Effect of 5-Iodo-2′-Deoxyuridine on Multiplication of Rous Sarcoma Virus *in vitro*.* (29378)

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Rous sarcoma virus (RSV) appears to contain ribonucleic acid (RNA), but not deoxyribonucleic acid (DNA)(1,2). Recently, Temin(3) showed that Actinomycin D inhibits production of RSV, a finding which suggested that the growth of the RNA-virus is somehow associated with the synthesis of DNA. The present work indicates that 5-iodo-2′-deoxyuridine (IUdR), an antagonist of DNA synthesis, not only inhibits RSV multiplication in primary cultures of chick embryo fibroblasts, but also appears to suppress the malignant transformation of the infected cells.

Materials and methods. Compound. IUdR was obtained from the California Corporation for Biochemical Research.

Tissue culture. The trunks of 10-day-old White Leghorn chick embryos, from RSV-susceptible flocks, were minced, washed with Scherer's solution and trypsinized with 0.25% trypsin (Difco, 1:250) for 5 minutes at room temperature. Tissue culture flasks (30 ml; Falcon Plastics) were seeded with 15×10^6 cells contained in 5 ml of growth medium, which consisted of 30% medium 199, 60% Scherer's solution, 10% horse serum (heat-inactivated at 56°C for 40 minutes), pH 7.2, and 100 units of penicillin G and 100 μg of streptomycin sulfate per ml. After incubation for 48 hours at 36°C, the cells were washed and refed for use with 5 ml of Scherer's solution (pH 7.2) containing 2.5% horse serum plus antibiotics (S-1).

Virus. Standard stock Rous sarcoma virus, lot CT-929, was kindy supplied by Dr. W. Ray Bryan, National Cancer Institute, Bethesda, Md.

Assay methods. Extracellular fluid of infected cultures was assayed for RSV by titrating 10-fold dilutions of each sample (10^{-1} to 10^{-6}) by subcutaneous inoculation of 0.5 ml into the wing web of 6-day-old White Leghorn chicks. Five chicks per dilution were employed. In these experiments, the TID_{50}, as calculated by the method of Reed and Muench(4), is that dilution of Rous virus or RSV-infected cells capable of inducing tumors in 50% of the chickens within 14 days after infection.

For cell-associated virus assay, the cell sheet was washed once with S-1 and then treated with 2 ml of 0.25% trypsin in S-1 (pH 8.0) for 5 minutes at 37°C in order to release cell-bound virus particles. The contents of triplicate test flasks were pooled and centrifuged at $900 \times g$ for 10 minutes at 4°C. The supernatant fluid was titrated (TID_{50}) in chicks for cell-associated virus content.

Cells were washed twice then resuspended in S-1 and the cell count was adjusted to 5×10^6/ml. Individual cell suspensions were either diluted in S-1 and injected directly into chicks for whole cell titrations or were subjected to 2 cycles of freezing and thawing (—70°C to 37°C) to liberate intracellular virus for assay. Cellular debris was removed by centrifugation at $900 \times g$ for 10 minutes at 4°C. The cell-free supernatant fluid was diluted in S-1 and titrated in chicks. Similar treatment of stock RSV does not significantly affect its titer (TID_{50}).

Experimental results. Effect of IUdR on RSV multiplication in vitro. In 3 separate experiments, a series of duplicate 48-hour-old primary cultures of chick embryo cells were washed and refed with 5 ml of S-1 containing RSV (32,000 TID_{50}) and IUdR in 4-fold variations in concentration of from 1 to 256 μg/ml. IUdR at 256 μg/ml has no detrimental effect on cell number or microscopic appearance during a 96-hour period of maintenance. The infecting dose of RSV did not produce any cytopathogenic effects (CPE) in the chick fibroblasts.

Cultures were assayed at 96 hours, since

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TABLE I. Effect of 5-Iodo-2'-deoxyuridine (IUdR) on RSV Multiplication *in vitro*.

Virus sample	IUdR dose (μg/ml)	Virus titer (TID_{50}*/0.5 ml)	Log reduction
Extracellular	256	$<10^{-0}$	2.5
	64	$10^{-1.0}$	1.5
	16	$10^{-1.0}$	1.5
	4	$10^{-2.5}$	0
	1	$10^{-2.5}$	0
	Control	$10^{-2.5}$	—
Cell-associated	256	$<10^{-0}$	3.0
	64	$10^{-1.0}$	2.0
	16	$10^{-1.5}$	1.5
	4	$10^{-2.0}$	1.0
	1	$10^{-3.5}$	0
	Control	$10^{-3.0}$	—
Intracellular	256	$<10^{-0}$	4.0
	64	$10^{-0.5}$	3.5
	16	$10^{-2.0}$	2.0
	4	$10^{-2.0}$	2.0
	1	$10^{-3.5}$	—
	Control	$10^{-4.0}$	—

* Highest dilution of virus capable of inducing tumors in 50% of chicks 14 days after infection. Presence of nodules is considered as a positive indication of tumor formation.

neoplastic changes have been observed at this time in tissue culture after infection with RSV(5). It has been shown also that virus titers reach a maximum level at 4-5 days(6).

