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*INHIBITION OF CELLULAR RNA SYNTHESIS BY NONREPLICATING VESICULAR STOMATITIS VIRUS**

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Many viruses inhibit synthesis of cellular RNA and other macromolecules. Among the best studied is mengovirus, which rapidly shuts off cellular RNA synthesis in L cells, probably by coding for an early nonstructural viral protein that interferes with the activity of the DNA-dependent RNA polymerase.[1] Poliovirus appears to have a similar effect,[2] but there is some evidence that structural components of the mature virion may also inhibit RNA and protein synthesis in cells infected with massive multiplicities of nonreplicating virus.[3] Cytopathic effects caused by poliovirus cannot be attributed solely to formation of early viral protein, but are better correlated with appearance of capsid antigen later in the replicative cycle.[4] Experiments with adenovirus provide the best evidence for the existence of a cytotoxic viral protein ("E" or "C" antigen) that inhibits cellular macromolecular synthesis in the absence of viral infection.[5] It is also of interest that viruses rendered noninfectious by ultraviolet irradiation still retain their capacity to activate certain cellular properties, such as induction of uncoating protein by poxviruses[6] and interferon formation by myxoviruses,[7] and induction of syncytia formation by Newcastle disease[8] and measles[9] viruses.

We have recently reported that vesicular stomatitis virus (VSV) rapidly inhibits RNA synthesis in Krebs-2 mouse ascites cells.[10] As expected, cellular DNA and protein synthesis is also compromised as are other cellular functions, including the capacity to synthesize interferon.[10] The cytoplasmic membrane subsequently becomes permeable to the dye eosin, and the cells undergo marked metabolic and morphologic changes. Other investigators have shown that VSV is toxic for cells infected at high multiplicities[11, 12] even after viral infectivity has been inactivated by ultraviolet irradiation.[13, 14] The present report extends these observations by demonstrating that replication of VSV is not essential for inhibiting cellular RNA synthesis. The data also suggest, but do not prove, that the toxic activity resides in a structural component of the virion.

Materials and Methods.—Virus-cell system: The large plaque variant of the Indiana serotype of VSV used throughout these studies has been described in detail.[14] Titrations were made by plating virus on monolayers of chick embryo cells and the results expressed as plaque-forming units (PFU). Chick embryo cell cultures were infected with VSV suspensions diluted to 10^{-3} to prepare stocks of high titer ($\sim 2 \times 10^9$ PFU/ml) or with undiluted VSV suspensions to produce stocks of low titer ($\sim 2 \times 10^7$ PFU/ml). As described in a previous report,[10] RNA synthesis was studied in VSV-infected or uninfected Krebs-2 mouse ascites cells suspended in a chemically defined maintenance medium for suspended cells, designated MM-S.[15]

Ultraviolet (UV) irradiation of virus: In 100-mm plastic Petri plates 4-ml suspensions of high-titered VSV stocks were irradiated at room temperature and with gentle rotation under a 15-w General Electric germicidal lamp. The UV dose was 85 ergs mm^{-2} sec^{-1} as measured by a Latarjet dosimeter. Residual infectivity after varying periods of irradiation was measured by plating on chick embryo monolayers. Multiplicities of infection with irradiated virus (VSV_{uv}) refer to original infectivity before irradiation.

Chemicals: Nuclear-Chicago Corp., Des Plaines, Illinois: uridine-5-H^3 (specific activity, 21.5–22.2 c/mmole). Cyclo Chemical Corp., Los Angeles: deoxycytidine and thymidine. Nutritional Biochemical Corp., Cleveland: puromycin dihydrochloride.

