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Action of Adenine and Related Compounds on Replication of Encephalomyocarditis Virus in Tissue Culture.* (27110)

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The study by McFall and Magasanik(1) on control mechanisms of purine biosynthesis in L cells and Ehrlich ascites cells encouraged us to examine the effect on virus growth of purines that alter metabolic pathways in host cells.

Because of extensive published data on the action of purine analogues on virus growth, it was of interest to find that adenine itself could suppress the growth of encephalomyocarditis virus (EMC) in mouse ascites cells. This positive finding was in striking contrast to essentially negative results in some other systems and with other purine nucleic acid precursors.

Materials and methods. Primary tissue cultures consisting of suspensions of 7-day Krebs 2 (K2) and Ehrlich ascites tumor cells (EAC) propagated by IP inoculation in mice were used with a final cell concentration of 1.3-1.5 $\times$ 10^6/ml and maintained up to 48 hours in roller tubes in a simple medium of Hanks' BSS plus 0.1% glutamine 0.1% bovine albumen, 0.1% lactate, 0.05% Na_2HPO_4, 0.02% glucose, 0.05% $NaHCO_3$, and antibiotics. For K_2 media, glucose and $NaHCO_3$ were omitted. Roller tubes in groups of 3 or 4, each containing 0.1 ml 10% cell suspension, 2 ml appropriate medium, 0.1 ml virus dilution or control inoculum, were set up and incubated at 35°C in a roller drum at ½ rpm. At appropriate time intervals, pH and eosin counts(2) were recorded whenever needed and the contents of tubes in each group pooled for titration.

The L cell clone 929 was used as monolayers in both bottles and stationary tubes. Waymouth's medium(3) was used for stock cultures, but bactopeptone was omitted during experiments because of its high purine content.

The L-adapted EMC virus, obtained from Dr. Habel (with a titer of 8.5-9.0 log $TCID_{50}$/ml) was used. In addition, 4 to 6 passages were done in K_2, EAC, and mouse brain.

Virus quantitation was done by a) LD_{50} by IC inoculation of mice and b) $TCID_{50}$ in L cells as determined by cytopathic effect and presence of hemagglutinins. Titrations were usually in duplicate and all readings were done by coding to eliminate any bias by the observer. In the earlier experiments, cells and fluids were combined, but in later experiments they were separated. Cells were washed with BSS, frozen and thawed three times with BSS to give a 10% cell extract considered as 10^{-1} dilution.

Results. The toxicity of the compounds under study was determined. For the adenine experiments the eosin counts(2), by 24 hours 5-20% stained and at 48 hours 15-30% stained, were not significantly different in non-infected cells except at the toxic level of 1000 μg/ml. The same result was obtained with the infected groups, but cells were killed by large inocula of virus. Sometimes, in the presence of virus or under adverse conditions such as high pH, adenine seemed to have a protective effect on the cells. From this pre-

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TABLE I. Summary of Adenine Effect on EMC Replication in Ascites Cells.

Cells	EMC strain	Virus input log $TCID_{50}$	Material tested	Adenine conc., μg/ml	Log difference LD_{50} or ID_{50} control/adenine at hour 3	6	12	24	48
EAC	Brain pass.	3.0	Cells & fluid	100					*3.5
"	" "	4.5	" "	500				2.0	*1.0, 0
"	" "	4.5	Fluid	500	0	0		2.0	0
			Cells		0	2.0		2.5	−1.0
"	L pass.	2.5	Cells & fluid	100				*1.2	*2.0
"	EAC pass.	3.0	" "	100					*2.0
									*>1.8, 1.5
K_2	L pass.	6.0	Fluid	500	−1.0	−.5	1.5	3.5	1.5
					0	.5	1.0	1.5	1.5
					0	.5		1.5	
					.5	1.8	.6	2.8	
			Cells	500	.5	−.5	1.5	2.2	0
					0	0	.3	1.0	.7
					0	−.5		3.0	
					0			1.8	
K_2	K_2 pass.	4.5	Fluid	500	.5	0	.7	1.0	1.0
			Cells		.3	.3	>1.2	1.7	2.0

* Titrations done IC in mice, all others in tissue culture of L cells.

liminary work a level of 500 μg/ml or less of adenine was chosen as essentially non-toxic for K_2 and EAC as judged by eosin counts. This was also true for short-term experiments with L cells lasting 24-48 hours, but growth failed over 14 days in the presence of 500 μg/ml of adenine. Even levels of 50 μg/ml, though not cytocidal, prevented cellular propagation; these same cells placed in 25 μg/ml adenine grew at the same rate as control cultures. Thus for the long-term L cell experiments 5-25 μg/ml of adenine were used.

