EFFECT OF 5'-DEOXY-5'-*S*-ISOBUTYL ADENOSINE ON POLYOMA VIRUS REPLICATION

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1. Introduction

5'-deoxy-5'-*S*-isobutyl adenosine (SIBA) was shown to inhibit cell transformation induced by oncogenic RNA viruses, such as Rous sarcoma virus [1] or murine sarcoma virus (Yoshikura et al. unpublished results). Further studies showed that SIBA inhibits also mitogen induced blastogenesis of human and rabbit lymphocytes [2].

SIBA is a synthetic analogue [3] of *S*-adenosyl-homocysteine (SAH), the natural inhibitor of *S*-adenosylmethionine dependent transmethylases [4]. It inhibits the incorporation of [^{3}H]methyl from methionine into chick embryo fibroblasts (Berneman et al. unpublished results) but it is not yet known whether the primary action of SIBA is on methyl transfer reaction, as the compound inhibits also protein RNA and DNA syntheses in chick embryo cells.

To explore further the activity of SIBA as an oncostatic agent we investigated its effect on some biochemical events resulting from the lytic infection of mouse cells by polyoma (PyV), an oncogenic DNA virus.

Winocour et al. [5] have found a several fold increase of [^{3}H]thymidine and [^{3}H]methyl incorporation into DNA of mouse kidney cultures upon infection by PyV. The amount of transmethylation was proportional to the amount of DNA synthetised, but there was no evidence that the virus induces hypermethylation of cellular DNA.

In this communication we report on the strong inhibitory effect of SIBA on PyV replication and on its effect on methylation and on macromolecular synthesis in infected cells.

Abbreviations: SIBA, 5'-deoxy-5'-*S*-isobutyl adenosine; PyV, Polyoma virus; PFU, Plaque forming unit; PBS, Phosphate buffered saline

2. Materials and methods

2.1. *Cells*

Secondary mouse embryo cells were cultivated in Dulbecco's modified medium containing antibiotics and 10% horse serum. Cultures were incubated at 37°C in an environment continually flushed with a gas mixture of 95% air + 5% CO_2.

2.2. *Effect of the inhibitor on cell replication*

Cells were seeded at 4.35×10^5 cells/dish and 24 h later SIBA (Sefochem, Fine Chemicals, Emek Hayarden, Israel) was added at the desired concentration.

The cultures (in duplicate) were then incubated at 37°C. Cell count was performed after trypsinisation of the cultures with and without inhibitor every 24 h. For the infected cells the same procedure was followed. SIBA was added 1 hour after infection. The inhibitor was dissolved in the medium and sterilised by filtration.

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2.3. *Virus infection of mouse cells and plaque titration*

Polyoma virus, small-plaque Toronto strain was used. 7×10^5 mouse embryo fibroblasts were seeded in 35 mm Falcon Petri dishes. One day later cells were washed twice with PBS and infected with 2 PFU/cell, 0.1 ml/plate. After one hour adsorption time at 37°C, the monolayers were washed with PBS. The action of the inhibitor on virus replication was studied using the same method by adding SIBA at various concentrations to the infected cells. 48 h later, cells from control and inhibitor containing cultures were removed and stored with the culture supernatants at −20°C until use. The cell suspension was then sonicated, diluted 10^6-fold and 0.2 ml of this suspension was added in triplicate to secondary mouse cells seeded as described above. After one hour the monolayers were overlaid with 0.9% Difco agar, Eagle's medium and 2.5% horse serum. Cultures were incubated at 37°C for 14 days, when 3 ml of overlay medium containing neutral red (0.008%) was added. The plaques were counted on the following day.

The conditions for the preparation of cell-free extract and the determination of the level of tRNA methylase activity were the same as before [1].

2.4. *Rate of penetration of ^{14}C-labelled SIBA* into normal and infected cells*

Secondary mouse cells were seeded as described above at 2.5×10^6 cells/6 cm Petri dish. One day later, medium containing respectively 100 μM and 500 μM unlabelled SIBA in 5 ml was added to each monolayer. The concentration of labelled SIBA was 0.7 μCi/ml (specific activity: 9 mCi/mM, CEA Saclay, France). Incubation at 37°C was carried out for 5, 10, 30, 60 min and 5 h. Supernatants were then discarded and cells washed three times with 3 ml of ice-cold PBS. To measure the uptake into the soluble pool and the incorporation of labelled material into the nucleic acids and proteins the method of Schmidt and Tannhauser [6] was used. For the infected cells the same method was used 24 or 48 h after infection.

