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EFFECT OF POLYAMINES ON THE UPTAKE OF POLY (2′-FLUORO-2′-DEOXYURIDYLIC ACID) BY A MAMMALIAN CELL LINE

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VERO cells can take up poly(dUfl)[1] from the medium. The uptake involves surface adsorption and, most probably, intracellular penetration. Part of the poly(dUfl) is hydrolyzed during incubation with the cells but the hydrolysis products are not incorporated into de novo synthesized nucleic acids. The uptake is reduced by serum and stimulated by polycationic ionenes. The magnitude of stimulation depends on the structure of the ionene and the treatment regimen.

INTRODUCTION

A large volume of experimental data has been accumulated to show that not only viral (1) but also nonviral nucleic acids and synthetic polynucleotides can be taken up by mammalian cells in vitro (2–7). Some of the evidence has met with skepticism (6, 7) because of inadequate characterization of both the polynucleotide and the recipient cells. Maintenance of a high percentage of viable cells should be considered a very important factor in uptake studies, since observations were made that damaged or dead cells had taken up considerably more nucleic acids and other macromolecules than had healthy cells (6, 8, 9). In our experiments, the viability of cells used was maintained at a high level (> 98%), minimizing thus the complicating effects of nonviable cells on the data. Poly(dUfl) (10) became of interest because it inhibits RNA dependent DNA polymerase of the avian myeloblastosis virus (11). Ionenes, polyamines of a general formula $\{(CH_2)_x\text{-}N(CH_3)_2\text{-}(CH_2)_y\text{-}N(CH_3)_2\}^+_z\ X^-$ (12), were examined for their anticipated uptake-stimulating properties.

METHODS

Poly(dUfl) (10) was prepared from ^{3}H-labeled 5′-diphosphate or by tritiation of poly(dUfl). Ionene bromides were gifts from Dr. A. Rembaum (Caltech).

VERO cells (CCL-81, American Type Culture Collection) were grown in monolayers at 37°C in humidified air-CO_2 with MEM which was supplemented with 5% heat-inactivated

[1] Abbreviations: poly(dUfl), poly (2′-fluoro-2′deoxyuridylic acid); MEM, minimum essential medium containing Hanks' balanced salt solution with penicillin and streptomycin; PBS, phosphate-buffered saline. The numbers in ionene names, e.g., in 6, 10-ionene, refer to the x and y multiples of the (CH_2) repeating unit in the general formula $\{(CH_2)_x\text{-}N\ (CH_3)_2\text{-}(CH_2)_y\text{-}N(CH_3)_2\}^+_z X^-$.

fetal bovine serum. The same conditions were used in uptake experiments; serum was omitted when required. Viability was determined by trypan blue dye exclusion in cell suspensions obtained by trypsinization (counts made with a hemocytometer) and/or in monolayers (viewing random fields in an inverted microscope).

In uptake experiments, log phase cell monolayers were incubated in a medium containing test substances for up to 5 hr. The medium was then removed, the cells were rinsed three times with PBS and all solutions, and the cells were assayed for ^{3}H-labeled low molecular weight degradation products (extractable into cold 0.2 N $HC1O_4$) and ^{3}H-labeled polymeric material (0.2 $HC1O_4$ precipitable) (procedure I). In experiments with ionenes, which cause ^{3}H-poly(dUfl) to strongly adsorb onto glass or plastic surfaces, an alternative was used to separate the cells from noncell-associated ^{3}H-poly(dUfl). In procedure II, the cell monolayers were incubated and rinsed as above; the cells were then removed from the surface by a mild trypsinization, recovered from a diluted suspension by centrifugation, and assayed for ^{3}H-labeled high and low molecular weight fractions. The uptake data were related to cellular protein as determined by the Lowry method (13 Concentrations of poly(dUfl) are given as concentrations of the repeating unit.

RESULTS

General Aspects

Poly(dUfl) undergoes considerable degradation when exposed to cells with serum present in the medium. After 0.5, 2, and 5 hr of incubation, the amount of degradation products found in the medium corresponded to approximately 35, 55, and 80%, respectively, of the input amount of poly(dUfl); in absence of serum, the respective amounts were 10, 20, and 25%. Generally, less than 1% of the total input label was extractable from cells into cold 0.1 N $HC1O_4$, regardless of whether the cells had been incubated with ^{3}H-poly(dUfl) or with corresponding labeled nucleoside or nucleoside diphosphate.

