Inhibition of Shope Fibroma Virus Replication by Polyriboinosinic Acid: Polyribocytidylic Acid[1] (34876)

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The production of infectious vaccinia virus and vaccinial hemagglutinin is sensitive to inhibition by interferon (1). Vaccina DNA synthesis is also inhibited by interferon in chick cells (2, 3). Shope fibroma virus (SFV), a DNA-containing poxvirus resembles vaccinia virus in several respects. However, it induces tumors in rabbits. The sensitivity of SFV to interferon has not been investigated. The purpose of this study was to study the sensitivity of SFV replication and DNA synthesis to an interferon inducer, polyriboinosinic acid: polyribocytidylic acid (poly I:poly C) (4, 5).

Materials and Methods. Viral DNA synthesis. Experiments on SFV DNA synthesis were carried out in primary rabbit kidney (PRK) cells. PRK cells were prepared by trypsinization of kidneys of week-old New Zealand white rabbits. They were grown in Falcon plastic flasks (75 cm^2) in medium 199 + 5% inactivated horse serum (medium). Confluent monolayers, formed usually in 1 week, were used.

Effect of poly I:poly C. Dilutions of sterile poly I:poly C (Microbiological Associates, Bethesda, Maryland) were made in medium just prior to use. PRK cultures were incubated with poly I:poly C (0.5 mg/flask) for 11 hr at 37°. Medium was removed prior to infection. The poly I:poly C-treated cultures were washed once and were infected with SFV at a multiplicity of infection (MOI) = 4 cell-infecting units per cell (6). Two control groups were included: one was not pretreated wtih poly I:poly C but infected with the same inoculum of SFV and the other was neither pretreated nor infected. Virus was allowed to adsorb for 2 hr. Unadsorbed virus was washed off and cells were fed with fresh medium. At different times postinfection (pi) two flasks of each group were pulse-labeled for 1 hr by replacing old medium with 1 ml fresh medium containing 5 μCi/ml ^{3}H-thymidine (^{3}H-TdR) (Amersham/Searle). Cells were next washed twice with cold medium, frozen, and processed as soon as possible. Since SFV replicates in the cytoplasm (7), the rate of SFV and host DNA synthesis was measured indirectly by the amount of ^{3}H-TdR incorporated into acid-insoluble material in the cytoplasm or nuclei. The separation of cytoplasm and nuclei was achieved using the method of Vesco and Penman (8): Cells were scraped and suspended in 1 ml hypotonic buffer RSB (0.01 *M* Tris (pH 7.4), 0.01 *M* KCl, 0.0015 *M* $MgCl_2$) and the suspension made 0.05% NP40 (Nonidet, Shell Oil Co.). After stirring vigorously for 30 sec, nuclei were separated from the cytoplasm by centrifuging at 1000*g* for 3 min. Samples of cytoplasm were resuspended in SDS buffer (0.05% SDS, 0.1 *M* NaCl, 0.001 *M* EDTA, .01 *M* Tris, pH 7.4) and precipitated with 5% cold trichloracetic acid (TCA). The precipitate was filtered onto a Millipore filter (23 mm), and the radioactivity was measured in a liquid scintillation counter.

Assay for inclusion body. After SFV infection, cytoplasmic inclusion bodies ("viral factories") could be shown by staining with May–Grünwald–Giemsa stain (MGG) (8). To measure the effect of interferon inducer

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and interferon-like substance (IFLS) on the development of SFV, the appearance of cytoplasmic inclusion bodies was followed. For this purpose, a continuous line of rabbit kidney cells (LLC-RK_1) (6) was used. RK_1 cells were grown on coverslips (10 × 35 mm) in Leighton tubes in medium 199 + 10% horse serum (HS). Identical cultures were divided into two groups: one group was treated with 0.1 mg per tube of poly I:poly C for 5 hr at 37° and then infected with SFV at a MOI = 0.7; the other group was infected with the same dose of SFV without prior treatment. At different times postinfection, two coverslips from each group were removed for MGG staining. The number of cytoplasmic inclusion bodies per 50 microscopic fields were enumerated and compared. Assay of IFLS from the medium was done in essentially the same way.