Measurement of RNA synthesis: The methods used were previously reported modifications[10] of procedures for uridine-H^3 incorporation and perchloric acid (PCA) precipitation described by Baltimore and Franklin.[16] VSV or medium alone was mixed with 1–2 × 10^7 packed Krebs-2 cells to give a final volume of 1 ml and incubated at 37° for 30 min with intermittent shaking. After virus attachment, the cells were washed twice, suspended in MM-S to a concentration of 10^6 cells/ml, transferred to siliconized glass-stoppered flasks, and incubated with gentle rotation in a water bath maintained at 37°. At intervals thereafter, 4 ml of cells were withdrawn from the incubation flask, centrifuged, and resuspended in MM-S containing uridine-H^3 (1.2–1.8 μc/ml) and 1–2 × 10^{-4} *M* each of deoxycytidine and thymidine. After incorporation of uridine-H^3 for 30 min at 37°, the cells were immediately chilled in an ice-water bath and washed with ice-cold acetate buffer (0.01 *M*, pH 5.1) containing 10^{-4} *M* unlabeled uridine. The cells were then suspended in acetate buffer containing 6 × 10^{-4} *M* unlabeled uridine, divided into two equal parts, and disrupted by ultrasonic vibration. The cellular contents were precipitated with 0.5 *M* cold PCA, washed twice with PCA, and once with ethanol-ether (1:1). The precipitates were dissolved in hyamine, and radioactivity was measured in a Nuclear-Chicago liquid scintillation spectrometer with an average counting efficiency of 28%. Nonspecific adsorption of tritium label on Krebs-2 cells was less than 1% of incorporated counts. Protein concentration was measured by the method of Lowry using bovine serum albumin as a standard. The results of uridine-H^3 incorporation into the cold PCA-precipitable fraction were expressed as cpm/μg protein of VSV-infected cells divided by cpm/μg protein of uninfected cells at each corresponding time point.

Results and Discussion.—Effect of infectious VSV at different multiplicities: RNA synthesis in Krebs-2 cells was inhibited by infection with diluted-passage stocks of VSV at multiplicities ranging from 0.1 to 100 PFU/cell. Figure 1 shows that

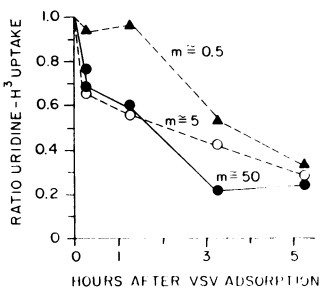

FIG. 1.—RNA synthesis in Krebs-2 cells infected at different multiplicities (*m*) with high-titered VSV stock. The data are recorded as the ratio of uridine-H^3 incorporation by infected cells to incorporation by control uninfected cells determined at each time point. Uptake of uridine-H^3 by uninfected cells during the first 30-min pulse was 65.4 cpm/μg protein. In this and subsequent experiments, time 0 refers to the end of 30-min VSV adsorption, and the results are plotted at the midpoints of the 30-min pulse intervals.

the onset of inhibition depended on the input ratio of VSV. At multiplicities sufficiently high to infect the majority of cells at the end of the adsorption period (time 0), uridine-H^3 uptake was reduced to 75 per cent during the first 30-min pulse interval and gradually declined to 20–25 per cent of the levels in control uninfected cells. When only a small proportion of the cells was infected initially, no significant decrease in total RNA synthesis could be detected until 2–3 hr. Maximal inhibition of RNA synthesis occurred by 5 hr after low-multiplicity infection, presumably owing to cross infection of cells by release of new progeny virus which could be detected 2–3 hr after adsorption. This effect on RNA synthesis could be prevented by

adding antiviral antibody to the medium after low-multiplicity infection. It could also be shown that viral RNA synthesized 1–3 hr after high-multiplicity infection represented only 10 per cent of total cellular RNA.

Effect of UV-irradiated virus (VSV_{uv}) on RNA synthesis: The rapid decline in cellular RNA synthesis after high multiplicity infection suggested that viral replication might not be essential for producing this effect. As one test of this hypothesis, stock VSV was exposed to 25,500 ergs mm^{-2} of UV irradiation, which reduced its infectivity from 10^9 PFU/ml to undetectable levels. Figure 2 reveals that this undiluted preparation (original multiplicity $\cong$ 50) inhibited uridine-H^3 uptake as efficiently as an equivalent amount of fully infectious virus. Early inhibition of RNA synthesis was also evident in Krebs-2 cells infected with VSV_{uv} diluted to give a multiplicity of 5, but the decline was not sustained as was the case with a comparable preparation of infectious virus (10^8 PFU/ml). As expected from the data in Figure 1, a low multiplicity of VSV_{uv} did not impair cellular RNA synthesis during the observation period of 5 hr.

