Effect of Purine Analogs on Encephalomyocarditis and Vaccinia Virus Growth in Analog Sensitive and Resistant Cells.* (28854)

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It was shown previously(1) that 2 purine analogs, 2,6-diaminopurine (DP) and 8-azaguanine (AZA), inhibit the intracellular multiplication of the psittacosis agent in the mouse fibroblast cell strain L of Earle which is susceptible to the toxic effect of these analogs. However, DP does not inhibit the growth of this agent in strain L cells which have been selected for resistance to the analog (2). This could be explained by the fact that L cells resistant to DP have no detectable adenosine 5-phosphate pyrophosphorylase (AMPP)(3) and are incapable of converting the analog base to its nucleotide derivative which produces the inhibitory activity. The growth of the psittacosis agent in DP-resistant L cells therefore takes place in a host system that no longer is able to produce the active intracellular form of the antimetabolite.

On the other hand, AZA inhibits growth of the psittacosis agent in strain L cells resistant to the toxic effect of AZA(2). Because the AZA resistance in bacteria(4) and in mammalian cells(5,6) has also been shown to be related to the lack of the guanosine 5-phosphate pyrophosphorylase (GMPP) and therefore of the ability to convert the free analog base to its nucleotide derivative, it has been suggested(2) that the psittacosis agent may have an independent enzymatic activity related to the metabolism of this analog as it does in other metabolic functions(7,8).

This report presents evidence that similar results are observed when DP-resistant and AZA-resistant L cells are infected with encephalomyocarditis virus (EMC), an RNA virus and vaccinia, a DNA virus, in the presence of the two analogs.

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Materials and methods. The EMC virus used in these experiments is the large plaque mutant obtained from Dr. K. K. Takemoto and was passed several times on L cells. The vaccinia virus strain was serially passed in testicular tissues of rabbit and grown for 3 passages on L cells.

Earle's mouse fibroblast strain L cells, clone 929, was obtained from Dr. H. Eagle and maintained in a modified Eagle medium (BME) containing twice the normal concentration of amino acids in vitamins, with 5% bovine fetal serum. The overlay medium for plates contained, in addition to the above ingredients, 0.9% Difco Noble Agar. Protamine at a final concentration of 0.05% was added to the overlay medium for EMC virus plaque titrations(9). All media contained 100 units of penicillin and 100 μg of streptomycin per ml.

The analog-resistant lines of the L cells were obtained by exposing cell monolayers to increasing concentrations of the analogs as reported(2) and maintained with a concentration of 100 μg/ml of the analogs.

The experiments with EMC virus were performed with both the analog-sensitive and the analog-resistant cells by testing the ability to form plaques on cell monolayers in 6 cm plastic Petri dishes. Plates were seeded with 10^6 cells per plate. When a complete monolayer was formed, usually 48 hours after seeding, the cells were washed and the bare monolayers were infected with 0.1 ml of proper dilutions of the stock virus calculated to give a countable number of plaques per plate. After allowing 90 minutes for adsorption of the virus, the plates were overlaid with 5 ml of agar medium. When DP or AZA was added, the cells were treated 3 hours prior to the infection with medium containing 100 μg/ml of the analogs and overlaid with agar medium containing the analogs at the same

concentration. Two ml of overlay medium, containing neutral red 1:5000, were added to all the plates 24 hours after infection and the plaques were counted the following day. During the 48 hours required by the plaque assay, analog-sensitive cells did not show a toxic effect due to the analogs.

The experiments on vaccinia virus multiplication in analog-resistant cells were performed essentially in the same way except that 0.5 ml of inoculum was used and a 3-hour period was allowed for adsorption of virus. Two days after infection, the plates were refed with 2 ml of overlay medium, and 2 days later 2 ml of overlay medium containing neutral red were added. The plaques were counted the following day.

The experiments with vaccinia virus in analog-sensitive cells could not be carried out in the same way due to the toxic effect of the analogs on the cells in the 5-day period required for the plaque assay. Such experiments were carried out in 2-oz. prescription bottles seeded with 10^6 cells. The day after seeding, the cell monolayers were washed and the bare monolayers infected with 0.5 ml of a virus dilution containing about 10^6 PFU. A 3-hour period was allowed for adsorption, then 4 ml of medium were added, and the cells incubated for 24 hours. At this time, the cells were rapidly frozen and thawed 3 times. When the analogs were added, the cells were treated 3 hours prior to the viral infection with medium containing 100 μg/ml of DP or AZA. The same concentration of the analogs was present in the medium added after the viral adsorption period. The infected fluids were titrated by the plaque method using analog-sensitive strain L cells with the same procedure described above.

Results. Effect of DP on EMC virus growth in sensitive and DP-resistant L cells. Plate cultures of sensitive and DP-resistant L cells were infected with dilutions of EMC virus. Half of the sensitive and DP-resistant cultures were pretreated with DP before infection and overlaid with agar medium containing the analog. Neutral red was added 24 hours after infection and the plaques counted the following day. The results of 2 experiments are presented in Table I. Essentially the same number of plaques is formed on sensitive and DP-resistant L cells when DP is not present. When the analog is added to the medium, plaque formation on sensitive cells is completely abolished, whereas on DP resistant cells, plaques are formed to the same extent as on plates without the analog.

