Inhibition of Vesicular Stomatitis Virus Replication by Poly(2'-Fluoro-2'-Deoxyuridylic Acid)

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Poly(2'-fluoro-2'-deoxyuridylic acid) is known to be an effective inhibitor of the deoxyribonucleic acid polymerase found within the oncornaviruses. This synthetic polynucleotide was found to inhibit the replication of vesicular stomatitis virus in mouse L cells. The polymer was shown to be capable of inhibiting the viral ribonucleic acid (RNA)-dependent RNA polymerase, and it is proposed that this is the mechanism of antiviral activity. The following observations support this viewpoint: (i) the polymer is most active when added after virus adsorption; (ii) the antiviral activity is not species specific; and (iii) the polynucleotide is nontoxic to the host cell. Conventional methodologies designed to increase nucleic acid uptake by cultured cells do not show an increase in antiviral potency.

The antiviral properties of synthetic polynucleotides have, in the past, been associated with either their ability to induce interferon (23) or to enhance the immune response against viral antigens (3). Recently, a more direct mechanism of antiviral activity has been attributed to this class of polymers by the demonstration that certain single-stranded polynucleotides can block the activity of virus-associated nucleic acid polymerases (8, 16, 17, 22). The majority of these data was obtained in studies utilizing the ribonucleic acid (RNA)-dependent deoxyribonucleic acid (DNA) polymerase of the oncornaviruses and indicated that the inhibitory polynucleotides blocked the association of enzyme and active nucleic acid template (7). Although the extension of these enzyme analyses to cell culture (16, 19, 21) and in vivo virus inhibition studies (20) did demonstrate significant antiviral activity, the actual mechanism of inhibition remains unclear. The replication of the oncornaviruses is a complex process that is strongly dependent on host cell metabolism (18), and mechanistic studies involving these viruses may remain difficult and equivocal.

Efforts to find a less complex virus-host cell system in which to study the direct inhibitory activity of the single-stranded polymers demonstrated that the replication of vesicular stomatitis (VS) virus in mouse L-cell culture was sensitive to poly(2'-fluoro-2'-deoxyuridylic acid) [(dUfl)_n], a potent inhibitor of the avian myeloblastosis virus RNA-dependent DNA polymerase (8). This system appeared to be amenable to our studies since VS virus carries a viral-specific, RNA-dependent RNA polymerase, viral

replication is relatively independent of host cell DNA and RNA synthesis, and (dUfl)_n was found to be nontoxic to the host cells. The present study demonstrates that the VS virus RNA polymerase is sensitive to (dUfl)_n inhibition and the polynucleotide is active when administered postadsorption and presents data which suggest that the inhibition is not mediated by interferon.

(A preliminary report on these investigations was presented at the Fourteenth Interscience Conference on Antimicrobial Agents and Chemotherapy at San Francisco, 11 to 13 September 1974.)

MATERIALS AND METHODS

Chemicals and radiochemicals. Unlabeled (dUfl)_n was prepared as previously described (13). The labeled polymer was synthesized by phosphorylation and polymerization of the nucleoside, which had been tritiated by New England Nuclear Corp. Nucleoside triphosphates were purchased from Pabst Laboratories; [³H]uridine, [³H]thymidine, and [³H]adenosine 5'-triphosphate were products of Schwarz/Mann. Diethylaminoethyl-dextran (molecular weight, ca. 5 × 10⁶) was obtained from Pharmacia, Triton X-100 was from Packard, and (A)_n was a product of the Research Products Department of Miles Laboratories.