SFV. An Indiana strain of SFV (laboratory designation, FV-X_2) was propagated by subcutaneous injection in suckling domestic rabbits. Skin tumors were excised about 7 days after infection. Tissues were minced in 10 vol of phosphate-buffered saline, pH 7.2, in a Virtis homogenizer, and the cellular debris was sedimented by low-speed centrifugation. The low-speed supernate was next centrifuged at 17,000 rpm in a Servall centrifuge for 1 hr. The pellet was then resuspended in a volume five times the original weight of the tissues. Virus was ampouled and stored frozen at −100°. Virus infectivity (cell-infecting units) was assayed on RK_1 cells in Leighton tubes according to the methods described previously (6).

Preparation of IFLS. For poly I:poly C induced-IFLS, PRK cultures were exposed to 0.2 mg of poly I:poly C per flask for 1 hr. Unadsorbed poly I:poly C was then removed by washing with 10 ml of medium. Cultures were refed with medium 199 + 10% HS and incubated for 11 hr at 37°. The medium was then saved and assayed for IFLS in RK_1 cells in Leighton tubes.

Results. Poly I:poly C-induced resistance. When PRK cultures were pretreated with 0.5 mg/flask of poly I:poly C and then infected with a MOI of 4, there was little change in the cytoplasmic DNA synthesis throughout the experiment (Fig. 1). In contrast, the nontreated cultures which were infected with the same dose of SFV, had an enhanced cytoplasmic incorporation of ^{3}H-TdR (four to five times that of the poly I:poly C-treated cultures) by 12 hr p.i. However, the cytoplasmic incorporation of ^{3}H-TdR in the uninfected control at 9 and 12 hr pi was greater than that of the poly I:poly C-treated cultures. The reason for that could be due to a slight toxicity of poly I:poly C in the treated cultures. Nevertheless, there was a significant difference in the cytoplasmic DNA synthesis between SFV-infected cultures and the uninfected controls at 12 hr pi.

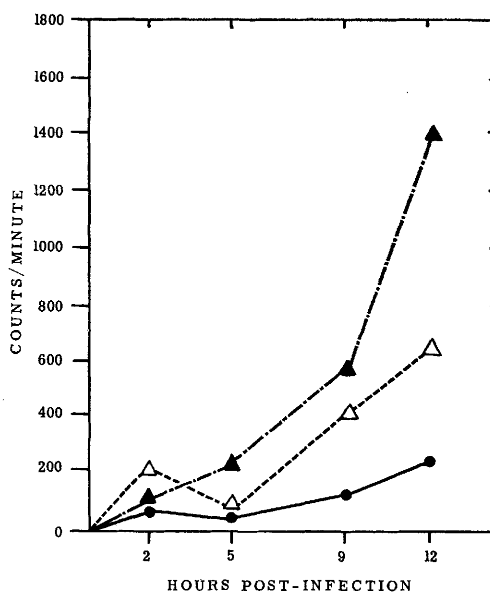

FIG. 1. Effect of poly I:poly C on the cytoplasmic DNA synthesis in SFV-infected PRK cells. Group 1 was preincubated with 0.5 mg of poly I:poly C for 11 hr and then infected with a MOI of 4 of SFV (●—●); group 2 was infected with the same dose of SFV without pretreatment (▲—.—▲) and group 3 was neither pretreated nor infected (Δ---Δ).

Poly I:poly C and the development of cytoplasmic inclusion bodies. Perhaps the most convincing data on the effect of poly I:poly C came from experiments using the inclusion-body assay technique (6). In RK_1 cells infected with SFV, there was a sharp increase in the number of cytoplasmic inclu-

