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ANTIVIRAL ACTION OF 5-BROMO-2'-DEOXYURIDINE AND POLYOMA VIRUS-SPECIFIC RNA SYNTHESIS

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The influence of 5-bromo-2'-deoxyuridine on the synthesis of polyoma virus-specific RNA in mouse embryo cells at a late phase of infection was assayed by molecular hybridization. The effect of the analogue on virus yields in terms of infectivity (p.f.u.) and haemagglutination activity (h.a.u.) was also quantitated. 12.68–126.80 μg/ml bromodeoxyuridine inhibited viral RNA synthesis to 15–10%, h.a.u. titers of virus yield to 50–10%, p.f.u. titers of virus yield to 0.10–0.01%, and p.f.u./h.a.u. ratios to 1.0–0.1% of the respective controls. The inhibition of viral RNA synthesis was reversed by thymidine.

Since i) bromodeoxyuridine inhibited viral RNA synthesis and h.a.u. titers only partially and to a different extent, and ii) it reduced p.f.u. titers and p.f.u./h.a.u. ratios by several orders, it is likely that viral RNA synthesis represents only one of the sites of the antiviral action of the analogue.

5-bromo-2'-deoxyuridine RNA synthesis polyoma virus

INTRODUCTION

The replication of polyoma (Py) virus is efficiently inhibited by bromo- or iodo-analogues of thymidine [11,24,30]. The analogue is phosphorylated and incorporated into replicating viral DNA. Such DNA, containing bromouracil or iodouracil instead of thymine may then lead to the formation of non-infectious, defective viral particles [15,16] and to various changes in the metabolism of infected cells, e.g. in the activity of some enzymes of DNA synthesis [14,39,40]. Bromodeoxyuridine (BrdUrd) disturbs also the gene expression in 'normal' and virus-non-producer cells in various ways [9,10, 21,23]. Nevertheless, the mechanism of the antiviral and anticellular action of the analogue is still far from clear.

In this work we present the results of our studies on the effect of BrdUrd on the Py virus-specific RNA synthesis in productively infected cells. A preliminary report of a part of this work has appeared earlier [33].

MATERIALS AND METHODS

Virus

The plaque-purified A2LP strain of Py virus, kindly supplied by Dr. M. Fried from ICRF, London, was propagated in primary cultures of mouse embryo (ME) cells. Virus, passaged at a multiplicity of infection (m.o.i.) 0.2 p.f.u./cell and extracted by the neuraminidase method [4], was purified by one cycle of differential centrifugation. The stock virus, resuspended in basal Eagle's medium–10% calf serum (BEM-CS_{10}) titered up to 10^5 h.a.u./ml.

Cells

Primary cultures of ME cells were prepared from 18–20 day old mouse embryos by the standard trypsinization procedure. Sixty mm plastic Petri dishes Koh-i-noor and 200 ml roller bottles were seeded with 6×10^6 and 65×10^6 cells, respectively.

Virus yields

Intracellular virus was assayed after extraction at pH 8.5 [4]. Cells from duplicate Petri dish cultures were scraped into medium, pelleted and resuspended in Tris buffer, pH 8.5. The cell-bound virus was extracted at 37°C for 18 h and then titrated. The haemagglutination titration was carried out with guinea pig red cells [38]. For plaque titration primary cultures of ME cells in Petri dishes were used. The original procedure [37] was modified in that the agar overlay contained 20% calf serum and plaques were counted 12–15 days post infection (p.i.).

Isolation of [^{3}H] RNA

Five to seven day old confluent bottle cultures of ME cells were washed with BEM and infected at m.o.i. 25–50 p.f.u./cell. After 3 h at 37°C the inoculum was changed for BEM-$CS_{0.5}$. The addition of virus was taken for zero time of the infection. 30 min before the addition of the radioactive label, actinomycin D was added (at a final concentration of 0.01 μg/ml) to reduce the synthesis of ribosomal RNA [3]. Labelling of RNA with [^{3}H]uridine (7.4 MBq/ml) was carried out for 2 h. To isolate RNA [20] cultures were washed and lysed with 0.5% SDS buffer. The lysate was extracted with an equal volume of phenol buffered to pH 5.2 three times, precipitated with ethanol, redissolved in 0.01 M Tris, pH 7.5, and digested with DNase (25 μg/ml) at 37°C for 30 min. Then the phenol extraction was repeated and RNA was precipitated with ethanol, redissolved in 2 × SSC and dialysed. Usually two bottle cultures were employed for the preparation of one RNA sample.

