In Vitro Sensitivity of Measles Virus to 6-Azauridine[1] (35380)

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(Introduced by E. H. Lennette)

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Measles virus has been implicated, by serological studies (1, 2), demonstration of measles virus antigen in brain sections (1, 3), and by virus isolation from tissue culture derived from brain biopsies (4, 5), in the etiology of a severe disease of the central nervous system, subacute sclerosing panencephalitis (SSPE). Three drugs, 5-bromo-2-deoxyuridine, pyran copolymer and amantadine, have been employed in clinical trials in the treatment of SSPE patients. Neither BUDR, a DNA virus inhibitor, nor pyran copolymer, an interferon inducer, significantly altered the course of the disease (6). Amantadine, an inhibitor of RNA virus penetration, has been reported to stabilize the disease process in a limited clinical study of 5 patients with disorders of the central nervous system diagnosed as SSPE (7). Since it should appear that progression of this disease may be due to viral proliferation, it seemed rational to attempt to find a selective inhibitor of viral RNA synthesis that will interfere with measles virus replication. A uridine analog, 6-azauridine, has been shown to inhibit lymphocytic choriomengitis virus (8) and dengue virus (9) *in vitro*, the inhibitory effect being brought about by an inhibition of the synthesis of pyrimidines (10). It was the purpose of this study to examine the *in vitro* sensitivity of several measles virus strains to 6-azauridine for its possible use in the treatment of SSPE.

Materials and Methods. Virus. Two strains of measles virus were employed in this study. The Edmonston strain was used; it was in its 56th and 57th tissue culture passage and had been plaque-purified 3 times in Vero cells monolayers. The second strain, Woodfolk, isolated in primary monkey kidney cells in this laboratory from a child with measles, was used in its 7th passage. Stocks of both strains were prepared in Vero cells. Viral infectivity was determined by a plaque assay method similar to that previously described (11), using Vero cells grown in disposable petri dishes (60 mm).

Tissue culture. A continuous monkey kidney cell line (Vero) derived from African green monkeys was employed in the 150th through 170th tissue culture passage. Cells were gown in Eagle's MEM in Earle's balanced salt solution supplemented with 10% fetal bovine serum. After the formation of monolayers, cells were maintained with Eagle's MEM with 2% fetal bovine serum.

Drug studies. The inhibitory effect of 6-azauridine (6-AZ, Calbiochem, Los Angeles) was tested by two methods. In the first method, 10-fold dilutions of virus were prepared in maintenance medium and 0.1 ml of each dilution was inoculated onto Vero cell monolayers in 60-mm petri dishes, two cultures per dilution. After a 1-hr adsorption period at 37°, agar overlays were added which contained various concentrations of 6-AZ. The cultures were then incubated at 35° for 4 days, a neutral red agar overlay was applied, and the plaques were enumerated

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on days 6 or 7. In the second test method cells in suspension were infected with measles virus at a multiplicity of infection of approximately 0.01. The suspension was incubated with frequent shaking for 1 hr at 35°. A noninfected control cell suspension was treated in the same manner. The cells were centrifuged, resuspended in maintenance medium and seeded into duplicate 4-oz prescription bottles (approx 10^6 cells/bottle) or on coverslips in petri dishes (approx 10^4 cells/coverslip). After either 1 or 18 hr of incubation, various concentrations of 6-AZ were added twice daily to duplicate infected as well as noninfected cell cultures in bottles and on coverslips in petri dishes. At 72 hr postinfection, cell free and cell associated virus were harvested by removing cells from the glass surface of the bottles by alternate freezing and thawing and scraping with a rubber policeman. The resulting virus suspension was concentrated 5-fold by centrifugation at 30,000 rpm for 1 hr at 5°. The infectivity titer of the concentrate was assayed on Vero cell monolayers. Cells from the noninfected, drug-treated control bottles were trypsinized and the total number of cells in each bottle was counted in a hemocytometer. The virus/total cell yield was calculated on the basis of the total infectivity as measured in plaque-forming units (PFU) and the total number of cells per bottle as determined in the drug-treated, noninfected bottle cultures. Coverslips seeded with infected cells were treated with varying drug concentrations in an identical manner, and 72 hr following infection they were fixed in acetone and stained by the indirect fluorescent antibody (FA) method for measles virus. The percentage of FA positive cells (*i.e.*, infected cells) on coverslip cultures, treated with the same concentrations of 6-AZ as the bottle cultures, was determined by counting the number of FA positive cells in a total of 500 cells. This percentage was then employed to determine the number of infected cells in the corresponding bottle culture, which, when compared with the total number of PFU from each bottle, gave an approximation of the virus yield per infected cell.

