The Effect of 6-Azauridine upon Subacute Sclerosing Panencephalitis Virus in Tissue Cultures[1] (36622)

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Subacute sclerosing panencephalitis (SSPE) has been the subject of intensive clinical and laboratory studies during the past 4 years. As a result of these efforts, a relationship between this disease and a measles-like virus has been established (1–3). Although the exact steps of the pathogenesis of SSPE are not yet understood, this virus probably plays a major role in the etiology of this disease. The nature of the SSPE virus has been studied intensively, and it resembles measles virus in many respects (1–4). There are, however, some differences between SSPE virus and measles virus, *in vivo* and *in vitro*, but they may only reflect an inadvertent selection of a viral strain and not be sufficient to establish the SSPE virus as an agent separate from measles virus (4). Comparative studies of measles and SSPE viruses have been made with respect to the steps of viral synthesis, range of susceptibility of animals and cell cultures, antigenic structure, rate of virus production (4) and ultrastructural characteristics (5). The effect of viral inhibitors has not yet been tested on SSPE *in vitro*, and the *in vivo* effects of several such drugs have not been impressive (6). In a recent study on the effect of an inhibitor of RNA synthesis, 6-azauridine (7), upon two strains of measles virus, it was demonstrated that the drug inhibited virus replication *in vitro* (8). This has prompted us to consider its effect upon the SSPE virus *in vitro*, both as a possible means for differentiating the SSPE and measles virus, and as a possible therapeutic agent for this neurological disorder.

Materials and Methods. Tissue culture. A continuous line (CV-1) derived from African green monkey kidney cells was employed in the 35th through 42nd tissue culture passages. Cells were grown in Eagle's minimum essential medium (MEM) in Earle's balanced salt solution supplemented with 10% fetal bovine serum. Cells were maintained in Eagle's MEM with 2% fetal bovine serum.

Viruses. Two SSPE viruses, LEC and JAC, isolated from brain tissues of two SSPE patients, by the technique of cell culture and fusion as previously described (3), were maintained in CV-1 cells and used in passage 16. An attenuated measles virus, the Edmonston strain, the vaccine seed virus, obtained from Merck, Sharp and Dohme through the kindness of Dr. Maurice Hilleman, was used in its 56th passage in the CV-1 cells. A wild strain of measles virus (Woodfolk), originally isolated from a child with clinical measles, was maintained in CF-1 cells and used in its 7th passage.

Studies with the RNA inhibitor. The inhibitory effect of 6-azauridine (Calbiochem, Los Angeles, CA) was tested by the following methods: (a) Serial dilutions of virus were inoculated into monolayers of CV-1 cells in

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Petri dishes. After absorption for 1 hr at 37°, agar overlayer, containing different concentrations of the drug, was added. After 4 days of incubation, neutral red agar overlayer was applied and plaques were counted on day 6 or 7.

(b) Cells were infected in suspension at an input multiplicity of 0.01 PFU/cell, incubated, and shaken frequently. After 1 hr, cells were concentrated by gentle centrifugation and seeded into appropriate bottles or onto cover slips. After either 1 or 18 hr of incubation, different amounts of 6-azauridine were added twice daily to infected, as well as noninfected, cell cultures to produce concentrations of 1, 10, and 100 μg/ml of the inhibitor. Seventy-two hours after infection, cell-free and cell-associated viruses were harvested by removal of cells from the glass surface of the bottles by alternate freezing and thawing. Infectivity titers were determined by plaque assay. Cover slips treated in an identical manner were stained by the indirect fluorescent antibody technique, with measles hyperimmune serum used to determine the percentage of infected cells.

Results. Incorporation of 100 μg/ml of the drug into the agar overlayer completely inhibited plaque development of all four viruses tested. At a concentration of 10 μg/ml, the infectivity titer of the Woodfolk and Edmonston strains, as measured in CV-1 cells, was reduced approximately tenfold, whereas at the same concentration the SSPE viruses were totally inhibited. A concentration of 1 μg/ml had no effect on the infectivity of either strain of the measles virus but resulted in a 10- to 100-fold reduction in the infectivity of the SSPE viruses (Table I). The drug also affected the time of plaque development. In the presence of the drug, plaque size never reached the dimensions seen in control cultures, and the plaques appeared 1 or 2 days later than in the control cultures.