Cells and viruses. Mouse L cells (strain LVD) and hamster BHK cells (strain BHK-21) were grown in Eagle minimal essential medium (Grand Island Biologicals) supplemented with 10% fetal calf serum (Colorado Serum). VS virus (Indiana strain) stock preparations were prepared by infecting monolayers of LVD cells with dilutions of unpurified virus (0.1 plaque-forming units [PFU]/cell) and harvesting the virions from the culture medium by centrifugation

at $54,000 \times g$ for 90 min. The pellet was resuspended in TKMD buffer [0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.4; 0.01 M KCl; 0.0015 M MgSO_4 ; and 0.001 M dithiothreitol]. Dispersion of viral aggregates was facilitated by sonication, and the sample was then layered on a linear (5 to 40%) sucrose gradient, the sucrose being dissolved in TKMD buffer. The preformed 26-ml gradients were placed in an SW25.1 Spinco rotor and centrifuged at 17,500 rpm for 3 h at 4 C. The highly infectious bottom (B) band of virus (12) was removed and diluted in TKMD buffer, and PFU per milliliter were determined. These purified B particles were then used to reinfect monolayers of LVD cells at a multiplicity of infection of 0.01 PFU/cell, and the virus was again purified as described above. This second purification yielded a single prominent B band upon gradient centrifugation. Virus samples to be utilized for infectivity and inhibition studies were stored in Eagle minimal essential medium containing 10% calf serum at -20°C , whereas virus preparations to be used in the transcriptase assay were stored at 4°C or used immediately.

Virus assays. In the inhibition studies to be described, the host cells were added to treated plastic dishes (15 by 60 mm; Lux) at a concentration of 10^6 cells/dish. The cells were incubated in a 5% CO_2 humidified atmosphere at 37°C and used after 18 h. The cells were growing and not confluent at the time of the experiment. Virus was added in a volume of 0.5 ml, and 45 to 60 min were allowed for virus adsorption and penetration. Nonadsorbed material was removed, and the experimental protocol was initiated. Virus released into the medium at defined intervals was assayed for PFU per milliliter on confluent monolayers as described (12), using crystal violet staining to help delineate plaques.

RNA-dependent RNA polymerase assay. Purified B particles were solubilized in a solution of Triton X-100 (1.87%), dithiothreitol (6×10^{-4} M), glycerol (9.4%), and NaCl (0.72 M). After 45 min of incubation at 30°C , the preparation was diluted by the addition of 1.5 volumes of TKMD buffer. This procedure was as described previously (6), except the solutions were not separated into soluble and particulate fractions. The diluted solubilized virus preparation was then mixed with an equal volume of RNA polymerase reaction mixture (6) which contained tris(hydroxymethyl)aminomethane-hydrochloride, 5×10^{-2} M, pH 7.4; $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$, 8×10^{-3} M; dithiothreitol, 6.5×10^{-3} M; cytidine 5'-triphosphate, uridine 5'-triphosphate, and guanosine 5'-triphosphate, all 1.4×10^{-3} M; and adenosine 5'-triphosphate, 2.8×10^{-4} M. The labeled substrate, [^3H]adenosine 5'-triphosphate, was added at a level of 35 $\mu\text{Ci}/\text{ml}$, which gave a specific activity in our experiments of 55 counts/min per pmol of adenosine 5'-triphosphate. The extent of polymerization was assessed by precipitating the acid-soluble product in 10 volumes of a mixture of trichloroacetic acid (100%-saturated NaH_2PO_4 -saturated $\text{Na}_4\text{P}_2\text{O}_7$ in a volume ratio of 1:1:1 at 4°C . The precipitate was collected on nitrocellulose filters, washed with cold 5% trichloroacetic acid, and dried, and radioactivity was quantitated in a liquid scintillation spectrometer.

Assay for polynucleotide degradation. The [^3H](dUfl) $_n$ was prepared at a specific activity of 3.4×10^5 counts/min per μg and mixed with unlabeled polymer to a final specific activity of 7.0×10^4 counts/min per μg . The [^3H](dUfl) $_n$ was added to the cells at the desired concentration in 2 ml of Eagle minimal essential medium in a plastic dish (60 by 15 mm). At various times the medium was removed from the cells, and both medium and cells were assayed for acid-precipitable isotope. The medium was assayed by simply precipitating material from 0.05 ml of medium using the acid solution described above. Cell-associated nucleic acid was extracted by the procedure of Marmur (14), and the liquid phase of the first Sevag test was assayed by acid precipitation as above. This procedure is specific for polymerized (dUfl) $_n$, since the [^3H]nucleotide has been found not to be incorporated in tissue culture cells (B. Janik, unpublished data).