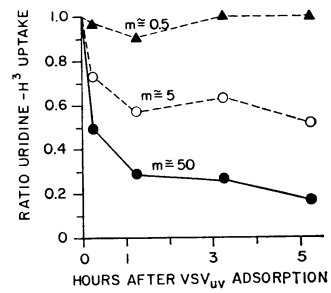

FIG. 2.—Inhibition of RNA synthesis in Krebs-2 cells infected with UV-irradiated VSV at different multiplicities (*m*). See Fig. 1 and *Materials and Methods* for details of procedures. Uridine-H^3 uptake by uninfected control cells at time 0 was 145.4 cpm/μg protein.

This disparity between the effect of UV irradiation on VSV infectivity and on its ability to inhibit cellular RNA synthesis prompted a more detailed comparison of the relative sensitivities to UV irradiation of these two viral functions. Figure 3 shows an exponential decline in infectivity with increasing irradiation up to a dose of 5000 ergs mm^{-2}. No residual infectivity could be detected at higher doses, but the early portion of the curve conforms to first-order kinetics. In sharp contrast,

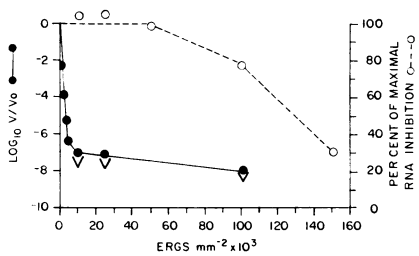

FIG. 3.—Comparative effects of UV-irradiation on VSV infectivity and ability to inhibit cellular RNA synthesis. Infectivity is expressed as the ratio (V/V_0) of residual PFU after increasing doses of irradiation divided by PFU of unirradiated stock virus. The effect of VSV_{uv} on RNA synthesis is expressed as % residual inhibitory activity compared with maximal inhibition produced by unirradiated VSV. RNA synthesis was measured by 30-min incorporation of uridine-H^3 beginning 3 hr after viral infection. Uninfected control cells incorporated 90.8 cpm/μg protein, whereas cells infected with unirradiated VSV incorporated 21.2 cpm/μg protein.

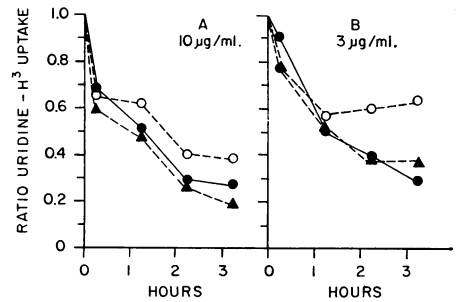

FIG. 4.—Effect of puromycin (10 or 3 μg/ml) on inhibition of cellular RNA synthesis by VSV. Krebs-2 cells were: (1) infected at a multiplicity of 100 with VSV and incubated without puromycin (●—●), (2) infected with VSV and incubated in puromycin-containing MM-S (▲—▲), or (3) not infected and incubated in the presence of puromycin (○—○). The ratios of uridine-H^3 uptake are based on 30-min values at each time point for uninfected control cells incubated without puromycin. Radioactivity of control cells at time 0 in both experiments averaged 118.3 cpm/μg protein.

the capacity of VSV to inhibit cellular RNA synthesis was not affected by a UV dose of 50,000 ergs mm^{-2} and was reduced only 23 per cent by 100,000 ergs mm^{-2}.

