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Sidney Halle

APRIL 1968

DEPARTMENT OF THE ARMY
Fort Detrick
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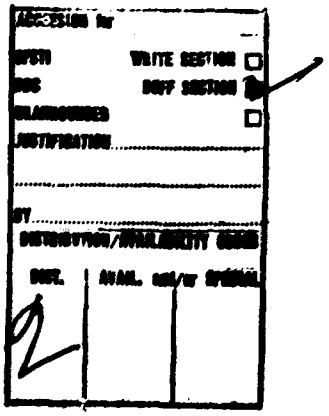

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BIOLOGICAL SCIENCES LABORATORY

Project 1C014501B71A

April 1968

ABSTRACT

A new cytidine analogue, 5-azacytidine (5-AzaC), not previously shown to be a viral mutagen, was highly mutagenic for an attenuated strain of Venezuelan equine encephalitis (VEE) virus and possibly for other strains. Treatment of the T strain, a small-plaque, attenuated variant of VEE virus, with 25 μg 5-AzaC/ml gave approximately a 2 $\log_{10}$ loss in titer compared with untreated controls, but the frequency of large-plaque revertants among survivors was increased 77- to 220-fold. The induced large-plaque formers were genetically stable mutants, and not unstable, phenotypic variants. Several types of reconstruction experiments indicated that the increased frequency of large-plaque formers resulted from induced mutation and not from selective inactivation of either variant in vitro (in the absence of cells) or from selection during plaquing or growth in chick embryo fibroblast cultures. Isolates with new growth-temperature characteristics also were derived from several different viral strains treated with 5-AzaC. Uridine reverses both the lethal and mutagenic action of 5-AzaC. The early stages of the viral growth cycle appeared to be particularly sensitive to both 5-AzaC action and uridine reversal.

MUTAGENESIS IN GROUP A ARBOVIRUSES BY 5-AZACYTIDINE

A relatively new cytidine analogue, 5-azacytidine (5-AzaC), has been reported by Sorm et al.* It is known to be inhibitory for growth, and one of its proposed modes of action is that it is incorporated into RNA, which subsequently disintegrates. It has also been shown by Fucik et al.** to be an effective mutagen for one of two strains of Escherichia coli. These reports led us to study the mutagenic effect of 5-AzaC on some selected arboviruses.

Most experiments were performed with strain T, the attenuated, small-plaque variant derived from the large-plaque-forming Trinidad strain of Venezuelan equine encephalitis (VEE) virus. The mutagen was usually incorporated in a minimal medium described by Zebovitz,*** which contains histidine and cystine as the only additives to a buffered, basal salt solution. This medium was used as a liquid overlay for chick embryo (CE) monolayer cultures held at 37 C after infection with virus. Supernatant fluids harvested at 24 hours were assayed for total plaque-forming-unit (pfu) titer and frequency of large plaques among survivors.

Concentrations of 5-AzaC as low as 5 μg/ml significantly increased frequency of large plaques in the T strain population when compared with controls lacking 5-AzaC. To maximize the effect, concentrations of 10 or 25 μg/ml were usually used. Treatment with 25 μg/ml under conditions just described gave a virus titer approximately 2 $\log_{10}$ lower than that of untreated controls (Fig. 1).

Table 1 shows that in several experiments using 25 μg of 5-AzaC/ml, the induced large-plaque frequency among survivors varied from 77- to 220-fold higher than that of untreated controls.

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* Sorm, F.; Piskala, A.; Cihak, A.; Vesely, J. 1964. 5-Azacytidine, a new, highly effective cancerostatic. Experientia 20:202-203.

** Fucik, V.; Zadrazil, S.; Sormova, Z.; Sorm, F. 1965. Mutagenic effects of 5-azacytidine in bacteria. Collect. Czech. Chem. Commun. 30:2883-2886.

*** Zebovitz, E. 1965. A defined maintenance medium for supporting chick fibroblast monolayers and for plaque formation by Venezuelan and eastern equine encephalitis virus. J. Infect. Dis. 115:77-82.