RESULTS

Inhibition of VS virus replication in L cells. The growth of VS virus in L cells is a rapid, reproducible process that, using our conditions of growth and assay, is illustrated in Fig. 1. Since our purpose for utilizing this virus-cell system was to present data indicating the intracellular inhibition of viral replication, our inhibitor, (dUfl) $_n$, should neither interfere with virus adsorption and penetration nor with the processes of virus release. With respect to the growth curve shown in Fig. 1, such an inhibitor would not alter the eclipse phase (e.g., increasing the PFU per milliliter by blocking

FIG. 1. Replication of VS virus in L cells. Monolayers of L cells growing in 60-mm plastic petri dishes in 5% CO_2 were infected with VS virus at a multiplicity of infection of 0.5. After a 45-min adsorption period, the cells were rinsed two times with growth medium and incubated in growth medium. The growth of VS virus was measured extracellularly by removing and pooling the growth medium from duplicate dishes at various times postinfection.

penetration), and the degree of inhibition should be relatively constant during both the rise phase of rapid virus production and the plateau region (e.g., virus infection has been aborted and not simply delayed in virus release).

The inhibition by $(dUfl)_n$ of VS virus production was characterized as regards these parameters in the experiments summarized in Table 1. The cells were treated with the drug by two regimens. In treatment A the cells were exposed to the virus at a low multiplicity of infection (4×10^{-3} PFU/cell) for 90 min and then $(dUfl)_n$ was added at a level of 50 $\mu\text{g}/\text{ml}$. In treatment B the cells were pre-exposed to 50 μg of $(dUfl)_n$ per ml for 60 min prior to virus challenge, and the polymer was added back to the cells after the 90-min adsorption period. The results show that the virus titer during eclipse phase (2- and 3-h samples) was not increased by the presence of the drug and that the degree of inhibition was relatively constant at both the rise phase (7 h) and plateau (23 h). It is also significant that in treatment A, in which the $(dUfl)_n$ was added after virus adsorption, the extent of inhibition was slightly higher than using regimen B. This result strengthens the hypothesis that $(dUfl)_n$ interferes with postadsorption events.

Inhibition of VS virus RNA-dependent RNA polymerase. If this system is to be analogous to that reported for the oncornaviruses (8, 16, 19, 21), then $(dUfl)_n$ should inhibit the activity of the virus-encapsulated nucleic acid polymerase. Purified B particles were solubilized by the high salt method of Emerson and Wagner (6) and assayed for RNA polymerase activity using the endogenous viral genome as template. The polymerization of [^3H]adenosine 5'-monophosphate proved linear for 120 min (Fig. 2), and this incorporation of label was blocked by the addition of pancreatic ribonuclease

FIG. 2. Inhibition of VS virus RNA-dependent RNA polymerase activity by poly($dUfl$). The reaction mixture is described in Materials and Methods. A 40- μl volume of the solubilized virus preparation was used in a total volume of 100- μl reaction. The reactions were incubated at 33 C. Symbols: ○, RNA polymerase activity of VS virus preparation; ●, RNA polymerase activity of VS virus preparation with 100 μg of $(dUfl)_n$ per ml added; Δ, RNA polymerase activity of VS virus preparation with 50 μg of pancreatic RNase per ml added.

(RNase). The inclusion of $(dUfl)_n$ in the assay mixture reduced the level of synthesis to that found in the RNase-treated samples and, hence, $(dUfl)_n$ can block the RNA-dependent RNA polymerase found in B particles of VS virus (Fig. 2).

