Inhibition of Foot-and-Mouth Disease Virus Replicase by Frog Virus 3 Particles

By R. BARZILAI AND L. H. LAZARUS
Department of Virology, Hebrew University – Hadassah Medical School, Jerusalem, Israel

SUMMARY
A cell-free system synthesizing FMDV RNA has been shown to be inhibited efficiently by purified FV3 particles. Neither vaccinia nor herpes virus particles produced this effect. The results obtained provide direct evidence that a structural component (or components) of FV3 is responsible for the inhibition.

INTRODUCTION
Frog virus 3 (FV3), a polyhedral cytoplasmic deoxyribovirus isolated from Rana pipiens, is distinct from the herpes-type and the pox virus groups (Granoff, 1969) and resembles structurally the iridescent viruses of insects (Bellett, 1968). One characteristic of FV3 is its ability to inhibit nucleic acid synthesis in many systems at 37 °C, which is a non-permissive temperature for FV3 replication (Granoff, 1969). In addition to its ability to inhibit DNA replication (Maes & Granoff, 1967; McAuslan & Smith, 1968; Guir, Braunwald & Kirn, 1970, 1971) and transcription in host cells (Maes & Granoff, 1967; Constanzo et al. 1970; Guir et al. 1971; Campadelli-Fiume et al. 1972) FV3 also inhibits the synthesis of the DNA and RNA vaccinia virus (Aubertin & Kirn, 1969; Vilagines & McAuslan, 1971), SV40 DNA (Barzilai et al. 1974) and the RNA of several RNA viruses (Guir & Braunwald, 1971; Barzilai & Lachmi, 1973).

Since these effects are observed in the absence of detectable FV3-directed synthesis it is inferred that a structural component(s) of the FV3 particle is responsible for this inhibitory effect (Vilagines & McAuslan, 1970; Guir et al. 1971). The demonstrated nuclease activities associated with FV3 particles which cause the degradation of single- and double-stranded RNA (Kang & McAuslan, 1972; Palese & Koch, 1972) as well as DNA (Kang & McAuslan, 1972; Palese & McAuslan, 1972) may be implicated in the inhibitory effect.

To obtain evidence that a structural component(s) of the FV3 particle is directly involved in the inhibition of at least RNA viruses, we have investigated the effect of purified FV3 particles on the cell-free RNA synthesis of foot-and-mouth disease virus (FMDV) (Lazarus et al. 1972a, b). It has been demonstrated that FMDV is inhibited by superinfection with FV3 (Barzilai & Lachmi, 1973). This communication presents evidence for the direct interaction between FV3 and the synthesis of FMDV RNA.

METHODS
FV3 was obtained from Dr A. Granoff (St Jude’s Children’s Research Hospital, Memphis, Tenn., U.S.A.). FMDV strain SAT/1 was obtained from the Veterinary Institute, Beth-Dagan, Israel. Both viruses were grown and titrated in monolayer cultures of BHK/21 cells (Flow Laboratories, U.K.). This cell line is permissive for both viruses (Maes &
Granoff, 1967; Polatnick & Arlinghaus, 1967). FMDV was purified by sedimentation through viscosity-density gradients (Barzilai, Lazarus & Goldblum, 1972) after concentration from growth medium of infected cultures by the aqueous polymer two-phase system of Norrby & Albertson (1960). FMDV replicase (Arlinghaus & Polatnick, 1967; Polatnick & Arlinghaus, 1967) was prepared according to Lazarus et al. (1972a, b). Enzyme activity was assayed (Lazarus et al. 1972a, b) by the incorporation of [\(^{3}H\)]-UTP (sp. act. 2.02 Ci/mmole; The Radiochemical Centre, Amersham, U.K.) into TCA-precipitable material. The standard reaction mixture (100 \(\mu\)l) contained 1 \(\mu\)mol tris-HCl, pH 8.1, 20 \(\mu\)mol KCl, 2 \(\mu\)mol MgCl\(_{2}\), 80 nmol each ATP, GTP, and CTP, 0.2 \(\mu\)mol 2-mercaptoethanol, 0.2 \(\mu\)mol phosphocreatine, 5 \(\mu\)g creatine phosphokinase, 42 \(\mu\)g protein of FMDV replicase preparation containing endogenous virus RNA template, and 2.5 \(\mu\)Ci of [\(^{3}H\)]-UTP.

FV3 was harvested from the cytoplasm of infected cells. Monolayer cultures of BHK cells were infected with 2 to 5 p.f.u./cell of FV3. After 20 to 24 h at 26 °C, the cells were shaken into the medium and recovered by brief sedimentation. The cell pellets were resuspended in 10 mM-tris-HCl buffer, pH 7.4, containing 10 mM-KCl and 1.5 mM-MgCl\(_{2}\). After swelling for 20 min in an ice bath, the cells were disrupted by 30 strokes of a tight-fitting Dounce homogenizer. Nuclei were discarded after sedimentation at 3000 rev/min for 10 min in a refrigerated centrifuge. The cytoplasmic extracts were then treated with ultrasound for 1 min in the cold, using an MSE sonicator at a wavelength of 8 to 9 \(\mu\)m. Extracts were then layered on to 20 to 40 % (w/w) sucrose gradients in 10 mM-tris-HCl buffer, pH 7.8, 1 mM-EDTA (ET buffer) and 10 % (v/v) glycerol. These gradients were then spun in a Spinco rotor SW 2.5 or SW 25.2 at 24,000 rev/min and 4 °C for 30 min. Virus bands were withdrawn with a syringe through the side of the cellulose nitrate tubes and diluted with two vol. of ET buffer. The virus particles were then pelleted by sedimentation in Spinco rotor type 30 at 27,000 rev/min. Virus pellets were scraped into a small vol. of ET buffer solution, dispersed by 5 to 10 strokes in a Dounce homogenizer, treated by ultrasound and banded in sucrose gradients as before. The amount of FV3 protein was estimated absorptiometrically [2.8 \(E_{260}\) per mg FV3 protein (McAuslan & Smith, 1968)].

