Effect of Actinomycin D and Halogenated Deoxyuridines on the Replication of Simian Virus 5.* (30629)

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SV5(1) is a simian virus which belongs to the Newcastle disease—mumps—parainfluenza subgroup of myxoviruses on the basis of both biological(2-4) and morphological(5) properties. Previous studies showed that primary cultures of rhesus monkey kidney cells infected with SV5 continued to produce high yields of virus for long periods of time with little cytopathic effects and without viral interference(4). Evidence has been obtained in the present studies employing actinomycin D and halogenated deoxyuridines that SV5 replication does not require DNA synthesis or DNA-dependent RNA synthesis, but that continued production of virus for many hours is inhibited by actinomycin D presumably through deleterious effects on the host cell.

5-Fluoro-2′-deoxyuridine (FUDR) inhibits DNA synthesis by inhibiting the enzymatic synthesis of thymidylic acid(6); 5-bromo-2′-deoxyuridine (BUDR) and 5-iodo-2′-deoxyuridine (IUDR) are incorporated into DNA (7,8). All three of these halogenated deoxyuridines inhibit DNA virus replication(9-12). Actinomycin D inhibits DNA-dependent RNA synthesis(13,14) and has been shown to inhibit the multiplication of DNA viruses, but not to inhibit several RNA viruses(13, 15-17). However, some RNA viruses have been found to be inhibited by this compound, including reovirus, which contains double-stranded RNA(18), influenza virus(19-21), Rous sarcoma virus(22-25), and, under certain conditions, Newcastle disease virus(21).

Materials and methods. Virus. The W3 strain of SV5 isolated in this laboratory was employed. The procedures used in the propagation and plaque assay of the virus have been described(4).

Cell cultures. Primary cultures of rhesus monkey kidney cells were prepared as previously described(4).

Results. Lack of effect of halogenated deoxyuridines. To determine the effect of the halogenated deoxyuridines on SV5 multiplication, FUDR, (10^{-5} M), IUDR, (10^{-5} M), or BUDR, (10^{-4} M) was added at time of infection and was present throughout the experiment. Cells were inoculated at a multiplicity of 40 plaque-forming units (PFU) per cell in order to obtain infection of all cells approximately simultaneously, and virus was harvested at 28 hours. None of these compounds inhibited the multiplication of SV5; virus yields of 103, 95 and 91% of control values were obtained in the presence of FUDR, IUDR, and BUDR, respectively. Under similar conditions, vaccinia virus, a DNA virus included as a control, was inhibited $>$ 93% by each compound. Thus, DNA synthesis does not appear to be required for the replication of SV5.

Effect of actinomycin D. To investigate the effect of actinomycin on SV5 replication, cells were inoculated at a multiplicity of 40 PFU/cell, and actinomycin was added either at time of inoculation or 2 hours prior to infection and was present throughout the experiment. By 10 hours an average virus yield of 96 PFU/cell is usually produced by untreated cells under these conditions; by 22 hours an average yield of 950 PFU/cell is obtained, due to continued production of virus. As shown in Table I, actinomycin did not reduce the yield of SV5 at 10 hours,

TABLE I. Effect of Actinomycin D on Replication of SV5.

	Time of harvest (hr)	Virus yield, % of control: Actinomycin (μg per ml) 10	1	.1	.01	.001
A.	10	95	98	115	105	107
	22	34	37	54	83	92
B.	22	21	28	38	83	99

Cells were inoculated at a multiplicity of 40 PFU /cell. Actinomycin was added either at time of inoculation (A) or 2 hr prior to inoculation (B), and was present throughout the experiment. Average yield from control cells at 10 hr was 96 PFU/cell, and at 24 hr, 950 PFU/cell.