Noninvolvement of interferon. The role of interferon in the virus inhibition studies outlined in Table 1 is doubtful in view of the facts that $(dUfl)_n$ and $(dUfl)_n \cdot (A)_n$ are poor interferon inducers (4) and that L cells do not form significant levels of interferon in response to polynucleotides in the absence of diethylaminoethyl-dextran (5). To add additional support to the noninvolvement of interferon in the $(dUfl)_n$ -mediated VS virus inhibition, the virus inhibition studies were carried out in a prescribed manner in mouse L cells, but the virus was titrated on both the L cells and hamster BHK-21 cells. Since interferon is known to be species specific, an interferon-induced resistance to viral infection would not be evident when assayed on the BHK-monolayers. The data presented in Table 2 show a reduction in virus titer in the samples grown in the presence of $(dUfl)_n$ when assayed on both the mouse and hamster cells. This inhibition is again observed in samples exposed to $(dUfl)_n$ after virus adsorption and at various multiplicities of infection.

TABLE 1. Effect of $(dUfl)_n$ on VS virus replication in L cells

Time after virus adsorption (h)	Virus titer (PFU/ml)		
	Control ^a	Treatment A ^a	Treatment B ^b
2	10	0	17
3	40	0	4
5	160	2	20
7	2.2×10^4	1.2×10^3	6.0×10^3
23	3.4×10^7	9.0×10^6	1.5×10^7

^a $(dUfl)_n$ (50 $\mu\text{g}/\text{ml}$) was added after a virus adsorption period of 90 min.

^b $(dUfl)_n$ (50 $\mu\text{g}/\text{ml}$) was added 60 min prior to the 90-min virus adsorption period and also was included in the postadsorption growth medium.

Nontoxicity of (dUfl)_n to L cells. Although the production of VS virus is not affected by inhibitors of host cell RNA and DNA synthesis such as actinomycin and cytosine arabinoside (2), (dUfl)_n could induce toxic alterations in host cell metabolism that might be reflected in decreased virus titer. To demonstrate the nontoxicity of (dUfl)_n, L cells were grown in the presence of the polymer and RNA and DNA syntheses were monitored over an extended period of time. No significant change in the rates of DNA or RNA synthesis was observed, and the cells were capable of forming healthy monolayers after 48 h of incubation (Fig. 3).

Fate of (dUfl)_n is tissue culture environment. Although (dUfl)_n is capable of inhibiting the production of VS virus, it is obviously not a potent antiviral substance, and a reduction of more than 90% in the virus titer is seldom observed. The polymer has been found to be resistant to degradation by pancreatic RNase and deoxyribonuclease I (13), but its fate in the presence of growing L cells was unknown. To investigate the possible degradation of (dUfl)_n, [³H](dUfl)_n was prepared and added to cells grown in the described manner. The acid-precipitable label was then quantitated as a function of time of incubation in both the medium and the cells. Surprisingly, (dUfl)_n was found to be degraded quite rapidly and had a half-life of only 40 to 45 min in the presence of growing L cells (Fig. 4). The amount of acid-precipitable (dUfl)_n associated with the cells appears constant during the time course of the experiment and amounts to approximately 2% of the total input isotope. This is an indication of only that material that is cell associated, and no attempt was made to discriminate between surface adsorption and intracellular uptake; however, it is an indication of the inefficient uptake of the polymer by the L cells.

Attempts to potentiate antiviral properties of (dUfl)_n. Several methods exist that stimulate the uptake of biologically active viral nucleic acids. Two of these procedures, diethylaminoethyl-dextran exposure of the recipient cell (15) and using nucleic acid-calcium phosphate aggregates (10), were utilized to attempt to enhance the antiviral properties of (dUfl)_n. In one set of experiments the diethylaminoethyl-dextran (50 μg/ml) was added to the virus-infected L cells for 15 min, cells were washed, and (dUfl)_n (50 μg/ml) was added subsequently. This procedure greatly increased the amount of [³H](dUfl)_n bound to cells (10- to 20-fold) but did not significantly increase the potency of the polymer as an inhibitor of VS virus.