Ascites cell with EMC virus. Both the K_2 and EAC with every strain of EMC virus tested in the presence of adenine in concentration of 100-500 μg/ml showed reduced yields of infective virus during the first 24 hours after initiation of infection. The data are summarized in Table I. The reduction in virus growth averaged 2.1 logs and proved to be statistically significant by the t test with a probability between .01-.005. After 24 hours adenine suppression was less evident and virus yields in some experiments approached control levels. Virus growth curves with K_2 cells, (Fig. 1), depict the appearance of cell-associated and supernatant virus with and without 500 μg/ml adenine.

L cells with EMC virus. In dramatic contrast to the results with ascites cells was the finding that adenine, with the experimental procedures described above, did not affect the replication of EMC virus in L cells. In addition, 36 hour pre-treatment with adenine in standard cells and also in cells depleted in BSS for 18 hours was without effect. Long-term experiments using L cells grown with and without adenine for 14 days were suggested by McFall and Magasanik's finding that there is not an immediate inhibition of *de novo* purine synthesis upon addition of adenine or guanine. Though results with

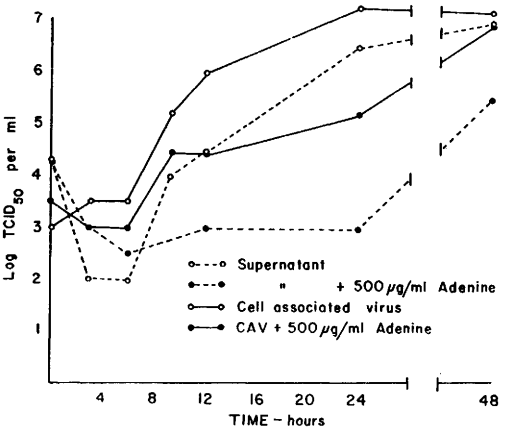

FIG. 1. EMC (L pass.). Growth curve in K_2 cells with and without adenine.

the adenine-grown cells were negative, the cells grown without adenine (*i.e.*, bactopeptone-free media) for the 2-week period and then infected in the presence of 5-25 μg/ml showed in one experiment a depressed viral growth of 1-2 logs as compared with controls. However, additional experiments with successive batches of cells failed to support these findings. Further work is under way on this problem.

Related compounds and EMC virus. The adenine results were considered of enough interest to justify examination of related purine nucleic acid precursors in both ascites cells and L cells. These included guanine, guanosine, adenosine, adenylic acid, adenosine-5-PO_4 (muscle adenylic acid), ATP, and deoxyadenosine. Most of these compounds had no effect on EMC replication except for adenosine and guanine at levels that may be just at the borderline of toxicity.

Results with adenine in chorioallantoic tissue cultures and de-embryonated eggs infected with PR_8 influenza were essentially negative except at 1000 μg ml where some virus suppression was evident. However, these levels did inhibit outgrowth of both fibroblastic and epithelial elements.

Discussion. Though the literature abounds with studies in tissue cultures of purine antagonists(4,5) and adenine is mentioned as reversing inhibition by 2,6 diamino purine (6), there is only one study on the role of adenine in virus growth. Visser, Lagerborg, and Pearson(7) found in mouse brain tissue culture a suppressive effect for Theiler's virus of adenine at 1 mg/ml.

The delaying effect of adenine on EMC replication in ascites cells is surprising, especially against a background of essentially negative findings in another cell line (L cells) or another virus (Influenza PR_8). The adenine effect seems to take place mainly during the first cycle(s) of virus multiplication without harming the virus-synthesizing machinery in many of the cells so that virus yields tend ultimately to reach control titers.