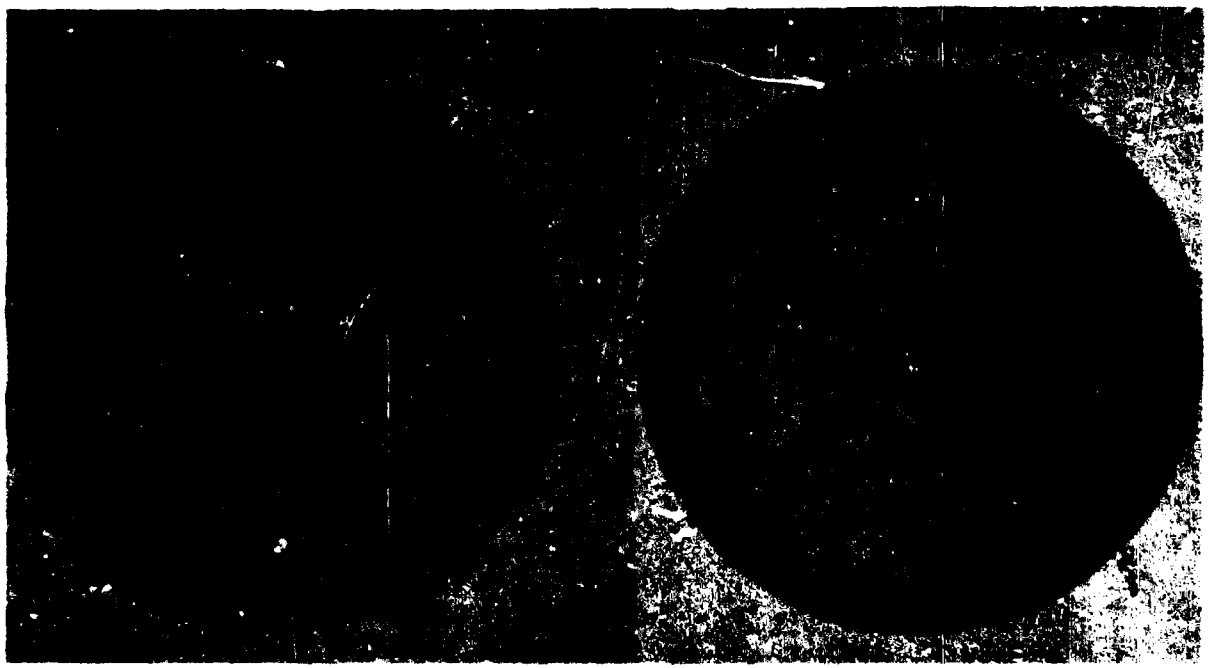

Figure 1. Effect of 5-AzaC on Large Plaque Frequency in T Strain of VEE Virus.

TABLE 1. EFFECT OF 5-AzaC ON FREQUENCY OF LARGE PLAQUES IN STRAIN T

Experiment	5-AzaC Concentration, μg/ml	Number of Large Plaques per 10^5 pfu of T	Increase in Large Plaque Frequency
1	0	9	-
	25	2,000	220X
2	0	30	-
	25	2,300	77X
3	0	13	-
	25	1,600	123X

To test whether the observed large plaques represented unstable phenotypic changes or stable genotypes, random large-plaque isolates were picked, and seeds were prepared and then checked for plaque size heterogeneity. Of the several isolates tested, all were true large-plaque mutants rather than phenotypic variants. Tests for sensitivity of the small- and large-plaque variants to the inducing agent (5-AzaC), as an inhibitor during growth in CE cells, showed no significant differences between them. Table 2 shows that, in reconstruction experiments, each virus alone or in mixtures gave similar survival (5 to 8.4%) after exposure of infected cultures to 25 μg 5-AzaC/ml. In these experiments, cultures were inoculated with seeds of stocks made from several independently isolated large- and small-plaque survivors of 5-AzaC treatment. Inoculum ratios of small- to large-plaque seeds varied from 21:1 to 4.8:1 in separate experiments.

As a further check on the possibility that selection rather than induction of new mutations occurred, the increased frequency of large plaques was followed in reconstruction experiments, the results of which are shown in Table 3. When compared with controls, the 5-AzaC-treated cultures showed no increase in the percentage of large-plaque formers that could be attributed to selection. Another type of reconstruction experiment in vitro, in which the viruses were treated (alone and in mixtures) with the drug in the absence of cells, also showed no selective inactivation by 5-AzaC in our system.