Protein concentration was determined by the Lowry method [7].

* 5′-deoxy-5′-*S*-[1-^{14}C]isobutyl adenosine

2.5. *Effect of SIBA on macromolecular synthesis*

Secondary mouse cells were seeded and half of the cultures were infected as described above. After allowing virus to adsorb for 1 h, 500 μM SIBA in the usual medium with 2% horse serum was added to all cultures (3 ml/plate). Infected and normal cells in medium without SIBA were used as control. Cells after infection were incubated for 4, 14, 24, and 48 h. To estimate protein in DNA and RNA syntheses (separate cultures), at the end of the incubation time an appropriate radioactive precursor was added for one hour to the medium. (L-[4-^{3}H]Leucine 30 Ci/mmol (4 μCi/ml). [5-^{3}H]Uridine 24 Ci/mmol. [*Methyl*-^{3}H]thymidine 46 Ci/mmol (2 μCi/ml). CEA Saclay, France.) To measure the extent of methylation, cells were labelled during 5 h at 37°C with [*methyl*-^{3}H]methionine 20 Ci/mmol (30 μCi/ml) in the presence of unlabelled sodium formate 20 mM, adenosine 20 μM, to inhibit methyl incorporation into purine and thymine via the 'one carbon' pool [8]. The supernatants were discarded after incubation, and the monolayers washed three times with 3 ml ice-cold PBS. Radioactive label in the different fractions, (acid soluble, nucleic acid and protein) was determined as above. Total uptake was defined as the sum of radioactive label in the acid-soluble and acid-precipitable fractions.

3. Results and discussion

Table 1 shows that 10 μM SIBA, (3.4 μg/ml) inhibits by 92% the replication of polyoma virus and consequently the lysis of the infected cells. The replication of this DNA virus seems to be more readily inhibited by SIBA than the production of the oncogenic RNA virus, Rous sarcoma virus by the virally transformed cells [1]. The question, whether SIBA inhibits specifically the replication of the virus or affects its infectivity is now under investigation. These results prompted us to study the effect of SIBA on the replication of the host cells. Figure 1b shows that 100 μM SIBA slows down only slightly the growth of infected cells whereas 500 μM inhibits it. Normal cells are not affected by these concentrations of the compound (fig.1a). Thus, to affect cell division, even that of infected cells, a ten times higher concentration of SIBA is necessary than for the inhibition of the replication of polyoma virus.

Table 1
Inhibition of polyoma virus replication by various concentrations of SIBA

[SIBA] μM	PFU/plate	% inhibition
–	2.45×10^9	–
10	2.00×10^8	92
50	5.50×10^7	98
–	3.00×10^8	–
100	3.50×10^6	98.8
500	1.25×10^6	99.6
1000	3.00×10^6	99

Mouse embryo fibroblasts were infected one day after seeding with 2 PFU/cell of PyV. Then SIBA at various concentrations was added to the cultures (except to the control cultures). 48 h later all cell suspensions were collected and used after adequate dilution to infect other mouse cells seeded as before. The plaques were counted 15 days later. (Experimental details are given in Materials and methods.)

Preliminary to a time-course experiment to determine whether SIBA acts on macromolecular synthesis in host cells, the rate of penetration of labelled SIBA in normal and infected cells was studied. It was observed, that the drug enters the cells in 5 min and a plateau is reached in 60 min. The initial rate of penetration was nearly identical in normal and in infected cells after 5 h the same amount of radioactivity was measured in both cultures. With 500 μM SIBA in the medium, the value at the plateau was 17 nmol/mg of protein.