Results. Initially the effect of 6-AZ on the plaque titration of measles virus (Edmonston) was determined. In Vero cell cultures, incorporation of 100 μg/ml of 6-AZ into the agar overlay completely inhibited plaque development, 10 μg/ml of 6-AZ reduced the infectivity titer approximately 2 logs, and 1 μg/ml of the drug had no effect on infectivity. In this experiment it was apparent that the drug not only affected the final level of viral infectivity, but also delayed plaque development. In the absence of 6-AZ, measles virus plaques can be enumerated on day 5, reaching a size of approximately 2 mm; in the presence of 6-AZ, however, plaque development was delayed and plaques could not be counted until day 6 or 7, reaching a size of approximately 1 mm.

Interest in the drug originally stemmed from its possible use in the treatment of established infections. Consequently, experiments were designed to test the effect of the drug on the amount of infectious virus produced by cells treated with 6-AZ at two different periods during the replicative cycle of the virus. The results obtained when cells were infected in suspension and treated with 6-AZ at different time intervals following infection are presented in Tables I and II. Cells treated with 6-AZ at 1 hr after infection produced less virus than did nondrug-treated, infected cells. In the case of the Edmonston strain, 100, 10, and 1 μg/ml of 6-AZ reduced the virus yield per total cell population by 4.3, 4.2, and 3.6 logs, respectively, while the virus yield per infected cell was reduced 2.8, 3.9, and 2.7 logs, respectively. The same concentrations of 6-AZ also affected the replication of the Woodfolk strain, decreasing the virus yield per total cell population by 2.9–2.6 logs and the virus yield per infected cell by 3.7–2.2 logs. When the replication cycle was allowed to proceed for approximately 18 hr before treatment, the inhibitory effect of 6-AZ on infectious virus production was less marked. The decrease in the amount of infectious Edmonston virus produced on the basis of the total cell population or on the basis of only the infected (*i.e.*, FA positive) cell population was approximately the same, from 2.9–0.9 logs by 100 μg/ml of 6-AZ. The Woodfolk stain was not significantly affected by addition of the

TABLE I. Effect of 6-Azauridine on Measles Virus Replication When Added 1 hr Postinfection.

Measles virus strain	Drug conc (μg/ml)	Virus yield (PFU)	Av virus yield (PFU)	No. of cells/bottle (noninfected, drug-treated controls)	Virus yield/ cell (PFU)	Log_{10} decrease in virus yield/cell	Percent infected cells by FA	Virus yield/ infected cells (PFU)	Log_{10} decrease in virus yield/ infected cells
Edmonston	100	4×10^1 4×10^1	4×10^1	9.4×10^5	4×10^{-5}	4.3	1	4.3×10^{-3}	2.8
	10	1.2×10^2 4×10^1	8×10^1	1.7×10^6	4.7×10^{-5}	4.2	5	9.4×10^{-4}	3.9
	1	9.4×10^2 1×10^3	9.7×10^2	2.8×10^6	3.5×10^{-4}	3.6	10	3.5×10^{-3}	2.7
	None	1.8×10^6 2.7×10^6	2.3×10^6	3.2×10^6	7×10^{-1}	—	10	7.2	—
Woodfolk	100	1.4×10^2 1.6×10^2	1.5×10^2	9.4×10^5	1.6×10^{-4}	2.9	1.2	1.4×10^{-2}	2.2
	10	4.6×10^2 3×10^2	3.8×10^2	1.7×10^6	2.2×10^{-4}	2.7	4.8	4.7×10^{-3}	3.7
	1	1×10^3 6.8×10^2	8.4×10^2	2.8×10^6	3.0×10^{-4}	2.6	8.0	3.8×10^{-3}	3.6
	None	3.8×10^5	3.8×10^5	3.2×10^6	1.2×10^{-1}	—	12.4	1	—

TABLE II. Effect of 6-Azauridine on Measles Virus Replication When Added 18 hr Postinfection.