TABLE I. Plaque-Forming Ability of EMC Virus Assayed in Sensitive and Diaminopurine (DP)-Resistant L Cells.

Exp	Cells	DP, 100 μg/ml			Control		
1	DP-sensitive	0	0	0*	80	81	87
	DP-resistant	87	79	83	57	80	84
2	DP-sensitive	0	0	0	95	84	86
	DP-resistant	97	90	95	90	101	95

* No. of plaques on replicate plates infected with 0.1 ml of virus dilution 10^{-6}.

Effect of AZA on EMC virus growth in sensitive and AZA-resistant L cells. Using the same experimental procedure described above, the ability of EMC virus to form plaques was assayed on sensitive and AZA-resistant cells with and without the analog. Two different concentrations of AZA were tested for their ability to inhibit the plaque formation by the virus. The results of 2 different experiments are presented in Table II. When the analog was not present, the plaquing efficiency of EMC virus was slightly lower in AZA-resistant cells than in sensitive cells. The plaque formation was inhibited by AZA at concentrations of 100 μg and 10 μg/ml in both sensitive and AZA-resistant cells. However, when 10 μg/ml of AZA were used, a few very small plaques appeared on both sensitive and AZA-resistant cells with a 24-hour delay period.

Effect of DP and AZA on vaccinia virus

TABLE II. Plaque-Forming Ability of EMC Virus Assayed on Sensitive and Azaguanine (AZA)-Resistant L Cells.

Exp	AZA, μg/ml	Cells	AZA			Control		
1	100	AZA-sensitive	0	0	0*	138	131	130
	100	AZA-resistant	0	0	0	100	97	96
2	10	AZA-sensitive	0	0	0†	65	73	62
	10	AZA-resistant	0	0	0†	49	50	45

* No. of plaques on replicate plate infected with 0.1 ml of virus dilution 10^{-6}.

† A few very small plaques appeared on these plates with a 24-hr delay.

TABLE III. Titer of Vaccinia Virus Grown on Analog-Sensitive L Cells.

Exp	DP, 100 μg/ml	AZA, 100 μg/ml	Control
1	9.0×10^2*	1.3×10^5	6.2×10^6
2	9.0×10^2	5.4×10^3	4.6×10^5

* Plaque-forming units per ml.

growth in analog-sensitive cells. The experiments on vaccinia virus growth, in analog-sensitive cells, were performed in prescription bottles and the fluids were titrated on analog-sensitive cells as described in the *Material and Methods* section. The results of 2 experiments are reported in Table III. The analogs significantly inhibit vaccinia virus multiplication, although inhibition by AZA was not as great as that by DP.

Effect of DP and AZA on vaccinia virus growth in analog-resistant cells. The experiments carried out with analog-resistant cells were performed by assaying the ability to form plaques of vaccinia virus on plates with and without DP or AZA. The results of 2 different experiments with DP-resistant cells are reported in Table IV, (Exp. 1 and 2). Plaque formation is not significantly reduced by 100 μg/ml of DP on analog-resistant cells. In sharp contrast to the absence of inhibition by DP, AZA inhibited vaccinia plaque formation in AZA-resistant cells, as seen in Table IV, Exp. 3 and 4.

Discussion. The data presented here demonstrate that 2 purine analogs, diaminopurine and azaguanine, affect in a different way the ability of both an RNA and a DNA virus to replicate or form plaques on analog-resistant cells. It is understood, however, that inhibition of plaque formation does not exclude limited multiplication of the virus.

TABLE IV. Plaque-Forming Ability of Vaccinia Virus Assayed on Diaminopurine (DP)- or Azaguanine (AZA)-Resistant L Cells.

Exp	AZA or DP, 100 μg/ml	Cells	Analog		Control	
1	DP	DP-resistant	137	147*	179	163
2	DP	"	93	101	111	140
3	AZA	AZA-resistant	0	0	55	70
4	AZA	"	0	0	28	39

* No. of plaques on replicate plates infected with 0.5 ml of virus dilution 10^{-4}.