RESULTS

Inhibition of FMDV replicase activity by FV3 in a cell-free system

Nuclei-free extracts of cells infected with FMDV incorporate [\(^{3}H\)]-UTP into TCA-precipitable material when supplemented with all nucleoside-triphosphates and an energy-generating system under suitable ionic conditions (Lazarus et al. 1972a, b). A standard reaction mixture yielded about 3000 ct/min in the acid precipitate. A marked decrease in the amount of the radioactivity incorporated was observed when various concentrations of purified FV3 particles were added to the mixture at zero time. The degree of inhibition by FV3 was determined by the virus particle concentration (Fig. 1). The non-linearity of the response may have been due to the factor responsible for non-genetic complementation (Gravell & Cromeans, 1971, 1972) and to the formation of virus aggregates which were readily observed after storage of concentrated preparations.

The observed inhibition may have been caused either by cellular material remaining with the FV3 particles or by a virus component common to FV3 and other viruses. However, we failed to simulate these results with vaccinia virus particles, vaccinia virus cores, herpes simplex virus particles, or cytoplasmic extracts from uninfected BHK cells. Duplicate reactions, with the above materials added at various levels, deviated from control reactions by no more than 4 % with respect to incorporated UMP.
FV3 inhibition of FMDV replicase

Fig. 1. Inhibition of FMDV replicase as a function of the amount of FV3 proteins present. Standard reaction mixtures (final vol. of 100 μl) for the incorporation of [3H]-UTP by FMDV replicase were supplemented with various amounts of purified FV3 particles added at zero time. After 30 min incubation at 38 °C the reactions (in duplicates) were terminated by the addition of TCA and sodium pyrophosphate to final concentration of 10 % and 0.02 M, respectively. Acid precipitates were collected on Whatman GF/C glass fibre filters, washed, dried and counted in a toluene-based scintillation mixture. Zero time incorporation of 125 counts/min has been subtracted from all values.

The kinetics of FMDV-replicase inhibition by FV3

Several kinetic experiments were made on the mechanism by which FV3 particles inhibit replicase activity. Fig. 2a represents the comparison of the normal kinetics of the replicase reaction (without FV3), and the kinetics of reactions to which FV3 particles were added at 1 min or at zero time. Essentially no further change in incorporation took place. In order to corroborate this finding, we added FV3 particles to on-going reactions at various times (Fig. 2b) and allowed an additional 30 min of incubation before termination with TCA; synthesis was inhibited at each and every time by addition of FV3. The results thus obtained were almost identical to those obtained by sequential sampling of the control reaction and suggested that the reaction was stopped rapidly by the added virus particles.

The possible cause of the observed inhibition

The described inhibition of the in vitro [3H]-UTP incorporation may be due to (a) damage to the energy-generating system, (b) introduction of an exonuclease so as to balance the synthesis and degradation of polynucleotide chains, or (c) invalidation of the replicative complex itself. Although FV3 particles exhibit ATPase activity (Vilagines & McAuslan, 1971), we were able to rule out the influence of this by adding FV3 to replicase reaction mixtures to bring about 50 % inhibition. The addition of ATP at 20- to 400-fold excess did
not counteract the inhibition. In contrast, these levels of ATP prevented the inhibition caused by added potato ATPase. Although FV3 particles exhibit no exo-RNAse activity (Palese & Koch, 1972), our preparations may have been contaminated with exonuclease of cellular origin. However, the addition of excess purified RNA (325 μg) failed to reverse the inhibition by FV3. Moreover, after prolonged incubation of labelled RNA with FV3 no soluble material was detected.

Although an ATPase or an exonuclease do not seem to be implicated in the inhibition process, we have obtained preliminary evidence that a proteinaceous substance may inhibit. Purified FV3 particles were heated for 10 min at various temperatures, quenched in an ice bath and then assayed for inhibition of FMDV replicase activity. We found that the inhibitor was sensitive to elevated temperatures (Fig. 3) and was reduced to 50% activity by 10 min at 60 °C and undetectable after 10 min at 70 °C.

**DISCUSSION**

FV3 has been shown to be an inhibitor of several RNA viruses (Barzilai & Lachmi, 1973) under conditions which are non-permissive for FV3 replication or transcription. Thus, a structural component has been implicated in the inhibitory process. By investigating the effect of purified FV3 particles in the cell-free system of Lazarus et al. (1972a, b) for the synthesis of FMDV-RNA, we were able to demonstrate that the active inhibitor was a structural component of FV3.

The thermal inactivation curve may suggest that the active factor is a protein or includes
FV3 inhibition of FMDV replicase

Fig. 3. Thermal sensitivity of the inhibition by FV3. Samples of FV3 particles were heated for 10 min at various temperatures and quickly cooled in an ice slurry. Portions were then added to replicase reaction mixtures (12 µg per 100 µl) and the [H] incorporation determined as above.

4 a protein moiety (Scheraga, 1961). Virus ATPase or some exo-RNAase may not be involved in the observed inhibition of RNA synthesis.

The observed kinetics of the inhibition indicate the disruption of the FMDV RNA replicative complex itself. The endo-ribonuclease associated with FV3 particles (Kang & McAuslan, 1972; Palese & Koch, 1972) may participate in the inhibition by fragmenting the double-stranded replicative form of the FMDV RNA.

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REFERENCES


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