These data are open to two interpretations: (1) the noninfectious viral RNA still retains an intact cistron which codes for a protein that is the specific inhibitor of cellular RNA synthesis; (2) no proteins can be synthesized by heavily irradiated viral RNA, and the inhibitor is a preformed part of the penetrating virion. Some estimate of the likelihood of these two alternative hypotheses can be made from the data obtained in the foregoing experiment. By applying the equation for determining lethal hits resulting from incident UV light[17]

$$s = e^{-\phi \cdot QY \cdot q \cdot t},$$

where s is the surviving virus fraction, q is the incident energy, t is time of irradiation, ϕ is absorbed energy, and QY is the fraction of absorbed energy that is lethal, we can calculate for $\phi \cdot QY$ which represents the chance of a lethal hit per incident hit. When $s = e^{-1}$ (37% survivors, derived from the zero term of the Poisson distribution), then $q \cdot t = 307$ ergs mm^{-2} and $\phi \cdot QY = 1/307$ ergs mm^{-2} or 3.3×10^{-3} lethal hits per erg for the VSV population used in our experiments. If we assume that VSV is a typical single-stranded RNA virus[18] containing RNA of a molecular weight of 2×10^6 daltons, it should carry information to code for no more than 20 proteins of 100 amino acids each. If each of the 20 VSV cistrons were equally susceptible and reacted independently to UV irradiation, then 100 randomly distributed lethal hits per virion should inactivate all 20 cistrons with a probability of 99.3 per cent. This degree of inactivation should require only 30,700 ergs mm^{-2}, a dose which had no effect on the capacity of VSV to inhibit cellular RNA synthesis (Fig. 3). Even if we assume that VSV has 50 cistrons, then inactivation of all the cistrons (99.3%) would require a dose of 76,750 ergs mm^{-2}. This amount of UV irradiation only reduced the inhibitory effect of VSV on cellular RNA synthesis by about 10 per cent.

These calculations favor the second of our two hypotheses: that the inhibitor of cellular RNA synthesis is part of the virion rather than being newly synthesized after infection.

Failure of puromycin to reverse inhibition of RNA synthesis caused by VSV: To explore further the possibility that synthesis of new viral protein is required to inhibit cellular RNA synthesis, the effect of puromycin was tested on the inhibitory action of VSV. Unfortunately, in addition to its known action on protein synthesis, puromycin alone also markedly reduced the incorporation of uridine-H^3 into the cold acid-precipitable fraction of Krebs-2 cells. Table 1 shows that increasing concentrations of puromycin progressively inhibited both RNA synthesis in uninfected Krebs-2 cells and VSV growth. Holland[19] reported a similar inhibition of

TABLE 1

INHIBITION BY PUROMYCIN OF RNA SYNTHESIS AND VSV GROWTH IN KREBS-2 CELLS

Puromycin (μg/ml)	Uridine-H^3 uptake* (cpm/μg protein)	VSV yield† (PFU/ml $\times 10^3$)
0	61.7	875
1	59.0	3150
3	49.8	52
5	29.8	20
10	25.1	3
20	—	4

* Uninfected Krebs-2 cells suspended in MM-S containing puromycin and incubated at 37° for 3 hr before and during 30-min incorporation of uridine-H^3.

† Input multiplicity = 27, attachment period of 1 hr at 4°, cells washed twice and incubated at 37° for 6 hr in MM-S with puromycin at concentrations shown.

RNA synthesis, primarily affecting the ribosomal fraction, caused by 100 μg/ml of puromycin.

Figure 4 shows the results of two attempts to reverse VSV inhibition of cellular RNA synthesis with puromycin. At a dose of 10 μg/ml, a concentration sufficient to reduce VSV growth by 99.6 per cent, uridine-H^3 incorporation was slightly but consistently less in cells exposed to VSV or VSV plus puromycin than it was in cells exposed to puromycin alone (Fig. 4*A*). Somewhat greater differences were found when the experiment was repeated with a puromycin dose of 3 μg/ml. Despite 94 per cent reduction in virus yield, no reversal of RNA inhibition could be detected. These data lend additional support to the thesis that replication of VSV is not essential for inhibition of cellular RNA synthesis. However, no conclusions can be drawn concerning the requirement for new viral protein synthesis.