Isolation of viral DNA

Viral DNA for hybridization purposes was prepared from infected ME cells by the selective extraction method of Hirt [12] and then purified by phenol extraction and isopycnic CsCl density gradient centrifugation in the presence of ethidium bromide

[28]. Cells, infected at m.o.i. 25–50 p.f.u./cell were lysed 7–10 days p.i. with 0.6% SDS buffer, the cellular DNA was precipitated with 1 M NaCl at 4°C overnight, pelleted at 15000 r.p.m. for 30 min, and the supernatant was extracted with an equal volume of phenol buffered to pH 8.0 and then with isoamylalcohol–chloroform (1 : 24). After digestion with RNase (10 μg/ml) at 37°C for 1 h, the isoamylalcohol–chloroform extraction was repeated, DNA precipitated with ethanol, redissolved in SSC/10 and dialysed. To approximately 1 mg of DNA in SSC/10 CsCl was added to a final density of 1.598 g/cm^3 and ethidium bromide to a final concentration of 150 μg/ml in a total volume of 10 ml/tube. Tubes were then filled with paraffin oil and centrifuged in Ti_{50} angle rotor at 39,000 r.p.m. for 66 h at 20°C in a Spinco L2 50 B ultracentrifuge. The lower band containing Py DNA I was separated by drop fractionation, pooled, freed from ethidium bromide by 2–3 extractions with isopropanol saturated with CsCl, and dialysed against SSC/10.

DNA : RNA hybridization

The nitrocellulose membrane filter method [7] was employed. To immobilize viral DNA on filters, it was first denatured at 100°C for 10 min in SSC/100, then quickly diluted with cold 6 × SSC to 0.2 μg/ml. Ten ml aliquots, containing 2 μg of viral DNA, were then slowly filtered through the 25 mm Sartorius filters SM 11306 prewashed with 6 × SSC. The DNA-containing filters were then washed with 6 × SSC, dried at 80°C for 4 h and used in hybridization. Ten μg of [^{3}H] RNA in 1 ml of hybridization buffer was annealed with DNA on filter at 66°C for 20 h, washed with 2 × SSC, treated with RNase (20 μg/ml) at 37°C for 2 h, washed again, and dried. Their radioactivity was counted in a toluene-based scintillation cocktail in Packard Tricarb spectrometer Model 3390. Hybridizations were usually performed in duplicates and empty filters served as controls. The specificity of the hybridization assay, checked by using heterologous components (calf thymus DNA, mouse RNA) was found to be satisfactorily high.

Media and solutions

BEM-CS_{10} and BEM-$CS_{0.5}$ consisted of basal Eagle's medium (BEM) supplemented with 10% and 0.5% calf serum, respectively. SSC contained 0.15 M NaCl and 0.015 M trisodium citrate. Phenol, buffered to pH 5.2, was 66% phenol in 0.01 M EDTA, 0.01 M sodium acetate and 0.05% bentonite. Phenol, buffered to pH 8.0, was 66% phenol in 0.5 M Tris and 0.005 M EDTA. Stock solution of RNase (1 mg/ml) was heated to 80°C for 10 min before use. The hybridization buffer contained 0.1% SDS in 6 × SSC. The toluene-based scintillation cocktail contained 0.4% PPO and 0.05% POPOP. SDS buffers 0.5% and 0.6% were made in 0.01 M EDTA, pH 7.5.

Chemicals

Actinomycin D, pancreatic DNase I, and thymidine were from Calbiochem; pancreatic RNase, five times crystallized, and neuraminidase from Koch-Light; ethidium bromide and Tris from Serva; BrdUrd, SDS and calf thymus DNA from Fluka; bentonite from

BDH; PPO and POPOP from Packard; and Bacto agar and Bacto neutral red from Difco. [^{3}H] 5-Uridine of specific radioactivity 740–1110 GBq/mmol was supplied by the Institute for Production, Research and Use of Radioisotopes in Prague.