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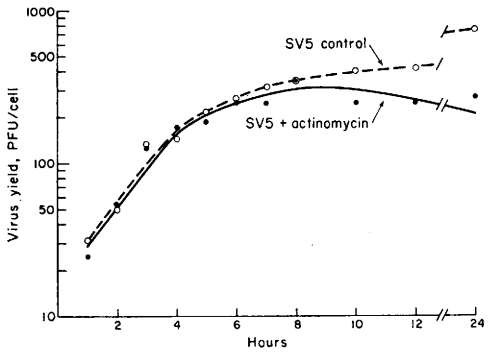

FIG. 1. Effect of addition of actinomycin D on the ongoing replication of SV5 in rhesus monkey kidney cells. Replicate monolayer cultures of cells were inoculated at a multiplicity of 28 PFU/cell 24 hr prior to time 0. At time 0, the medium was removed, the cells were washed twice, and control medium or medium containing actinomycin D, 5 μg/ml, was added. At intervals cells and medium were harvested and total virus assayed.

which suggests that virus-directed biosynthesis is not inhibited by the drug, and that cellular DNA-directed RNA synthesis is not required for virus production during the first 10 hours of viral growth. However, by 22 hours even very low concentrations of actinomycin caused inhibition of virus production. Although the degree of inhibition was moderate, it is significant and reproducible; the values shown are the means of 5-7 experiments. Pre-treatment of cells with actinomycin caused a slightly greater degree of inhibition. In these experiments there was no visible effect of actinomycin on the cells at 10 hours, and none at 22 hours at concentrations below 1 μg/ml; higher concentrations usually did cause some rounding up of cells by 22 hours, involving 20-30% of the cells. If virus yields were determined after longer periods of treatment with actinomycin, greater inhibition was obtained; 0.1 μg/ml caused 76% inhibition at 24 hours, 91% at 48 hours and 97% at 56 hours.

Addition of a very small amount of actinomycin D to the agar overlay in the plaque assay procedure inhibited plaque formation; 0.01 μg/ml caused a 60% reduction in the number of plaques obtained in 5 days. This provides additional evidence that SV5 production is very sensitive to indirect inhibition by actinomycin if the host cells are exposed to the drug for long periods. A possible explanation for this inhibition is suggested by the nature of the SV5 virus-cell interaction. Virus production in rhesus monkey kidney cells is a continuing process(4), and the assembly of myxoviruses occurs at the cell membrane as the virus particle emerges by a mechanism analogous to budding. Thus an intact cell with a membrane functionally capable of continuing this process is required for production of mature virus. It is probable that the inhibition by actinomycin of cellular RNA synthesis, and subsequently of protein synthesis, secondarily interferes with the continued elaboration of virus by the cell.

The ability of SV5-infected rhesus monkey kidney cells to produce SV5 over long periods made it possible to determine the effect of the compound on the ongoing synthesis of virus in cells infected prior to actinomycin treatment. Replicate monolayers of cells were inoculated at a virus multiplicity of 28 PFU/cell, and after 24 hours were washed, and control medium or medium containing actinomycin, 5 μg/ml, was added. At intervals thereafter, cells and medium were harvested and total virus yields assayed. Under these conditions, actinomycin causes > 95% inhibition of RNA synthesis in uninfected cells within 2 hours, as determined by incorporation of H^3-uridine into RNA. As shown in Fig. 1, there was no detectable inhibition of the ongoing SV5 replication until about 10 hours after the addition of actinomycin, which again suggests that cellular DNA-dependent RNA synthesis is necessary only for the continued production of virus for longer periods.

Discussion. Granoff and Kingsbury have suggested previously that the inhibition of Newcastle disease virus by actinomycin which they observed in chick embryo fibroblasts was due to secondary effects on cellular processes rather than direct inhibition of a DNA-dependent step in viral RNA synthesis(21), and the present findings with SV5 are in accord with this interpretation. The characteristics of the inhibitory effects of actinomycin on other RNA viruses vary. The inhibitory effect of actinomycin on influenza virus multiplication(19-21,26) differs from that obtained with SV5; in the growth cycle of influenza a process sensitive to actinomycin D occurs within 2 hours of infection(26). Although the available evidence is compatible with the

view that DNA-dependent RNA synthesis is required for influenza virus replication, the mechanism of actinomycin inhibition of this virus is still unknown(21,26).

Actinomycin inhibits the multiplication of reovirus in L cells(18). Since it also inhibits the enzymatic synthesis of RNA in a cell-free system in which the double-stranded reovirus RNA serves as a template, it is possible that the *in vivo* inhibition of viral replication may be due to interference with the template function of reovirus RNA by actinomycin D(18,27). However, it has also been pointed out that the long growth cycle of reovirus may be a factor in the inhibition in cultured cells(18). Actinomycin also inhibits the multiplication of Rous sarcoma virus when added at the time of infection (22-25), and prevents further virus synthesis when added 12 hours or more after infection (24). As with influenza and reoviruses, the exact mechanism of actinomycin inhibition of Rous sarcoma virus multiplication is not clear. Rous sarcoma virus, unlike SV5, NDV, influenza, and reovirus(10,18,20), is also inhibited by halogenated deoxyuridines, suggesting that DNA synthesis as well as DNA function is necessary for virus growth(24,25,28).