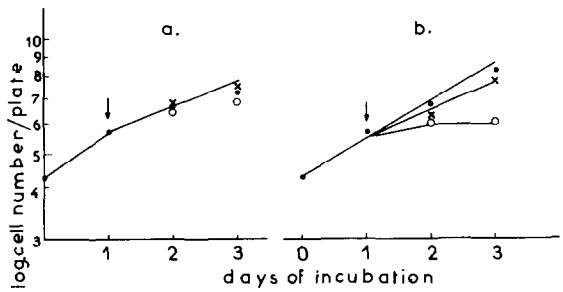

Fig.1. Effect of 100 μM and 500 μM SIBA on growth of normal (a) and polyoma infected (b) cells. 60 mm dishes were seeded with 4.35×10^5 mouse cells. 24 h later cells were counted. One series of cells were then infected as described in Materials and methods and one hour later the inhibitor was added to both normal and infected cells, as indicated by the arrow. Cells were counted in duplicate during two days. (•——•) Cells without inhibitor (×——×) with 100 μM SIBA (○——○) with 500 μM SIBA.

As 500 μM SIBA does not affect growth of uninfected, normal mouse cells (fig.1a) but stops completely the division of polyoma infected cells as well as the replication of the virus (fig.1b and table 1). This concentration of the substance was chosen to study its site of action at the cellular level.

The effect of SIBA on the macromolecular synthesis of cells following the infection is shown in fig.2. In absence of SIBA the first event to be stimulated 4 h following the infection seems to be

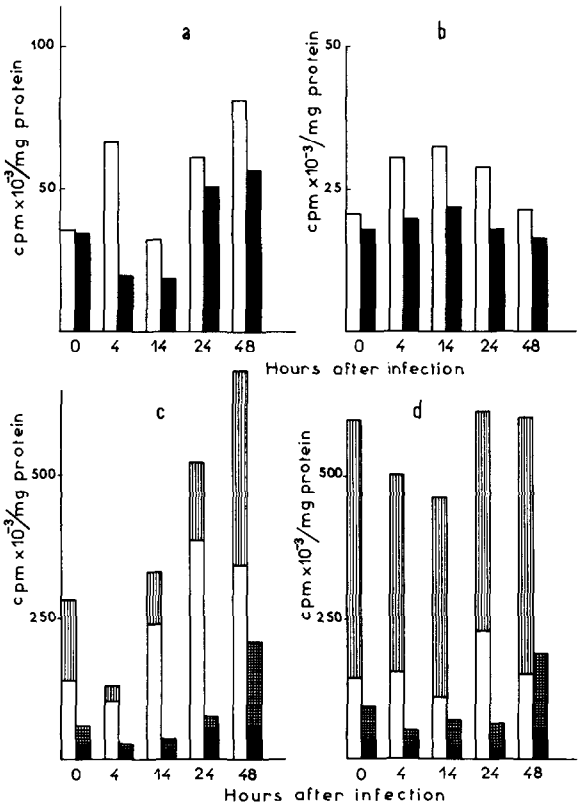

Fig.2. Macromolecular synthesis in mouse embryo fibroblasts after various times of infection by polyoma virus and effect of 500 μM SIBA on these events. (a) Incorporation of [^{3}H] methyl from methionine into the nucleic acid fraction of the cells. (b) Protein synthesis measured by [^{3}H]leucine incorporation into the trichloroacetic acid insoluble fraction. (□) Extent of incorporation in cells without SIBA; (■) extent of incorporation in cells with SIBA. (c) [^{3}H]Thymidine uptake and incorporation. (d) [^{3}H]Uridine uptake and incorporation. (▦) Uptake of the precursors into the trichloroacetic acid soluble fraction with SIBA; (▩) the same in the presence of SIBA; (▨) incorporation into the trichloroacetic acid insoluble fraction without SIBA; (▪) the same in the presence of SIBA.

methylation, as measured by [^{3}H]methyl incorporation from methionine into the nucleic acid fraction of the cells. This methylation is then depressed at 14 h and stimulated again after 24 h (fig.2a). The early increase in methyl incorporation is probably due to infection, as no such stimulation was observed in uninfected cells. The same figure shows that this early methylation is virtually not inhibited by 500 μM SIBA in uninfected cells whereas the same concentration produces 73% inhibition in cell infected for 4 h. The inhibitory effect decreases with time. In cells infected for 48 h, methylation is inhibited only by 30%. Further experiments will be necessary to study the substrate of this early methylation.