RESULTS

Inhibition of virus-specific RNA synthesis by BrdUrd

The virus-specific RNA (Py RNA) is defined in our work as the radioactivity present in RNA preparations isolated from [^{3}H] uridine-labelled infected cells, forming hybrids with viral DNA that are resistant to RNase. It apparently contains various molecular classes of functional messengers [35], as well as some of their precursors and breakdown products. A time period of 28–30 h p.i. was chosen for the labelling and assay of Py RNA. This approach was motivated by the fact that BrdUrd, when present only after virus adsorption to cells, can affect mainly the late events, depending on the newly synthesized and analogue-containing progeny DNA.

The influence of the analogue on Py RNA synthesis was investigated in the concentration range of 0.012–126.8 μg/ml. BrdUrd was added to the medium after virus adsorption and was present until the harvest of RNA. Control cultures were incubated in the absence of the analogue and RNA preparations, isolated from them, contained 1–4% of Py RNA in various experiments. The results of two representative experiments (Table 1) show that BrdUrd reduced Py RNA synthesis to 68.9–57.7% of control at a concentration of 0.063–0.254 μg/ml. It caused a more pronounced inhibition (reduction of Py RNA to 19.5–15.4% of the control value) at a concentration of 1.268–12.68 μg/ml. Further increase of the concentration of BrdUrd to 126.8 μg/ml brought about a reduction of Py RNA to 8.2% of its control value.

Although it is probable that BrdUrd is incorporated into viral DNA under our experimental conditions and exerts its effects primarily at the DNA level, it was of interest to attempt to reverse the BrdUrd effect by thymidine. Simultaneously with the analogue we added thymidine in equimolar or tenfold higher concentration to the cell cultures. Table 2 shows that thymidine caused an obvious reversal of the inhibition of Py RNA synthesis by BrdUrd.

Inhibition of virus multiplication by BrdUrd

We attempted to establish a relationship between the effect of the analogue on viral RNA synthesis and that on virus multiplication. Although there was already evidence for the antiviral action of BrdUrd in this respect [11,30], quantitative measurements using the same BrdUrd concentration as used in this work have not been reported. Therefore, we determined virus yields 72 h p.i. by both haemagglutination and infectivity titers. The virus yields of cultures which had been exposed to the compound were compared to those which had been obtained in the absence of the compound. In these experi-

TABLE 1

The influence of BrdUrd on the synthesis of Py RNA

Expt. No.	BrdUrd (μg/ml)	Input [^{3}H] RNA (c.p.m.)	Py RNA		
			Hybridized c.p.m.[a]	% Input RNA	% Control
1	0	229,010	3661	1.559	100.0
	0.012	310,380	4975	1.603	100.3
	0.063	307.220	3383	1.101	68.9
	0.254	311.740	2877	0.923	57.7
	1.268	499.530	1560	0.312	19.5
	6.34	276,250	724	0.262	16.4
	12.68	289,330	711	0.246	15.4
2	0	216,828	2173	1.002	100.0
	63.4	184,859	208	0.112	11.2
	126.8	126,704	103	0.081	8.2

[^{3}H] RNA was isolated 30 h p.i. with Py at 25–50 p.f.u./cell and 2 h after a pulse of [^{3}H] uridine. BrdUrd was present from 3 till 30 h p.i. Control cells were without BrdUrd.

[a] The values of empty filter controls, 59 and 47 c.p.m., were subtracted in Experiments 1 and 2, respectively.

ments, the short pretreatment with actinomycin D, used in the Py RNA synthesis assays, was omitted.

The results of two experiments (Table 3) show that the virus yields determined by haemagglutination and infectivity were inhibited by BrdUrd to a different extent [11]. Whereas the h.a.u. titers were reduced only partially by a relatively high concentration of BrdUrd (126.8 μg/ml), the p.f.u. titers were lowered by several orders. There was a BrdUrd concentration range (0.254–1.268 μg/ml), where the h.a.u. titers remained normal and the p.f.u. titers were significantly reduced. The ratio p.f.u./h.a.u., reflecting

TABLE 2

The effect of thymidine on the inhibition of Py RNA by BrdUrd

BrdUrd (μg/ml)	Thymidine (μg/ml)	Py RNA	
		% Input RNA	% Control
–	–	2.63	100.0
6.34	–	0.32	12.2
6.34	5.0	1.88	71.6
6.34	50.0	2.70	102.7

[^{3}H] RNA was isolated 30 h p.i. with 25–50 p.f.u./cell and 2 h after a pulse of [^{3}H] uridine. BrdUrd or thymidine were present from 3 till 30 h p.i.