TABLE 2. CELL CULTURE RECONSTRUCTION EXPERIMENTS: SENSITIVITY OF 5-AzaC-DERIVED ISOLATES OF VEE VIRUS TO 5-AzaC

Experiment	Isolate	5-AzaC Concentration, µg/ml	Survival, %
1	T-3[a]	0	
		25	5.8
	L-1[a]	0	
		25	8.0
	T-3 & L-1	0	
	(21:1)[b]	25	5.0
2	T-2	0	
		25	6.9
	L-2	0	
		25	8.4
	T-2 & L-2	0	
	(4.8:1)[b]	25	7.5

a. T-2 and T-3 are the designations for small-plaque (parental) isolates from T; L-1 and L-2 for large plaques from same T parent.
b. Ratio of each isolate used in inoculum.

TABLE 3. CELL CULTURE RECONSTRUCTION EXPERIMENTS: EFFECT OF 5-AzaC ON LARGE-PLAQUE FREQUENCY IN VEE VIRUS AMONG SURVIVORS OF 5-AzaC TREATMENT

Experiment	Isolate	5-AzaC Concentration, µg/ml	% Large Plaques	
			Input	24 hours
1	T-3	0		0 (<0.05)
		25		0.9
	T-3 & L-1	0	4.7	1.0
		25		0.9
2	T-2	0		0.2
		25		4.0
	T-2 & L-2	0	21.0	1.0
		25		4.0

Although large-plaque isolates derived by 5-AzaC treatment showed no special resistance to the drug when reincubated in its presence, such retreated populations exhibited heterogeneity in plaque size. Such plaque-size heterogeneity has also been seen in several experiments in which the parental VEE (Trinidad) strain and the Louisiana strain of eastern equine encephalitis were treated with 5-AzaC under the described experimental conditions. This evidence indicates that 5-AzaC may have a mutagenic effect on the parental large-plaque VEE strain and the intermediate-plaque-size EEE strain, as well as causing mutation again in the new induced large-plaque formers.

Random isolates from cultures treated with 5-AzaC and infected with parental strain VEE virus were examined for growth-temperature characteristics as another genetic marker. Figure 2 shows one such interesting large-plaque isolate (H3) with a maximum temperature for plaquing on CE monolayers of approximately 41 C, two degrees lower than normal for the parental strain of VEE virus. This isolate also had a lower temperature maximum for growth in liquid culture. Virus titers in cultures incubated at 42 C for 24 hours were more than 5 $\log_{10}$ lower than those obtained from cultures grown at 40 C. The parental, large-plaque, VEE virus titers at these temperatures usually show less than a 3 $\log_{10}$ difference. Characterization of large-plaque isolates from among survivors of 5-AzaC-treated T cultures also revealed strains with different characteristics, ranging from better growth at higher temperatures than wild type to various degrees of growth inhibition.

Initial studies on the mechanism of 5-AzaC action involved the addition of various nucleic acid precursors as antagonists to this potent analogue. The results in Table 4 show that cytidine and uridine were both good antagonists to 5-AzaC action, but deoxycytidine gave only partial reversal of both lethality and mutation. Neither uracil nor thymidine at concentrations up to 200 μg/ml reversed 5-AzaC lethality for strain T. Cytosine was also ineffective.

Further information on the action of 5-AzaC was obtained by adding uridine to CE cultures infected with strain T. When both uridine and 5-AzaC were added at time 0, i.e., immediately after viral adsorption, uridine reversed the lethal and mutagenic effects of 5-AzaC simultaneously, i.e., increased survival and lowered the large-plaque frequency in T virus cultures treated with 5-AzaC. Experiments in which inhibitor (10 μg 5-AzaC/ml) and antagonist (200 μg uridine/ml) were added at various times postinfection showed the early stages of the viral growth cycle to be most sensitive to both inhibition and reversal (Table 5). Treatment with 5-AzaC 2 hours postinfection was as effective as treatment at the time of infection. Simultaneous addition of uridine was best for reversing 5-AzaC damage, although it was still highly effective if added 2 hours later. If one of the current ideas of virus replication is correct, our results suggest that the replicative forms and replicative intermediate species of viral RNA, which are synthesized first, may be the primary sites of 5-AzaC action. Further studies on the action of this compound are in progress.