After the loosely adsorbed polynucleotide had been removed from the cells by rinsing with fresh medium, there was always ^{3}H-labeled polymeric material which remained cell associated. Actinomycin-D (5 μg/ml), which inhibited the DNA and RNA synthesis by 85% and 98%, respectively, did not significantly affect the levels of the polymeric cell-associated material with serum either present or absent from the medium.

The presence of serum in the incubation medium considerably affected the uptake; the amount of poly(dUfl) which was taken up by cells was reduced and the uptake appeared to reach saturation at lower poly(dUfl) concentrations (Fig. 1). The time course of the uptake (data not shown) indicated that the uptake leveled off at 3–5 hr of incubation in the absence of serum, while in its presence the uptake leveled off after 1 hr of incubation.

About 70% of the cell-associated ^{3}H-poly(dUfl) was released in a chase experiment with a 10-fold excess of unlabeled poly(dUfl) regardless of whether the uptake and cold chase were carried out in the presence or absence of serum in the medium or whether the chase period varied from 1 to 4 hr. Only negligible amounts of cell-associated ^{3}H-poly(dUfl) were released into the medium within up to 4 hr of incubation in poly(dUfl) free medium.

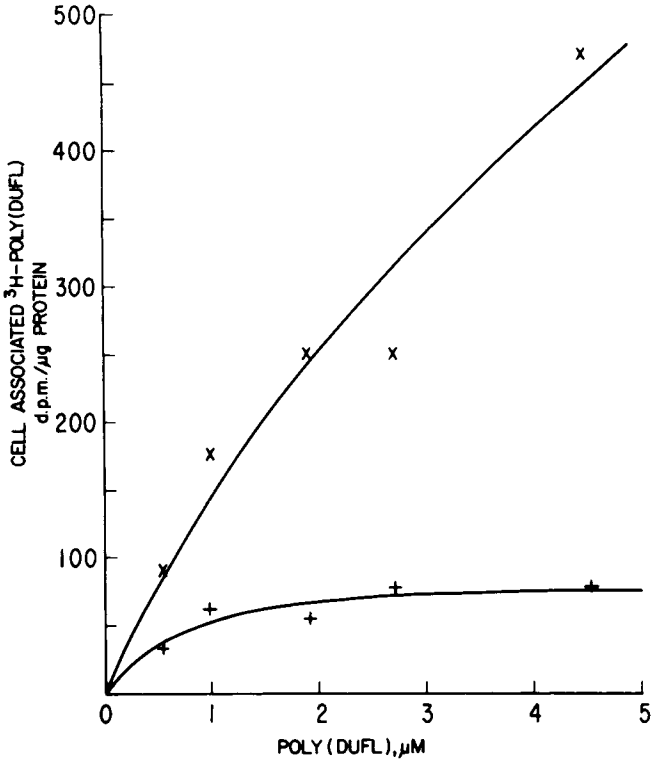

Fig. 1. The effect of poly(dUfl) concentration on its uptake by VERO cells. The cells were incubated for 3 hr with ^{3}H-poly(dUfl) (procedure I) at concentrations as indicated with (+) and without (x) serum in the medium. The ^{3}H-label was assayed in the acid-insoluble cellular fraction.

Effect of Ionenes

Hydrolysis of poly(dUfl) by cell- and serum-associated hydrolytic enzymes was inhibited to various degrees by ionenes depending on their structure; the inhibition was more effective when the ionene was added simultaneously with the polynucleotide (Fig. 2) than when the cells were pretreated with the ionene and its excess was removed (Table I) before addition of the polynucleotide.

Ionenes also stimulated uptake of poly(dUfl) by the cells. Treatment of the cells with 6, 10-ionene prior to incubation with the polynucleotide was more effective than simultaneous addition of the ionene and poly(dUfl) (Table I). Pretreatment of the cells with ionene followed by incubation with an ionene-poly(dUfl) mixture was the least effective regimen. The amount of poly(dUfl) which associated with cells increased with increasing concentration of 6, 10-ionene and leveled off above 5 μM (Fig. 3).