TABLE 3

The effect of BrdUrd on the multiplication of Py virus

BrdUrd (μg/ml)	Expt. No.	Titers of virus yields		Log
		h.a.u./ml	p.f.u./ml	p.f.u./h.a.u.
0	1	2048	3.45×10^8	5.23
	2	2048	1.40×10^8	4.84
0.012	1	2048	3.85×10^8	5.27
	2	4096	9.25×10^7	4.35
0.063	1	2048	4.05×10^8	5.30
	2	2048	1.10×10^8	4.69
0.254	1	2048	2.65×10^8	5.11
	2	1024	6.00×10^7	4.77
1.268	1	2048	2.75×10^7	4.13
	2	2048	4.75×10^7	4.37
12.68	1	1024	5.30×10^5	2.72
	2	512	5.05×10^5	2.99
126.8	1	512	8.00×10^4	2.19
	2	256	2.00×10^4	1.89

Virus yields were determined 72 h p.i. in 2 ml extracts from duplicate Petri dish cultures. BrdUrd was present from 3 till 72 h p.i. Controls were without BrdUrd.

the integrity or defectivity of Py virus particles [6], decreased from 10^5 to 10^3-10^2 with increasing the BrdUrd concentration from 12.68 to 126.8 μg/ml. This means that at this BrdUrd concentration range defective virus particles or at least non-infectious virus-specific material was produced which had retained haemagglutination activity.

The effects of BrdUrd on viral RNA synthesis, virus multiplication and p.f.u./h.a.u. ratios are illustrated in Fig. 1. It presents the concentration dependence curves of the different effects in a logarithmic plot. The weakest inhibition, detectable only at the highest concentration of BrdUrd, was observed for the haemagglutination activity. A somewhat stronger inhibition, apparent at a much lower concentration was noted for the viral RNA synthesis. Thus, it seems that these two inhibitory effects, although of similar magnitude at their maximum, do not correlate. On the other hand, the curves for the infectivity and p.f.u./h.a.u. ratio correspond to each other although they show an inhibition that was significantly greater than for the two former effects.

DISCUSSION

The level of viral RNA in ME cells found in our experiments is comparable to the data reported in similar systems [1–3,13]. We have also determined the inhibitory effect of 0.01 μg/ml actinomycin D on ribosomal RNA synthesis [3], but we could not detect any (results not shown).

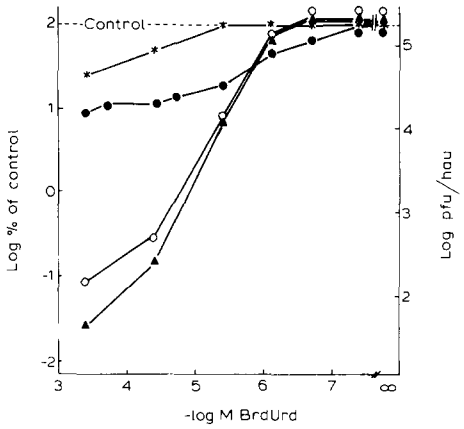

Fig. 1. Inhibition of Py RNA synthesis and virus multiplication in ME cells by BrdUrd. Py RNA (•), h.a.u. titers (*) and p.f.u. titers (▲) of virus yields expressed in log % of control; log p.f.u./h.a.u. (○). For explanation, see Tables 1 and 3.

The present results on the inhibition of virus-specific RNA synthesis, based only on its hybridization properties, do not allow any judgements as to the nature of the RNA molecules which were most severely inhibited in their synthesis. Thus, the observed inhibition of Py RNA synthesis by BrdUrd does not necessarily mean inhibition of formation of functional viral messengers; and if it does, one may question what class of messengers is affected to what extent [29]. Nevertheless, the observed inhibition probably reflects the late transcription products, which account for the great majority of total virus-specific RNA that specifies virus structure proteins.