Low grade toxicity cannot be completely ruled out by such rather crude measurements as eosin counts, total cell counts, or microscopic examination. Studies on the damaging effect of adenine on mouse embryo skin and mouse sarcoma in tissue culture(8), as well as on partial inhibition of anaphase activity in dividing cells in chick tissue culture (9), used levels above 500 μg/ml. Hakala and Taylor(10) showed that growth of HeLa cells over a 7-day period was inhibited by adenine concentrations above 10^{-4} M while mouse sarcoma (S180) cells were not affected by higher concentrations. There may be similar differences in adenine sensitivity between K_2, EAC, and L cells. Aronow(11) reported that adenine at concentrations of 10^{-3} M produced inhibition of L cell growth that could be reversed by uridine or cytidine. The author postulated that high levels of adenine might deplete the cells of their pool of available phosphoribosylpyrophosphate (PRPP).

Still to be considered is the original concept for the investigation, namely that feedback mechanisms, whether through direct inhibition or repression of enzyme formation may have an effect on viral replication. However, if L cells and ascites tumor cells have the same purine biosynthetic pathways, one should see the same adenine effect unless the continuous cell culture in a complete medium represents a more stable state less affected by changes in the medium. One other point of interest is that EAC and K_2 cells in the simplified medium used here are slowly degenerating while L cells continue to grow in a more complex medium. Whether this difference plays a role in the adenine effect on virus growth is an interesting speculation.

Summary. Addition of 100-500 μg/ml of adenine under a variety of experimental conditions to Krebs 2 and Ehrlich ascites tumor cell suspension in a simplified medium was found to delay growth of encephalomyocarditis virus. The approximately 2-log lower yield in both supernatant and cell-associated virus observable at 24 hours was less apparent after this time. The replication of EMC virus in L cells as well as influenza, PR_8, in CAM roller tubes, and deembryonated eggs was unaffected by adenine under the experimental conditions tested. Other related compounds were essentially without effect at nontoxic levels.

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Effect of Adrenalectomy and Corticoid Replacement on Mammary Gland Growth in Rats.* (27111)

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Evidence concerning the need of adrenocorticoids in normal mammary gland growth is positive but scanty. Lyons *et al.*(1) found that desoxycorticosterone at a level of 100 μg per day synergized with growth hormone and estrone in the hypophysectomized, ovariectomized, adrenalectomized rat to produce mammary growth comparable to that in the intact animal. A similar synergizing action of adrenocorticoids with growth hormone and estrone was reported by Cowie and Lyons (2).

The following experiments were conducted in adrenalectomized-ovariectomized (ADRX-OVARX) rats to determine the role of adrenocorticoids on mammary gland growth. It was felt that use of quantitative DNA index (3) of mammary growth would produce results more meaningful than those reported using qualitative or semi-quantitative morphological technics.

Materials and methods. Mature female albino rats of Sprague-Dawley-Rolfsmeyer strain weighing at least 200 g were maintained on standard laboratory pellet feed at constant temperature of 78 ± 1°F. Adrenalectomy and ovariectomy were performed in one operation. During a post-operative recovery period of 14 days, 1% saline drinking water was provided to maintain electrolyte balance. This was continued in treatment groups except those given desoxycorticosterone acetate (DOCA).

One μg estadiol benzoate (EB) and 3 mg progesterone (P) were injected subcutaneously in 0.1 ml carrier each day for 19 days. The levels and ratio were found by Moon *et al.*(3) to be optimal in rats. Carrier consisted of 10% benzyl alcohol and 90% olive oil.

Adrenocorticoids were suspended in physiological saline with aid of 5 mg gum arabic per 100 mg steroid and injected subcutaneously each day in 0.1 to 0.25 ml carrier. Prednisone (1,2-dehydrocortisone) was injected alone and in combination with EB and P at 100 μg per day. Hydrocortisone acetate (HC) was injected alone at 100 μg per day and in combination with EB and P at 100, 250, and 1000 μg per day. DOCA was injected alone and in combination with EB and P at 250 μg per day.

Animals were sacrificed on the day following the nineteenth day of injection. Six posterior mammary glands were removed and

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