In the second set of experiments a calcium phosphate-(dUfl)_n aggregate was formed by the

TABLE 2. Inhibition of VS virus production in L cells by (dUfl)_n assayed on L-cell and BHK-cell monolayers

Treatment	Multiplicity of infection	Virus titer 4.75 h postadsorption (PFU/ml)	
		L cell	BHK cell
Control	0.5	5.9×10^5	6.8×10^5
	0.05	2.8×10^4	4.8×10^4
Treatment A ^a	0.5	8.8×10^4 (0.83) ^b	2.2×10^5 (0.49)
	0.05	5.2×10^3 (0.73)	8.3×10^3 (0.76)
Treatment B ^a	0.5	2.7×10^5 (0.34)	4.2×10^5 (0.21)
	0.05	2.0×10^4 (0.15)	4.0×10^4 (0.08)

^a See Table 1 and text for explanation.

^b Number in parentheses represents log₁₀ reduction in virus titer.

procedure of Graham and VanDerEb (10), the only alteration in methodology being the substitution of (dUfl)_n for the adenovirus-5 DNA. As a control in this experiment, in order to create a similar aggregate for comparative purposes, (dA)_n was precipitated with the calcium phosphate. It has been reported that (A)_n does not inhibit the replication of VS virus (16). The (dUfl)_n aggregate inhibited VS virus production relative to (dA)_n-treated cells (Table 3), but this activity was comparable to results achieved in the absence of the calcium phosphate. Hence, procedures that are known to stimulate nucleic acid-cell association do not significantly increase the inhibitory potency of (dUfl)_n.

DISCUSSION

The concept of utilizing synthetic or modified nucleic acids to interfere with viral replication was introduced by Haruna and Spiegelman (11) when they observed that the bacteriophage Qβ RNA-dependent RNA polymerase (replicase) was inhibited by (U)_n and not affected by the presence of (G)_n. They suggested that their data might indicate that polynucleotides might be highly selective inhibitors of the "recognition mechanism between a replicase and homologous template." Subsequent studies by Spiegelman and associates (17) demonstrated that, when selective pressures (i.e., short periods of incubation) were applied to an *in vitro* Qβ RNA replicating system, variant small RNA molecules evolved that possessed a very high affinity for the replicase. It was again suggested that such modified RNA molecules might represent novel antiviral compounds.

The demonstration that (U)_n, (G)_n, and, to a lesser extent, (A)_n inhibited the RNA-dependent DNA polymerase of the Rauscher murine leukemia virus (22) has stimulated the poten-

FIG. 3. Effect of (dUfl)_n on RNA synthesis (A) and DNA synthesis (B) of L cells. (A) Duplicate monolayer cultures of L cells were incubated at 37 C in the presence of 50 μ g of (dUfl)_n per ml. These cells were pulsed with 0.35 μ Ci of [³H]uridine per ml for 1 h, and the labeled RNA was extracted at each of the indicated times. Symbols: ○, L cells grown in the presence of 50 μ g of (dUfl)_n per ml; ●, L cells grown without (dUfl)_n. (B) Triplicate monolayer cultures of L cells were incubated at 37 C in the presence of 50 μ g of (dUfl)_n per ml. These cells were pulsed with 0.1 μ Ci of [³H]thymidine per ml for 2 h, and the labeled DNA was extracted at each of the indicated times. Symbols: ○, L cells grown in the presence of 50 μ g of (dUfl)_n per ml; ●, L cells grown without (dUfl)_n.

tial application of these concepts to inhibiting the oncogenic potential of the RNA tumor viruses. This area of investigation gained additional impetus with the simultaneous development of a number of nuclease-resistant forms of synthetic polynucleotides including (1-vinyluracil)_n and (9-vinyladenine)_n (16), (2'-O-methyladenylic acid)_n (21), and (dUfl)_n (8), all of which have been found to inhibit oncornavirus DNA polymerase activity.