Measles virus strain	Drug conc (μg/ml)	Virus yield (PFU)	Av virus yield (PFU)	No. of cells/bottle (noninfected, drug-treated controls)	Virus yield/cell (PFU)	Log_{10} decrease in virus yield/cell	Percent infected cells by FA	Virus yield/infected cells (PFU)	Log_{10} decrease in virus yield/infected cells
Edmonston	100	1.7×10^3 1.0×10^3	1.4×10^3	6.5×10^5	2×10^{-3}	2.9	2	1×10^{-1}	2.2
	10	3.8×10^3 7.3×10^3	5.5×10^3	6.6×10^5	8×10^{-3}	2.3	5	1.7×10^{-1}	2.0
	1	1.4×10^5 2.5×10^5	2.0×10^5	10.0×10^5	2×10^{-1}	0.9	10	2	0.9
	None	1.2×10^6 2.0×10^6	1.6×10^6	1×10^6	1.5×10^0	—	10	15	—
Woodfolk	100	2.0×10^4 2.0×10^4	2.0×10^4	5.7×10^5	3.5×10^{-2}	0.8	9.6	3.7×10^{-1}	0.7
	10	1.1×10^5 8.6×10^4	9.0×10^4	7.8×10^5	1.1×10^{-1}	0.3	12.2	9.5×10^{-1}	0.3
	1	3.2×10^5 1.8×10^5	2.5×10^5	1.2×10^6	2.0×10^{-1}	0.04	11.9	1.8	0.02
	None	3.0×10^5 2.4×10^5	2.7×10^5	1.2×10^6	2.2×10^{-1}	—	12.4	1.9	—

drug 18 hr after infection, since the decrease in infectious virus was less than 1 log when compared with untreated controls.

Discussion. It was found by the two different experimental methods used here that the replication of measles virus within mammalian cells *in vitro* can be inhibited by a uridine analog (6-azauridine), which inhibits the *de novo* synthesis of pyrimidines by interfering with the decarboxylase conversion of orotidylic acid to uridine monophosphate (10). Reversal of the effects of 6-AZ by the addition of uridine has been conclusively demonstrated by other workers (8, 9). Therefore, the drug affects viral replication by interfering with viral RNA synthesis.

The effect of the drug on a one-step growth curve was not examined, since the objective of the study was to determine the effect of 6-AZ on virus at a low input multiplicity, more like that of natural infection in brain cells of patients with SSPE. The data indicate that 6-AZ not only decreases the amount of infectious virus produced in the total cell population, but will also decrease the amount of infectious virus produced by each infected cell (Table I and II). In the case of cells infected with the Edmonston strain and treated with 6-AZ at 1 hr post infection, there were approximately the same number of infected (FA positive) cells in the test cultures treated with 1 μg/ml of the drug as in the nondrug-treated, infected control. However, the amount of infectious virus produced by each cell in the cultures treated with the 1 μg/ml dose of drug was 2 logs less than in the nondrug-treated control. It thus appears that while certain drug concentrations may not affect antigen production within an infected cell, they may, nevertheless, reduce the amount of infectious virus produced within that cell.

The decrease in the amount of virus produced per infected cell when the drug was added 18 hr postinfection is apparently dose-dependent. On the other hand, when cells are treated with 6-AZ at 1 hr postinfection, 10 μg/ml of 6-AZ appear to inhibit viral replication to a greater extent than do 100 μg/ml of 6-AZ. An examination of the cell count in each of the drug-treated bottles showed that the drug was toxic at the 100 μg/ml level; toxicity lowered the cell count in both infected and noninfected cultures, thus adversely affecting calculations on the virus yield per infected cell.

The inhibitory action of 6-AZ should manifest its maximum effect early in the replication cycle of the viral agent, during viral RNA synthesis. This has been found to be true in the case of lymphocytic choriomeningitis virus and dengue virus (8, 9), and these earlier observations have been confirmed in the present experiments with measles virus, viz., 6-AZ was more effective when added 1 hr after infection than when added later in the infection cycle. The data also indicate that the Woodfolk strain of measles virus synthesizes its viral RNA faster than does the Edmonston strain, since the Edmonston strain is inhibited by 6-AZ added to 18 hr, while the Woodfolk strain is not.

Although it would appear that 6-AZ does inhibit viral replication *in vitro*, considerable work must be done before this drug can be utilized in the treatment of SSPE. Far more must be known about the toxicity of the drug, about its penetration of the blood brain barrier in infected and in noninfected animals, and about its activity and effects after intrathecal injection. Also, it will be important to test 6-AZ against measles virus or measles-like viruses isolated from SSPE patients, since many *in vitro* properties of these agents differ from those of wild-type measles virus (12).

Summary. The effect of 6-azauridine, an inhibitor of RNA synthesis, has been shown by 2 experimental methods to inhibit the replication of measles virus within mammalian cells *in vitro*. In a direct virus titration in which the drug was incorporated in the agar overlay, 6-AZ not only reduced the infectivity of measles virus but also delayed plaque development. Experiments designed to test the effectiveness of 6-azauridine when added to cultures at intervals after infection, showed that this uridine analog was capable of reducing the amount of infectious virus produced by each infected cell, and that the drug is more effective when added early in the infection cycle.

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