Inhibition of RNA synthesis by undiluted-passage stocks of low infectivity: Cooper and Bellett[11] reported that VSV suspensions prepared by undiluted passage of stock virus are cytotoxic despite their low infectivity. Hackett[20] found a preponderance of small defective virus particles rather than characteristic "bullets" in similar undiluted-passage VSV preparations examined by electron microscopy. These observations suggested still another means to test the postulate that nonreplicating VSV can inhibit cellular RNA synthesis. To this end VSV suspensions were prepared by successive undiluted passages in chick embryo cell cultures starting at an input multiplicity of 250 PFU/cell. The second undiluted passage (VSV_{up}) harvested at 17 hr resulted in a preparation containing only 2.5×10^7 PFU/ml compared with 2×10^9 PFU/ml in regular diluted passage stock (VSV_{dp}).

Figure 5 compares the effects of VSV_{up} and VSV_{dp} on cellular RNA synthesis. Despite its low infectivity (multiplicity $\cong$ 1 PFU/cell), VSV_{up} inhibited uptake of

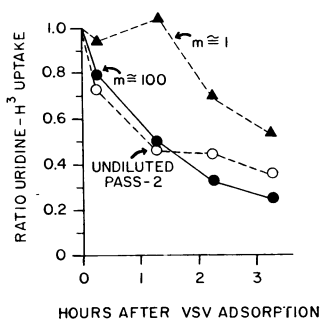

FIG. 5.—Comparative effects of undiluted pass-2 (VSV_{up} of low infectivity) and regular diluted passage stock (VSV_{dp} of high infectivity) on RNA synthesis in Krebs-2 cells. Multiplicities of infection (m) were 1 PFU/cell for VSV_{up} pass-2 compared with $m \cong 100$ or $m \cong 1$ for VSV_{dp}. Ratios of uridine-H^3 uptake were calculated from the radioactivity of uninfected cells similarly pulse-labeled at each time point. The control value for the first 30-min pulse was 111.2 cpm/μg protein.

uridine-H^3 at the same rate and to approximately the same extent as VSV_{dp} (multiplicity $\cong$ 100 PFU/cell). As expected, when Krebs-2 cells were infected with VSV_{dp} diluted 100-fold to give a multiplicity of 1, inhibition of cellular RNA synthesis was appreciably delayed.

These data support the postulate that the replicative form of VSV is not essential for inhibition of cellular RNA synthesis. Experiments now in progress reveal that the infectivity and the cellular RNA inhibitor of purified VSV_{dp} band at the same position after centrifugation in a sucrose density gradient. On the other hand, VSV_{up} is composed predominantly of small noninfectious particles, most of which band in a different position but which also switch off cellular RNA synthesis.

Summary.—Vesicular stomatitis virus was found to inhibit RNA synthesis in Krebs-2 carcinoma cells, even if replication of the virus was prevented by prior ultraviolet irradiation or by puromycin. Defective virus in undiluted passage stocks of low infectivity also retained the property of inhibiting cellular RNA synthesis. The rate and extent of this effect were dependent on multiplicity of infection. These experiments favor the hypothesis that a preformed toxic component of the virion is responsible for inhibition of cellular RNA synthesis, but they do not rule out the possibility of an inhibitory protein newly synthesized on an intact cistron of the infecting viral genome.

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*ON THE MECHANISM OF POLYOMA VIRUS-INDUCED SYNTHESIS OF CELLULAR DNA**

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It has recently been found independently in three laboratories[1–3] that infection with polyoma virus (PV) can induce the synthesis of cellular DNA in contact-inhibited mouse kidney cultures. The present paper presents results of experiments undertaken to elucidate the mechanism of this induction. In these experiments it was found that after PV infection there is an induction of cellular DNA synthesis in X-irradiated mouse and rat embryo cells. Using such a system, experiments were undertaken: (1) to determine the amount of cellular DNA synthesis induced per cell; (2) to test if there is an induction of histone synthesis; (3) to show