Ionenes at higher concentrations were toxic to VERO cells. The lowest concentration at which this effect became detectable, i.e., the cells began to dislodge from the surface and DNA synthesis began to cease, was 10 μM for 6, 10-ionene and about 100 μM for 3,3-, 3, 4-, 4, 6-, and 6, 6-ionene. In uptake studies, all ionenes were tested below the 50% level of their lowest toxic concentration.

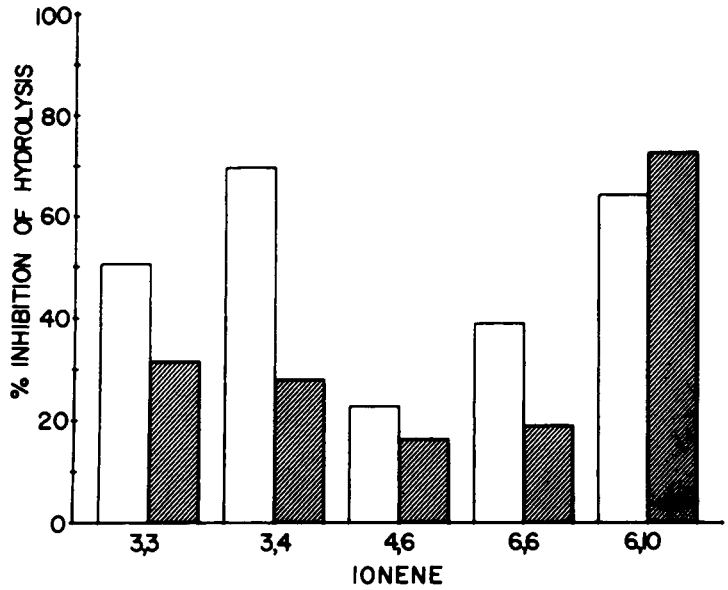

Fig. 2. The inhibition by ionenes of the hydrolysis of poly(dUfl). The cells were incubated with ^{3}H-poly(dUfl) and 3 μM ionene in the absence (empty bars) and presence (shaded bars) of serum (procedure I). The data are taken from the plateau region of the time course of hydrolysis plots and are averages of 2-, 3-, and 5-hr incubation times. Control = 0%.

TABLE I. The Effect of Method of 6, 10-Ionene Application on the Uptake of ^{3}H-poly(dUfl) by VERO Cells

Method	Cell-associated ^{3}H-poly(dUfl) (μmoles/μg protein) $\times 10^{8}$	Methods which differ from the given method at $p < 0.01$
A	27.6	B, C, D
B	21.4	A, C
C	60.5	A, B, D
D	18.7	A, C

Both the 6, 10-ionene and poly(dUfl) were 3 μM. The periods of pretreatment with ionene and incubation with poly(dUfl) were 1 and 3 hr, respectively. Procedure II was used to process the cells. The data were analyzed by analysis of variance and the studentized range statistic and the means from five parallel samples are shown. Methods: A, the cells were incubated with an equimolar mixture of ionene and poly(dUfl); B, pretreatment with ionene, the poly(dUfl) was added to the pretreatment media; C, pretreatment with ionene followed by two rinses with fresh ionene-free media and incubation with poly(dUfl); D, the same as C except an equimolar mixture of poly(dUfl) and ionene was added for the final incubation.

The magnitude of stimulation of uptake depended on the structure of the ionene (Table II). At 5 μM concentration, the 6, 10-ionene was by far the most effective while the 6, 6-ionene stimulated only slightly ($0.01 < p < 0.05$) and 3, 3-ionene hardly at all ($p > 0.05$). At higher concentrations, i.e., at those where 6, 10-ionene was toxic, the other two ionenes also caused a severalfold increase of uptake. The data obtained on four preparations of 6, 6-ionene indicated that within the range from 3×10^{4} to 4.2×10^{4} daltons the stimulation was independent of molecular weight.

