freed in the cytoplasm, it becomes translated, giving rise to several molecules of the virus-coded replicase that can be used to make a copy of minus-strand RNA on the parental template. These minus-strand RNAs cannot be transcribed to generate more copies of positive polarity in the presence of 3-MQ since this process requires the participation of additional proteins. We speculate that one of these proteins is the target of 3-MQ. Although the action of 3-MQ can be considered similar to that of guanidine, it seems that 3-MQ is more selective, whereas in the presence of 1.5 mM guanidine there is still synthesis of both replicative intermediates and replicative forms (6). In our study, 3 mM guanidine blocked replicative intermediates and replicative forms equally well (results not shown). It is also interesting that poliovirus mutants resistant to 3-MQ are not cross resistant to guanidine (unpublished results). Recently, some guanidine-resistant mutants have been clearly mapped in the region of the genome coding for the 2C protein (1, 16). It remains to be determined where the mutants resistant to 3-MQ map.

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