The results in Table I indicate that, in cultures treated with IUdR at 256 μg/ml, no extracellular or cell-associated virus was detected on assay at 96 hours after infection, whereas titers of $10^{2.5}$ and $10^{3.0}$, respectively, were reached in untreated controls. At concentrations of IUdR of 64 and 16 μg/ml, titer reductions of 1.5 to 2 logs were noted, as compared with the controls. Extracellular virus titers for cultures treated with 4 μg of IUdR per ml were equivalent to controls, while the cell-associated titer was reduced by 1.0 log. Viral titers did not appear to be inhibited by IUdR at 1 μg/ml.

Production of intracellular virus in IUdR-treated cells was inhibited by concentrations of 4 μg/ml or greater; the degree of inhibition was directly related to the concentration of drug. Little or no RSV was detected in cells treated with 64 and 256 μg/ml of IUdR, while virus titers were reduced 2 logs at levels of 16 and 4 μg/ml, as compared with the virus controls. No inhibition was noted with IUdR at 1 μg/ml.

It was concluded that IUdR, added to chick fibroblast cultures at time of infection, inhibits RSV multiplication.

Effect of delayed IUdR treatment on RSV multiplication. As an extension of the above findings, a study was made of the degree of virus inhibition when treatment with IUdR was initiated at various times following infection. In this experiment, medium was removed from triplicate chick embryo cell cultures at 24, 48 and 72 hours after RSV infection and replaced with an equal volume of S-1 containing IUdR at 256 and 64 μg/ml. Separate virus controls, refed with medium only, were included with each test group. Extracellular and intracellular virus assays were made 96 hours after infection in all test cases, using the chick wing web titration technique previously described.

As Table II shows, IUdR inhibits RSV multiplication only when administered to infected cells at time of infection.

Effect of IUdR on malignant transformation of cells. An attempt was made to evaluate the suppressive effect of IUdR on the neoplastic properties and morphological transformation of RSV-infected chick embryo fibroblasts *in vitro*. In a series of 3 experi-

TABLE II. Effect of Delayed IUdR Treatment on RSV Multiplication *in vitro*.

Hr infection before treatment	Virus titer (TID_{50}*/0.5 ml)—96 hr post-infection					
	Extracellular			Intracellular		
	IUdR, μg/ml		Virus control	IUdR, μg/ml		Virus control
	256	64		256	64	
0	$<10^{-0}$	$10^{-1.0}$	$10^{-2.5}$	$<10^{-0}$	$10^{-1.5}$	$10^{-4.0}$
24	$10^{-3.0}$	$10^{-3.0}$	$10^{-3.0}$	$10^{-3.0}$	$10^{-3.5}$	$10^{-3.0}$
48	$10^{-2.5}$	$10^{-3.0}$	$10^{-2.5}$	$10^{-2.5}$	$10^{-3.5}$	$10^{-3.5}$
72	$10^{-3.0}$	$10^{-2.5}$	$10^{-3.0}$	$10^{-3.5}$	$10^{-4.5}$	$10^{-3.5}$

* Highest dilution of virus capable of inducing tumors in 50% of the chicks within 14 days after infection.

TABLE III. Effect of IUdR on Tumor-Inducing Capacity of RSV-Infected Whole Cells Compared with Intracellular Virus.

Sample	*In vitro* IUdR dose (μg/ml)	*In vitro* material (TID_{50}/0.5 ml)*	Latent period (days)†	Avg tumor wt (g)‡	Infectivity titer of tumor extract (TID_{50}/0.5 ml)§
Intracellular virus	256	$<10^{-0}$	>14	0	—
	64	$10^{-0.5}$	12	1.0	—
	16	$10^{-2.0}$	8	8.0	—
	4	$10^{-2.0}$	7	11.0	—
	1	$10^{-3.5}$	6	14.0	—
	Virus controls	$10^{-3.5}$	6	15.0	—
Whole cells	256	$10^{-1.5}$	9	6.0	$<10^{-0}$
	64	$10^{-1.5}$	8	6.0	$10^{-1.5}$
	16	$10^{-2.5}$	7	13.0	$10^{-3.5}$
	4	$10^{-3.5}$	7	19.0	$10^{-4.0}$
	1	$10^{-4.5}$	6	25.0	$10^{-4.0}$
	Virus controls	$10^{-4.5}$	5	19.0	$10^{-4.5}$

* Highest dilution of whole cells or intracellular virus capable of inducing tumors in 50% of the chicks within 14 days after infection.