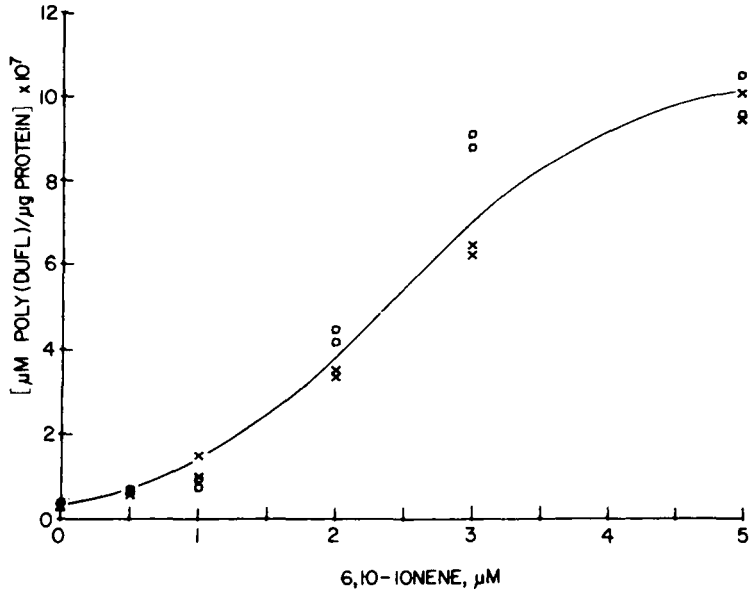

Fig. 3. Stimulation of poly(dUfl) uptake by various concentrations of 6, 10-ionene. The cells were pretreated for 1 hr with the ionene at concentrations as indicated. Then the cells were rinsed and incubated for 3 hr with ^{3}H-poly(dUfl) (3 μM) in fresh media containing serum. Procedure II was used to obtain the data. The data from two separate experiments are shown.

TABLE II. Effect of Ionenes on ^{3}H-poly(dUfl) Uptake[1]

	Distribution of Label			
	Supernatant and Washes		Cell associated	
Treatment	Polymeric	Low mol. wt.	Polymeric	
	%[2]	%[2]	(μmoles/μg protein) X 10^7	%[2]
Control	9	89	0.4	0.5
6, 10-ionene	10	68	8.0	9.5
6, 6-ionene	12	83	0.8	0.9
3, 3-ionene	16	85	0.6	0.6

[1] Procedure II was used for uptake determination. All values are the mean of three determinations.

[2] This is the percent of the total input label. The total of the experimentally determined label is close to 100% of the input label when the amount associated with the trypsinization supernatant, i.e., 1–3% for 3, 3- and 6, 6-ionene and 10–12% for 6, 10-ionene, and the amount of low molecular weight cell-associated label, i.e., < 1% of the total input, are taken into account.

DISCUSSION

Our results indicate that poly(dUfl) can be taken up by VERO cells despite extensive degradation during incubation particularly with serum in the medium. Since poly(dUfl) is resistant to pancreatic RNase (10), the data show that other nucleolytic enzymes associated with cells and serum (14, 15) are capable of hydrolysing poly(dUfl).

The degradation products essentially do not incorporate into de novo synthesized nucleic acid, which in part may be due to their insufficient transport into cells. The polymeric material which is found associated with cells thus represents the original poly(dUfl). The uptake involves surface adsorption and most probably also intracellular penetration, as evidenced by a fraction of poly(dUfl) which remains cell-associated after repeated washings, incubation in a poly(dUfl) free medium, cold chase with poly(dUfl), and separate or combined treatments (unpublished results) with nuclease and trypsin.

Ionenes generally reduce hydrolysis of poly(dUfl) by nucleolytic enzymes and stimulate its uptake by VERO cells. The stimulatory effect, similarly to poly-L-lysine (16), appears to be two-fold. Neutralization of the cell surface negative charge by these polycations leading to perturbation of the membrane seems to be a more effective stimulus, e.g., pretreatment of cells with ionenes, than neutralization of the polynucleotide phosphate groups, e.g., uptake of the stoichiometric mixture of poly(dUfl) and ionene. The stimulatory effectiveness of ionenes depends on their structure; the spacing of positive charges seems to be the important factor. Serum, in contrast, when present in the incubation medium substantially reduces the uptake (6). These adverse effects on uptake can be rationalized in the context that serum which contains nucleases promotes hydrolysis, is acting against cell impairment by providing a protective environment and stabilizing the cytoplasmic membrane, and is competing for surface sites with the polynucleotide.

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