The synthetic polymer (dUfl)_n proved to be of particular interest not only because of its resistance to pancreatic RNase and deoxyribonuclease I (13), but, in addition, (dUfl)_n was shown to be significantly more potent as an enzyme inhibitor than the naturally occurring polymers and demonstrated a degree of specificity with respect to its ability to inhibit various DNA polymerases (8). In regard to this latter observation, (dUfl)_n was found to inhibit various avian, murine, and primate oncornavirus DNA polymerases but not DNA polymerase activity extracted from *Micrococcus luteus*, calf thymus, or the DNA polymerase II (1) of human lymphocytes (8).

The extension of these enzyme inhibition

studies to the interference of virus replication in host cells demonstrated that virus production and cell transformation were inhibited (16, 19, 21), but the mechanism of action was not unequivocally demonstrated. Results obtained with (A)_n and related analogues suggested that these polymers blocked virus adsorption at high concentrations (19). In addition, (U)_n and related analogues, which have been shown to be significantly more potent enzyme inhibitors than (A)_n-related compounds (8, 16, 22), were less effective antiviral compounds (16, 21). Results obtained using (dUfl)_n on the inhibition of Rous sarcoma virus showed that this polymer was also capable of inhibiting the formation of transformed cell foci, but the mechanism of action was not elucidated (J. C. Grosch, R. L. Soehner, and R. J. Erickson, Abstr. Annu. Meet. Am. Soc. Microbiol., 1973, p. 235).

In studies assessing the sensitivity of various viruses to polynucleotide-mediated inhibition, it was noted that VS virus was reduced in titer when grown in the presence of (dUfl)_n. In this study we have shown that this inhibition is not due to gross toxicity on the host cell or to impaired adsorption or delayed release and is not

FIG. 4. Degradation of [^3H] (dUfl) $_n$ to acid-soluble isotope in the presence and absence of growing L cells in monolayer culture. The cells were treated as described in Materials and Methods. Symbols: Δ , L cells in Eagle minimum essential medium (MEM) alone; $\circ$, L cells in MEM plus 2% calf serum; $\bullet$, [^3H](dUfl) $_n$ in MEM plus 2% calf serum.

mediated by interferon in toto. The demonstration that (dUfl) $_n$ interferes with the VS virus RNA polymerase suggests that this interaction may be related to the mechanism of inhibition. If this interpretation is correct, then this virus-host cell system may prove a valuable tool in the study of synthetic nucleic acids as inhibitors of virus replication by the mechanism described.

If synthetic polynucleotides can function in this manner, then the results of our study indicate that at present this class of compounds are relatively inefficient antiviral agents. Numerous explanations exist for this inefficiency and the most obvious is the degradation observed in these experiments. A chain length requirement of approximately 150 to 200 nucleotide residues per polymer has been shown for (U) $_n$ -mediated inhibition of the avian myeloblastosis virus DNA polymerase (9), and, therefore, only a limited degree of hydrolysis would be permitted before a sample was rendered inactive. In addition, and perhaps more important, the polymerized material must be taken up by the host cell and transported to the intracellular site of virus replication. Different modes of uptake in conjunction with cellular compartmentalization may effectively separate the inhibitory polynu-

TABLE 3. Effect of diethylaminoethyl-dextran and calcium phosphate-nucleic acid aggregation on the antiviral properties of (dUfl) $_n$

Treatment	Multiplicity of infection	Virus titer (PFU/ml) ^a
None	0.005	2.7×10^3
Dextran alone	0.005	9.9×10^2
Dextran-(dUfl) $_n$	0.005	1.7×10^2
None	0.05	4.5×10^4
Dextran	0.05	6.6×10^3
Dextran-(dUfl) $_n$	0.05	3.1×10^3
None	0.5	2.9×10^5
Dextran	0.5	4.2×10^4
Dextran-(dUfl) $_n$	0.5	2.1×10^4
(dUfl) $_n$ -CaPO $_4$	0.5	1.9×10^5
(dA) $_n$ -CaPO $_4$	0.5	7.2×10^5

^a Experimental protocol was as reported in Table 2.

cleotide from the viral polymerase. Experiments to investigate different modes of polynucleotide uptake as related to virus inhibition are now in progress.

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