In summary, the growth of EMC and of vaccinia virus is inhibited by DP if the host cells are also sensitive to the toxic effect of the analogs. Viral growth is not affected, however, by DP in a subline of cells which are also resistant to this analog. The cellular mechanism of resistance to DP, therefore, prevents the inhibitory action of this analog on viral replication. It has been suggested that DP-resistant L cells owe their resistance to the lack of the AMP pyrophosphorylase and therefore would be incapable of converting the DP to its nucleotide(3). This mechanism of resistance would account for the results obtained when the DP-resistant cells are the host system of both an RNA and a DNA virus. On the other hand, AZA inhibits viral growth in AZA-resistant cells as in analog-sensitive cells. In this case, the mechanism of cell resistance does not prevent virus inhibition by the analog. It has been shown that the mechanism of cell resistance to AZA is correlated with the lack of GMP pyrophosphorylase(5). On this basis it has been proposed that growth inhibition of the psittacosis agent would result from synthesis of the enzyme by the agent(2). A similar possibility might be considered for EMC and vaccinia viruses, since virus-induced enzymatic activities have been demonstrated for vaccinia, mengo, polio and other viruses(10-14). Mengo virus has been shown to induce a RNA polymerase activity in infected L cells(10). A similar RNA polymerase has been demonstrated in HeLa cells infected with poliovirus Types 1 and 2(11). Thymidine kinase is induced by vaccinia virus in KB cells(12) and in mouse fibroblasts(13). Studies by Green(12), however, failed to show any increase in GMP pyrophosphorylase in vaccinia-infected cells.

On the other hand, induction of GMP pyrophosphorylase by virus genetic material is not the only mechanism that would explain the results presented here. Mechanisms of resistance to AZA that do not involve GMP pyrophosphorylase were suggested by the results of other studies(15,16). Szybalski *et al* (17) provided evidence of a cellular mechanism of resistance to AZA involving some reaction prior to ribophosphorylation. In our

experiments, however, it appears that AZA is converted in the resistant cell to a metabolically active compound able to inhibit the replication of such diverse intracellular parasites as an RNA virus, a DNA virus, and the psittacosis agent. The replication of both RNA and DNA viruses requires the synthesis of specific RNA and proteins. It is possible therefore to assume that AZA ribonucleotide interferes with the synthesis or function of RNA and/or proteins primed by the virus (11) without affecting, at least to an inhibitory extent, cellular metabolic functions. The mechanism by which the viability of AZA-resistant cells is not affected by this analog when the concentration of an AZA-derived antimetabolite inhibits the replication of intracellular parasites is not known. This problem is being investigated.

Summary. The effect of 2 purine analogs, 2,6-diaminopurine (DP) and 8-azaguanine (AZA) on the multiplication of encephalomyocarditis (EMC) and vaccinia viruses in analog-sensitive and -resistant strain L cells has been investigated. It was shown that DP and AZA inhibit the multiplication of both viruses in analog-sensitive cells. DP does not inhibit the replication of EMC and vaccinia viruses in DP-resistant cells. The mechanism of cellular resistance to this analog might prevent the inhibitory action of DP on viral replication. On the other hand, AZA inhibits virus growth in AZA-resistant cells as well as in analog-sensitive cells. The mechanism of cell resistance is not effective in protecting viral replication from the inhibitory effect of the analog. It is suggested that the cell resistance to AZA may be at a step following ribophosphorylation and that virus-specific RNA synthesis might be affected by the antimetabolite, whereas cellular RNA synthesis is not significantly inhibited by the analog nucleotide.

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1. Morgan, H. R., *J. Exp. Med.,* 1952, v95, 272.
2. Dalen, A. B., Morgan, H. R., PROC. SOC. EXP. BIOL. AND MED., 1962, v110, 251.
3. Lieberman, I., Ove, P., *J. Biol. Chem.,* 1960, v235, 1765.
4. Brockman, R. W., Debavadi, C. S., Stutts, P., Hutchinson, D. J., *ibid.,* 1961, v236, 1471.
5. Brockman, R. W., Bennett, L. L., Jr., Simpson, M. S., Wilson, A. R., Thomson, J. R., Skipper, H. E., *Cancer Res.,* 1959, v19, 856.
6. Brockman, R. W., Roosa, R. A., Law, L. W., Stutts, P., *J. Cell. Comp. Physiol.,* 1962, v60, 65.
7. Morgan, H. R., *J. Exp. Med.,* 1948, v88, 285.
8. Moulder, J. W., *Ann. N. Y. Acad. Sci.,* 1962, v98, 92.
9. Liebhaber, H., Takemoto, K. K., *Virology,* 1961, v14, 502.
10. Baltimore, D., Franklin, R. M., *Biochem. and Biophys. Res. Comm.,* 1962, v9, 388.
11. Baltimore, D., Eggers, H. J., Franklin, R. M., Tamm, I., *Proc. Nat. Acad. Sci. U. S.,* 1963, v49, 843.
12. Green, M., *Cold Spring Harbor Symposium on Quantitative Biology,* 1962, v27, 219.
13. Kit, S., Piekarski, L. J., Dubbs, D. R., *J. Mol. Biol.,* 1963, v6, 22.
14. Rogers, S., *Nature,* 1959, v183, 1815.
15. Davidson, J. D., Bradley, T. R., Roosa, R. A., Law, L. W., *J. Nat. Cancer Inst.,* 1962, v29, 789.
16. Szybalski, W., Szybalska, E. H., *Univ. Mich. Med. Bull.,* 1962, v28, 277.
17. Szybalski, W., Szybalska, E. H., Brockman, R. W., *Proc. Am. Cancer Res.,* 1961, v3, 272.

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