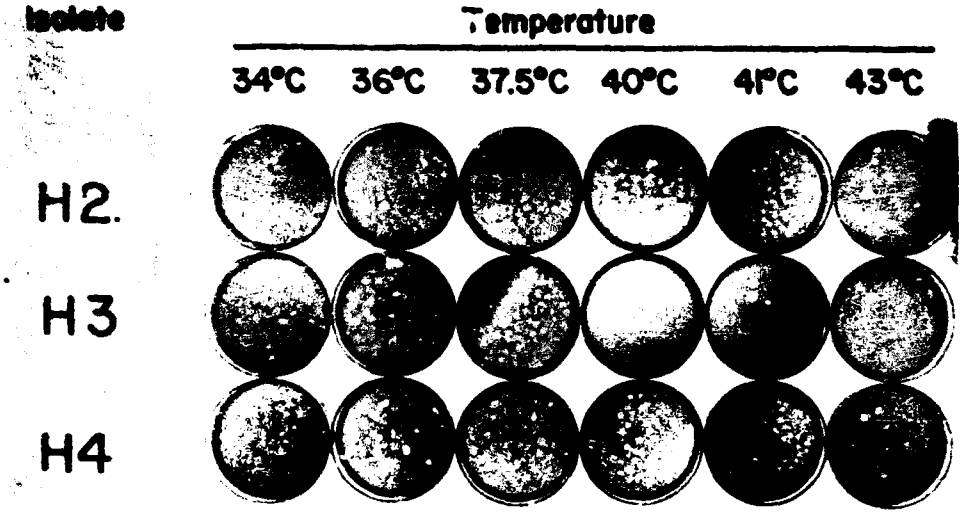

Figure 2. Plaquing Ability of Isolates from VEE Virus Parental Strain Treated with 5-AzaC. H2 and H4 are same as parental VEE virus in T_{f-max} for plaquing.

TABLE 4. REVERSAL OF 5-AzaC ACTION BY NUCLEIC ACID PRECURSORS

Experiment	5-AzaC Concn., μg/ml	Additions, 200 μg/ml	Survival, %	Number of Large Plaques per 10^5 pfu of T Strain
1	-	-	100.0	0 (<34)
	10	-	0.6	1,300
	10	Uracil	0.2	1,300
	10	Deoxycytidine	11.7	590
	10	Uridine	18.3	380
2	-	-	100.0	0
	10	-	0.2	830
	10	Thymidine	0.2	1,200
	10	Cytosine[a/]	0.05	1,500
	10	Cytidine	24.0	200

a. Cytosine control (in absence of 5-AzaC) gave only 8% survival. 5-AzaC and additions were added simultaneously at time 0, i.e., immediately after virus adsorption.

TABLE 5. INHIBITION AND REVERSAL CAUSED BY ADDING 5-AzaC AND URIDINE AT DIFFERENT TIMES AFTER INFECTION WITH T STRAIN

Time of Addition Postinfection		Exp. A	Exp. B	
5-AzaC, 10 μg/ml	Uridine, 200 μg/ml	Survival, %	Survival, %	No. of Large Plaques per 10^5 pfu T Strain
-	-	100[a]	100[b]	0 (<240)
0	-	2.3	3.2	1,900
1	-	3.1		
2	-		3.1	1,900
3	-	14.0		
4.5	-		18.8	630
5	-	23.2		
6	-		17.6	200
0	0	26.6	47	250
0	1	21.9		
0	2		18.8	630
0	3	8.4		
0	5	5.0		
0	6		9.4	1,600

a. Virus titer at 24 hours 3.2×10^8 pfu/ml.
b. Virus titer at 24 hours 1.7×10^8 pfu/ml.

In summary, 5-AzaC was highly mutagenic for one RNA-containing arbovirus strain studied in detail, and possibly for others. The compound induced a 77- to 220-fold increase above controls in frequency of large-plaque revertants in a small-plaque, attenuated strain of VEE virus. No selection by the compound of large-plaque formers was shown in vitro (in the absence of cells), nor during growth in cell cultures. Some large-plaque revertants showed new temperature characteristics different from the original large-plaque, Trinidad strain of VEE virus. Furthermore, mutants with new temperature characteristics were isolated from different viral strains treated with 5-AzaC. Uridine reversed both the lethal and mutagenic action of 5-AzaC. Experiments in which inhibitor and antagonist were added at various times postinfection showed the early stages of the viral growth cycle to be most sensitive to both inhibition by 5-AzaC and reversal by uridine.

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14. Key Words

*Arboviruses
*Mutagens
Venezuelan equine encephalitis
*Cytidine
*Uridine

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