When the drug was added 1 hr after infection, the cells produced less virus than the untreated infected cells. In the case of the Edmonston strain, concentrations of 1, 10, and 100 μg/ml of the drug reduced the virus yield between 1000 and 10,000 times, per total cell population. When these results were interpreted in terms of reduction of virus yield per infected cell, the reduction was 100- to 1000-fold. Essentially the same degree of decrease was observed when the cells were infected with the Woodfolk strain of measles virus. Furthermore, when cells were treated with 1 μg/ml of the drug and infected with the Edmonston strain, the percentage of infected cells, as determined by fluorescence microscopy, approximated that in the untreated infected cells. However, the amount of infectious virus produced by each cell in the treated group was 1000 times less than in the untreated group (Table II). When replication of the virus was allowed to proceed for 18 hr before the addition of the drug, the inhibitory effect of 6-azauridine on infectious virus production was less marked. The decrease in infectious Edmonston virus produced approximated that of Woodfolk since it was between 10- and 100-fold. The Woodfolk strain was not significantly affected by addition of the drug 18 hr after infection (Table III).

TABLE I. Effect of 6-Azauridine on Production of Measles and SSPE Viruses (Plaque Titration).

Conc. of 6-azauridine (μg/ml)	Virus yield (PFU/ml)			
	Measles		SSPE	
	Woodfolk	Edmonston	LEC	JAC
100	$<5.0 \times 10^{0}$	$<5.0 \times 10^{0}$	$<5.0 \times 10^{0}$	$<5.0 \times 10^{0}$
10	2.1×10^{3}	1.8×10^{3}	$<5.0 \times 10^{0}$	$<5.0 \times 10^{0}$
1	3.8×10^{4}	3.5×10^{4}	1.0×10^{3}	2.8×10^{2}
0	3.0×10^{4}	4.1×10^{4}	3.5×10^{4}	2.5×10^{4}

TABLE II. Effect of 6-Azauridine on Measles and SSPE Virus Replication when Added 1 hr Postinfection.[a]

Virus	Drug conc (μg/ml)	Virus yield (PFU/ml)	Virus yield/cell (PFU)	Percentage of infected cells (by FA)	Virus yield/infected cell (PFU)
Woodfolk	100	1.5×10^{2}	1.6×10^{-4}	1.2	1.4×10^{-2}
	10	3.8×10^{2}	2.2×10^{-4}	4.8	4.7×10^{-3}
	1	8.4×10^{2}	3.0×10^{-4}	8.0	3.8×10^{-3}
	None	3.8×10^{5}	1.2×10^{-1}	12.4	1
Edmonston	100	4×10^{1}	4×10^{-5}	1.0	4.3×10^{-3}
	10	8×10^{1}	4.7×10^{-5}	5.0	4.4×10^{-4}
	1	9.7×10^{2}	3.5×10^{-4}	10.0	3.5×10^{-3}
	None	2.3×10^{6}	7×10^{-1}	10.0	7.2
SSPE LEC	100	$<5.0 \times 10^{0}$	ND	0.2	ND
	10	$<5.0 \times 10^{0}$	ND	1.2	ND
	1	7×10^{1}	5.3×10^{-5}	7.2	7.5×10^{-4}
	None	3.0×10^{4}	1.2×10^{-2}	10.8	0.1
SSPE JAC	100	$<5.0 \times 10^{0}$	ND	0.1	ND
	10	$<5.0 \times 10^{0}$	ND	1.0	ND
	1	5×10^{1}	5.0×10^{-5}	5.0	1.0×10^{-3}
	None	4×10^{4}	2.6×10^{-2}	11.0	0.2

[a] FA = fluorescent antibody technique; ND = not determined.

On the other hand, production of the SSPE viruses was markedly affected. Cells infected with the LEC strain and treated 1 hr after infection with 10 or 100 μg/ml of the drug did not produce any infectious virus. A concentration of 1 μg/ml was sufficient to reduce the yield of infectious virus by 1000 plaque-forming units (PFU)/infected cell, in comparison with the untreated infected cells. In spite of this, the percentage of infected cells, as determined by fluorescence microscopy, was almost the same in the two preparations (Table II). The effect of treatment of infected cells with the drug 18 hr after infection was less marked than at 1 hr after infection. Concentrations of 10 and 100 μg/ml reduced the virus yield 100-fold/infected cell, whereas the effect of a 1 μg/ml concentration was comparable to that observed in cells treated 1 hr after infection. Here again the percentage of cells producing viral antigen was higher in the other two preparations (Table III).

The yield of infectious virus per total number of cells was approximately the same in each of the treated preparations, and the drug concentration of 1 μg/ml was as effective in inhibiting virus multiplication as that of 100 μg/ml.

The JAC virus behaved exactly like the LEC virus in its sensitivity to 6-azauridine. Treatment of infected cells with 10 and 100 μg/ml of drug, 1 hr after infection, completely inhibited the production of infectious virus. When the drug was added 18 hr after infection, the virus yield per infected cell again was markedly reduced (Tables II and III).