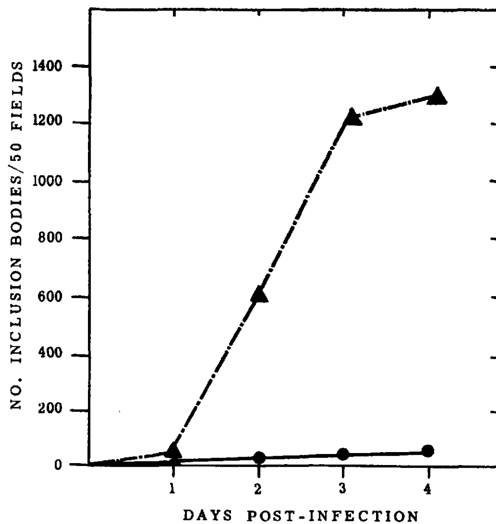

FIG. 2. Effect of poly I:poly C on the development of cytoplasmic inclusion bodies in SFV-infected LLC-RK_1 cells. Identical cultures were divided into two groups: Group 1 was pretreated with 0.1 mg of poly I:poly C for 5 hr and then infected with SFV (●—●); and group 2 was infected with the same dose of SFV without pretreatment with poly I:poly C (▲—.—▲).

sion bodies between Days 2 and 3 pi, as revealed by MGG stain (Fig. 2). In contrast, identical RK_1 cells pretreated with 0.1 mg/tube poly I:poly C had little or no increase in cytoplasmic inclusion bodies, up to Day 4 pi. An IFLS was found in medium of poly I:poly C-treated PRK, which also suppressed the development of inclusion bodies (Table I).

TABLE I. Inhibition of SFV Replication by Interferon-Like Substance (IFLS).

Source of IFLS[a]	Dilution	No. of inclusion bodies[b] per 50 fields at day 4 p.i.
Poly I:poly C medium	Undiluted	93
	1/2	125
	1/4	173
	1/8	1053
Medium[c]	—	1153

[a] See Materials and Methods (preparation of IFLS).

[b] LLC-RK_1 cells were incubated with IFLS for 5 hr at 37° before infecting with the same dose of SFV.

[c] Medium = medium 199 + 10% HS.

Poly I:poly C and cytopathic effects. Three to four days after infection with SFV, cytopathic effects (CPE) could easily be observed in RK_1 cells. Darkly stained aggregates of cells as well as empty areas were seen throughout the monolayer. Cytoplasmic inclusion bodies appeared in most of the cells. In contrast, few cell aggregates, and no empty areas were observed in poly I:poly C-pretreated, SFV-infected cultures. Cells bearing inclusion bodies were seen less frequently than in the infected cultures.

Discussion. In adult rabbits, subcutaneous injection of SFV leads to the formation of a fibroma in approximately 7 days. The tumor then regresses, and the host animal survives. As the result of experiments using immunosuppressive drugs, Allison and Friedman concluded that the regression was brought about by cellular immunity (9, 10).

The role of interferon in the regression of virus-induced fibroma has not been investigated. Indirect evidence exists that SFV is sensitive to interference by other viruses. Andrews (11) provided indirect proof that interference may cause early regression in the rabbit fibroma. He happened to note by chance intranuclear inclusions in the fibroma tumor cells. The inclusions were due to a contaminant of the SFV, virus III, a latent rabbit virus which causes no pathological reaction but is commonly harbored in American domestic rabbits.

Semliki forest virus also interferes with the production and development of SFV in rabbits (12). Recently, Takehara *et al.* (13) also reported the inhibitory effect of certain arboviruses on the development of fibromas in the skin of rabbits. The inhibition could be induced by simultaneous or prior injection of live, heated, or UV-inactivated virus. The authors interpreted the effect as "interference."

Our results indicate that SFV DNA synthesis and replication are sensitive to a nonviral interferon inducer, poly I:poly C. The inhibitor in our experiments resembles interferon in that it is released into the medium by PRK cells long after removal of the inducer.

Many tumor viruses have been shown both

to induce interferon synthesis and to be sensitive to its action (see review by Vilček, (14). It will be of great interest to see whether SFV is also capable of inducing interferon.

Summary. Pretreatment of PRK or RK_1 cells with poly I:poly C suppressed the DNA synthesis and the development of cytoplasmic inclusion bodies of SFV. An interferon-like substance was found in the medium which also inhibited development of SFV inclusion bodies.

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