† Day on which 50% of chicks developed tumors after infection with either undiluted whole cells (5×10^6/ml) or intracellular virus of these cells.

‡ Average grams of tumor weight 14 days after inoculation with either undiluted whole cells (5×10^6/ml) or intracellular virus.

§ Intracellular virus titer—refer to (*).

ments, 48-hr-old primary cultures of chick fibroblasts were infected with RSV (32,000 TID_{50}); IUdR was added immediately at varying 4-fold concentrations of 1 to 256 μg/ml. Four days after infection the cultures were examined microscopically for cellular morphology and assayed for intracellular virus. Whole cells were harvested from duplicate cultures and titrated in 5-day-old chicks.

Evidence has already been presented that cells infected *in vitro* are malignant and capable of producing tumors when injected into young chicks. The latent period, *i.e.*, the time it takes for a tumor to develop, was shorter after inoculation of whole cells into a chick than after inoculation of the intracellular virus alone(7,8). The decreased time difference was attributed to tumor induction by malignant cells.

The data presented in Table III indicate that the latent period after inoculation of equivalent numbers of whole cells of either RSV controls or IUdR-treated chick embryo cell cultures ranged from 5 to 9 days, as compared to 6 to more than 14 days after injection of the intracellular virus. The generally greater tumor induction titers (TID_{50}) and average tumor weights obtained with cell suspensions seem to bear out the theory that cells pre-transformed *in vitro* promote a more rapid tumor formation.

IUdR caused significant reductions in the whole cell titers (TID_{50}), which were related to drug dose. The decreased tumor weights and extended latent periods noted with the treated whole cell infection indicated that IUdR, particularly at 256 and 64 μg/ml, suppressed the number of chick fibroblast cells transformed to sarcoma cells.

All tumors that developed in the chick wing web after inoculation of RSV-infected whole cells treated with IUdR at 256 μg/ml were harvested and assayed for their intracellular virus content; RSV was not found. It seems reasonable to consider that the chick tumors consisted of converted, non-virus-producing cells(9).

Upon microscopic examination 96 hours after infection, neither IUdr nor virus control cultures evidenced altered morphology. The results of this and other studies(9,10) would seem to indicate that the acquisition of neoplastic properties by infected cells, their ability to produce RSV and morphological transformation are not necessarily associated.

Discussion. Evidence has been presented which indicates that IUdR, an antagonist of DNA synthesis, inhibits the replication of

the RNA-containing Rous sarcoma virus in 48-hour-old primary cultures of chick fibroblast cells. The present data with IUdR indicate, as did Temin's findings with Actinomycin D, that replication of RSV is somehow associated with DNA-synthesis. The mode of IUdR action in the described test system is not yet known.

Interpretation of data is complicated by the fact that standard stock preparations of RSV, and presumably ours, contain Rous associated virus (RAV) which is serologically identical with RSV(11,12). Although DNA was shown to increase in RAV-infected chick fibroblast cells, there is no definite information on whether this virus is a DNA virus (13). RAV is known to assist in the multiplication of RSV in transformed cells, while RSV itself causes malignant transformation, but no production of complete virus(14), so that RSV may be considered an incomplete or defective virus particle.

The data indicate that IUdR does inhibit RSV; for example, no tumors formed after inoculation of the extracellular or intracellular fluids from IUdR-treated (256 μg/ml) cells. It can be assumed that IUdR-treatment of infected cells prevented the production of tumor-inducing RSV particles. Because of the inter-relationship, RSV multiplication could presumably be affected by drug action on RAV. No attempt was made, however, to assay the stock virus or cell cultures specifically for incomplete and complete RSV or RAV. Consequently, it cannot be determined whether RAV, if present, was affected by IUdR.

The ability of IUdR to suppress the malignant transformation of chick fibroblast cells infected with RSV is assumed to reflect its activity as an inhibitor of the replication of RSV (tumor-inducing) particles.

Summary. IUdR, added to primary chick fibroblast cultures at the time of RSV infection, not only inhibits virus multiplication but also appears to suppress the malignant transformation of infected cells.

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Effects of Angiotensin on the Toad Skin. (29379)

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Whereas angiotensin generally produces a decrease in urine volume and in sodium excretion, it occasionally produces an increase in both. The mechanism responsible for these changes may entail a direct action of angiotensin on the renal vasculature(1) or on the tubule to inhibit the diffusion of water and/or the transport of sodium(2,3). Layssac *et al*(4) found that angiotensin produced an inhibition of sodium transport in isolated kidney slices, and proposed that angiotensin is concerned with a renal auto-regulatory

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