Discussion. Our results indicate that 6-azauridine decreases the amount of infectious SSPE virus produced by the cell culture and that it does so by inhibiting virus production by each infected cell. The inhibitory effect is maximal if the drug is allowed to act during the early part of the replicative cycle of the virus. This effect parallels that observed in the action of 6-azauridine against LCM (9) and dengue (10) viruses, as well as that reported by us against measles virus (8). Thus, 6-azauridine is a more effective inhibitor when added to the culture 1 hr after infection than later. However, it still has a substantial inhibitory effect even when added 18 hr after infection. In this respect, the

TABLE III. Effect of 6-Azauridine on Measles and SSPE Virus Replication when Added 18 hr Postinfection.

Virus	Drug conc (μg/ml)	Virus yield (PFU/ml)	Virus yield/cell (PFU)	Percentage of infected cells (by FA)	Virus yield/infected cell (PFU)
Woodfolk	100	2.0×10^{4}	3.5×10^{-2}	9.6	3.7×10^{-1}
	10	9.0×10^{4}	1.1×10^{-1}	12.2	9.5×10^{-1}
	1	2.5×10^{5}	2.0×10^{-1}	11.9	1.8
	None	2.7×10^{5}	2.2×10^{-1}	12.4	1.9
Edmonston	100	1.4×10^{3}	2×10^{-3}	2.0	1×10^{-1}
	10	5.5×10^{3}	8×10^{-3}	5.0	1.7×10^{-1}
	1	2.0×10^{5}	2×10^{-1}	10.0	2
	None	1.6×10^{6}	1.5	10.0	1.5
SSPE LEC	100	6.4×10^{1}	1.2×10^{-4}	1.6	8.0×10^{-3}
	10	9.8×10^{1}	2.4×10^{-4}	2.4	1.0×10^{-3}
	1	7.4×10^{1}	6.1×10^{-5}	8.0	7.7×10^{-4}
	None	1.5×10^{4}	1.0×10^{-2}	10.0	0.1
SSPE JAC	100	5.0×10^{1}	7.8×10^{-5}	1.5	5.3×10^{-3}
	10	8.0×10^{1}	1.3×10^{-4}	2.0	6.6×10^{-3}
	1	8.5×10^{1}	4.6×10^{-5}	7.0	7.0×10^{-4}
	None	1.0×10^{4}	1.0×10^{-2}	9.0	0.1

SSPE viruses differed from the measles viruses, because the latter were unaffected by addition of the drug 18 hr after infection. On the assumption that 6-azauridine inhibits RNA synthesis, our results suggest that the synthesis of viral RNA proceeds more slowly in SSPE than in measles viruses. On the other hand, the drug added at 1 hr after infection completely blocks the production of infectious SSPE virus, but only reduces, without fully blocking, production of infectious measles virus. This, too, may be explained by the slower rate of replication of SSPE viruses than of measles virus. Still, viral protein is synthesized in the presence of the inhibitor in the cultures, because small numbers of cells exhibited presence of intracellular antigen by the technique of immunofluorescence.

There was also an apparent effect of 6-azauridine on plaque formation by the viruses. When the drug was incorporated into the agar overlayer, one-tenth of the amount required to inhibit completely the two strains of measles virus, was sufficient to effect complete inhibition of the SSPE viruses. At the lowest concentration tested, the drug was still substantially able to inhibit synthesis of SSPE virus when it was no longer able to influence that of the two measles virus strains.

These findings support the results of our earlier comparison of the SSPE and measles viruses, in which we showed that under identical laboratory conditions measles-infected cell cultures produced virus earlier than did the SSPE-infected cultures, although the intracellular antigen (viral protein) was produced at the same time in both cultures (4).

The results we have reported provide additional evidence that SSPE viruses can be distinguished from measles virus. It is possible that 6-azauridine may ultimately be useful as a therapeutic agent in SSPE; however, the present state of our knowledge does not permit any specific predictions in this regard.

Summary. Four viruses, wild measles (Woodfolk), attenuated measles (Edmonston strain), and JAC and LEC SSPE viruses, were compared for their susceptibility to 6-azauridine, an RNA inhibitor. All four were found susceptible to the drug. When the drug was incorporated into the agar overlayer of the infected tissue culture, the SSPE viruses were inhibited by the concentation of 1

μg/ml and blocked by the concentration of 10 μg/ml, whereas the other two viruses showed inhibition and block at concentrations of 10 μg/ml and 100 μg/ml, respectively. When the drug was added 1 hr postinfection, the SSPE viruses were inhibited by 1 μg/ml and blocked by 10 μg/ml. Under the same circumstances the measles viruses showed some inhibition of synthesis, but could not be blocked, even by the concentration of 100 μg/ml. Addition of the drug 18 hr postinfection still substantially inhibited synthesis of SSPE viruses, but did not block it completely. The two measles viruses were only minimally affected by the drug added at the later time. The greater sensitivity of the SSPE viruses to the action of 6-azauridine serves as an additional characteristic distinguishing them from measles virus.

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