Comparing the effect of high concentration of BrdUrd (12.68–126.8 μg/ml) on viral RNA synthesis, haemagglutination activity and virus infectivity, we must conclude that these inhibitions do not correspond quantitatively. A similar discrepancy appears with low concentration of BrdUrd (0.254–1.268 μg/ml) which reduces viral RNA synthesis and p.f.u. titers of virus yields markedly, but leaves h.a.u. titers unchanged. We have no reason to assume that the formation of messengers coding for viral haemagglutinin is normal, because the structural form of the haemagglutinating activity in this particular situation is not known. Admittedly, various possible viral structures may possess haemagglutinating activity: mature virions, non-infectious virus particles with defective DNA, empty capsids, pseudovirions and viral haemagglutinin molecules in a more or less complex form. Our results on the inhibition of virus growth are in agreement with those of Smith et al. [30] and Hirt [11]. The latter observed a reduction of the p.f.u. titers by two orders and a reduction of the h.a.u. titers by one order, when using 4 μg/ml BrdUrd, but in their assay they had also added 6×10^{-5} M fluorodeoxyuridine.

The low infectivity and p.f.u./h.a.u. ratio of virus material produced in the presence of BrdUrd may be based on one or more of the following phenomena: i) BrdUrd-DNA containing virions which possess a reduced infectivity; ii) potentially infectious BrdUrd-

DNA is produced in normal amounts, but not assembled into virions; iii) inability to form mature virions because of a) faulty proteins, b) faulty DNA, or c) insufficient amounts of individual components available. The literature data show that individual parameters of the virus growth cycle are affected in a different manner according to the virus studied and the type and concentration of the compound tested. Thus, BrdUrd-T_4 phages are fully infectious [18,22], but animal DNA viruses grown up in the presence of BrdUrd or IdUrd are non-infectious [16]. Both viral DNA and antigen of herpes simplex and vaccinia viruses are 'normally' synthesized in the presence of BrdUrd, but their assembly is impaired [5,26,31]. On the other hand, BrdUrd-containing pseudorabies virus is 'normally' assembled, but is non-infectious [16].

The reversal of the BrdUrd effect by thymidine as shown here and previously [11] indicates that the analogue acts via its incorporation into DNA, where thymidine is substituted by bromouracil to a certain extent. Of course, BrdUrd could also interfere with thymidine metabolism in other respects, i.e. transport and phosphorylation.

It has been shown [11,36] that BrdUrd-DNA of Py virus is able to replicate without restriction, but it displays an altered behaviour and may be responsible for many of the effects mentioned above. Considering the mechanisms of BrdUrd action, we must take into account the possibility of mutagenesis and faulty transcription. BrdUrd is a known mutagen [22] and BrdUrd-DNA displays increased errors in base pairing [34], leading to altered transcription products [10]. However i) its mutation frequency is too low to account for the magnitude of the inhibition of Py RNA synthesis, and ii) the BrdUrd effects can be reversed by thymidine [10]. BrdUrd-DNA may mainly manifest its abnormal behaviour at the transcription level. This assumption is supported by several observations: constitutive enzymes are relatively resistant to the analogue [21]; lac repressor binds 10-times more firmly to the operator of BrdUrd-DNA [21]; non-histone proteins have a greater affinity for BrdUrd-DNA, which leads to increased condensation of chromatin [10]; transcription of repetitive eukaryotic BrdUrd-DNA is inhibited [8]; synthesis of specific proteins and their mRNAs is inhibited by the analogue [25, 27,32].

That BrdUrd can influence the gene expression also in the opposite sense is demonstrated by the induction of infectious retroviruses [23] and EBV [9] in non-producer cells and by the induction of alkaline phosphatase [17] and some enzymes involved in DNA synthesis [14,39,40]. The inhibition of viral RNA synthesis reported in this work may thus represent another case of interference of BrdUrd with the transcriptional machinery of the infected cell. However, further studies on the exact nature of the RNA molecules synthesized in the presence of BrdUrd will be required to unambiguously assess the effects of the analogue on viral transcription.

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