The present results with SV5, as well as those obtained previously with Newcastle disease virus(21), indicate that the replicative mechanism of the RNA of the Newcastle disease—mumps—parainfluenza subgroup of myxoviruses is insensitive to inhibition by actinomycin D. The multiplication of these viruses can, however, be inhibited by the drug under certain circumstances, presumably through deleterious effects on the host cell.

Summary. The replication of simian virus 5 in rhesus monkey kidney cells was not inhibited by 5-fluoro-2′-deoxyuridine, 5-bromo-2′-deoxyuridine, or 5-iodo-2′-deoxyuridine. Actinomycin D did not inhibit viral replication during the first 10 hours of viral growth, and the addition of the drug to cells which were already producing virus did not inhibit continuing virus production until after 10 hours. However, concentrations of actinomycin as low as 0.1 μg/ml significantly reduced the virus yield obtained at 22 hours or later. The results suggest that neither DNA synthesis nor DNA-dependent RNA synthesis is required for the synthesis of the virus, and that the inhibition of the continued production of virus is secondary to effects on the host cell.

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Effect of Diisopropyl Fluorophosphate on Gastric Secretion and Gastric ATPase.* (30630)

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Diisopropyl fluorophosphate (DFP) was first used as an inhibitor of cholinesterase(1). Since that time its inhibitory action has been extended to several other enzyme systems(2). In general, its mechanism of action is to form a diisopropyl phospho derivative of the enzyme, presumably at or near the active site. In many instances this combination has been shown to occur at a serine residue forming a diisopropyl phosphoryl serine derivative(3). Recently, Hokin and Yoda(4) described the inhibition of kidney ATPase (Na^+ and K^+ dependent) due to the phosphorylation of the serine residue. With this in mind, the effect of diisopropyl fluorophosphate on acid secretion and on the gastric ATPase was tested.

Methods. Rana pipiens were decapitated and the stomach was removed. The muscle layer was stripped off and the membrane was mounted into lucite chambers as previously described(5). Potential difference was measured using a vacuum tube voltmeter and short circuit current was defined as the current required to reduce the potential difference to zero after about 2 minutes. Resistance was calculated from the change of potential difference obtained by sending 10 microamps of current in either direction. Acid secretion was measured using the pH stat method of Durbin and Heinz(6). The nutrient solutions contained Na^+ 118 mM, K^+ 4 mM, Mg^{++} 0.8 mM, Ca^{++} 1.7 mM, HCO_3^- 18 mM, Cl^- 109 mM, glucose 10 mM, and the secretory solutions contained Na^+ 105 mM, K^+ 4 mM, and Cl^- 109 mM. For Cl^--free experiments, sulfate was substituted for chloride. DFP was added to the nutrient or secretory side and usually at 2×10^{-2} M final concentration.

Enzyme preparations were obtained by placing mucosae in 0.25 M sucrose, 0.1 M tris-HCl at pH 7.4 and 0°C. After scraping off the mucosal cells with the edge of a glass slide, the standard homogenization and fractionation techniques of Schneider and Hogeboom(7) were carried out and the microsomal fraction was retained for further study. In experiments where the effects of DFP on the intact tissue were studied, the enzyme was prepared from tissue in which DFP had been added on the nutrient or on the secretory side and incubated for 30 minutes or one hour. The protein analysis was performed by Lowry method(8), and enzyme activities were expressed per mg protein. For direct inhibition studies of microsomal enzymes, the microsomal fraction was incubated with 10^{-2} M DFP for varying times and the DFP was removed by centrifugation. A second washing was performed and the enzyme was resuspended in sucrose for assay. The ATPase system contained 3 mM ATP, 3 mM $MgCl_2$, 20 mM tris-HCl buffer pH 8.4; incubations were carried out for 20 minutes at 37° and results expressed as μM Pi/mg protein/hr.

Results. From Table I it can be seen that 2×10^{-2} M DFP within 30 minutes inhibited acid secretion, P.D., short circuit current and increased the resistance of the frog gastric mucosa. In Fig. 1, the time course

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