Figure 2b shows that protein synthesis measured by [^{3}H]leucine incorporation increases also 4 h after infection and begins to decrease after 14 h. In normal cells, protein synthesis is not significantly inhibited (15%) whereas in infected cells the inhibition is between 32% and 38%. Leucine transport into the cells was not affected by this concentration of SIBA. (Result not shown.)

It was shown by Cramer and Feinendegen [9] that thymidine incorporation was depressed in mouse cells between 4 and 7 h after infection by PyV, while after 12 h it was stimulated. As shown in fig.2c total thymidine uptake and incorporation are lower in cells 4 h after infection than in uninfected cells. The incorporation and the total uptake are then stimulated up to 48 h following the infection. Uridine uptake and incorporation increased slightly, and only 24 h after infection (fig.3d). In the presence of SIBA a dramatic inhibition of thymidine and uridine transport was observed (85–90%) (fig.3c and d). The uptake of thymidine and uridine by normal cells was also inhibited by SIBA to about the same extent and this effect on uptake was fully reversible after the elimination of SIBA from the culture (Raies et al. unpublished results). Besides the strong effect of SIBA on nucleoside uptake, the compound inhibited also the incorporation of thymidine into the trichloroacetic acid insoluble material of infected cells.

To check whether the increase of the incorporation of [^{3}H]methyl groups into the nucleic acid fraction is related to an increase in the activity of tRNA methylase, this activity was measured in cell free extracts, following the infection. Table 2 shows that the specific activity of the tRNA transmethylase is the same in normal and in PyV infected cells, whereas the endogenous protein methylase activity increases two-fold in cells infected for 24 h. *S*-adenosylhomocysteine and SIBA are competitive inhibitors of *S*-adenosylmethionine and inhibit both tRNA and protein methylases, but *S*-adenosylhomocysteine is a more efficient inhibitor than SIBA. Thus, the results of this study do not support the hypothesis of a polyoma virus induced increase in the tRNA methylase activity in the lytic cycle: However, the possibility that there are some qualitative changes in the methylated bases in infected cells, cannot be excluded.

Table 2
Specific activities of tRNA[a] and endogenous protein[b] methylases in normal and polyoma infected mouse cells – apparent kinetic constants of SIBA and *S*-adenosylhomocysteine in cell-free extracts

Methylase		Specific activity	K_m (SAM)[c] (μM)	K_i (SAH)[d] (μM)	K_i (SIBA) (μM)
tRNA methylase	Normal cells	98	14	3.3	100
	PyV infected cells after 24 h	91			
	PyV infected cells after 48 hours	94	3.0	7.5	300
Endogenous protein methylase	Normal cells	58	166	3.0	600
	PyV infected cells after 24 h	104			
	PyV infected cells after 48 h	86	50	12	1000

[a] pmol $^{14}CH_3$ incorporated in 100 μg tRNA *E. coli* B in 60 min at 30°C in the presence of 100 μM SAM/mg protein
[b] pmol $^{14}CH_3$ incorporated/mg protein in 60 min at 30°C in the presence of 100 μM SAM
[c] SAM, *S*-adenosylmethionine
[d] SAH, *S*-adenosylhomocysteine

It was shown by Weber and Rubin [10] that in density inhibited mouse cells, the entry of [^{3}H]uridine is greatly reduced. As shown in fig.1, 500 μM SIBA stops the growth of infected cells. One can speculate about the possibility that the inhibitor brings the cells to a physiological state, similar to a density dependent growth arrest. As uridine and thymidine are not essential metabolites for animal cells in culture, alteration in their transport cannot by itself explain the inhibition of growth, but may be an indicator of other alterations related to growth arrest. It was observed by Bader et al. [11] that natural heterologous nucleosides prevent nucleoside uptake in a variety of normal and tumor cells, without affecting nucleic acid synthesis. SIBA also inhibits protein synthesis and it is known that nucleic acid synthesis is simultaneously affected when protein synthesis is inhibited. Further studies are necessary to see if SIBA affects also nucleoside kinase activity.

In conclusion, we have shown that SIBA inhibits the early methylation of nucleic acids in whole cells following the infection by PyV. However it is not yet evident whether the primary effect of SIBA is indeed on methylation and if so, whether this interaction is decisive for the effect on virus replication, which is completely inhibited by 50 μM SIBA.

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