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MODE OF ACTION OF ALANOSINE*

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Abstract—Alanosine inhibits the synthesis of RNA adenine from formate-^{14}C and from glycine-^{14}C in Ehrlich ascites tumor cells *in vivo*. There is no appreciable depression of the rate of RNA guanine synthesis. The incorporation of preformed adenine-^{14}C into RNA purines is unaffected. Inhibition of RNA adenine synthesis is not antagonized by glutamine or aspartate. The incorporation of thymidine-^{3}H, uridine-^{3}H, and L-leucine-^{14}C into the acid-insoluble fraction of the cells *in vitro* is unaffected by the drug. Evidence indicates that alanosine may interfere with the conversion of inosine monophosphate to adenosine monophosphate.

A RECENT report by Murthy *et al.*[1] described the production by *Streptomyces alanosinicus* nov. sp. (ATCC 15710) of an antibiotic with antitumor and antiviral properties. Purification and characterization revealed the compound to be L(—) 2-amino-3-nitroso hydroxylamino propionic acid, subsequently designated "alanosine". Cytopathic effects induced by neurovaccinia, sheep pox, and cow pox viruses in a human embryo epithelial cell line were inhibited by 2–8 μg/ml of the drug, and significant protection from the neurovaccinia virus was evident even when treatment was delayed 24 hr after infection. Protection *in vivo* against neurovaccinia was clearly demonstrated. There was a marked antitumor activity against a transplanted fibrosarcoma induced by SV-40 virus in hamsters, even though the drug had no action *in vitro* against the virus. Well developed tumors after treatment regressed to such a size as to be no longer measurable. In a personal communication, one of the authors of the initial report[1] (Y.K.S.M.) related that the antitumor spectrum of alanosine encompasses other solid and liquid neoplasms.

The structural formula of alanosine shows certain similarities to that of azaserine, a known glutamine antagonist[2] (Fig. 1). The major pharmacological action of azaserine is inhibition of the conversion of formylglycineamide ribonucleotide to formylglycineamidine ribonucleotide, leading to a diminished rate of *de novo* purine synthesis.[3] The following work was initiated to determine if the similarities in chemical structures of the two antibiotics are accompanied by similar modes of action.

EXPERIMENTAL

Alanosine was furnished by Dr. Piero Sensi of Lepetit S.p.A., Milan, Italy. Azaserine was a gift from Parke, Davis & Co. through Dr. J. R. Dice. Other chemicals used were of reagent grade from commercial sources. Radioisotopes were from New

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England Nuclear Corp., and Eagle's Minimum Essential Medium with Hank's balanced salt solution (MEM) was from Microbiological Associates. BALB/C mice used for propagating the Ehrlich ascites tumor cells were from Dublin Farms, Dublin, Va.

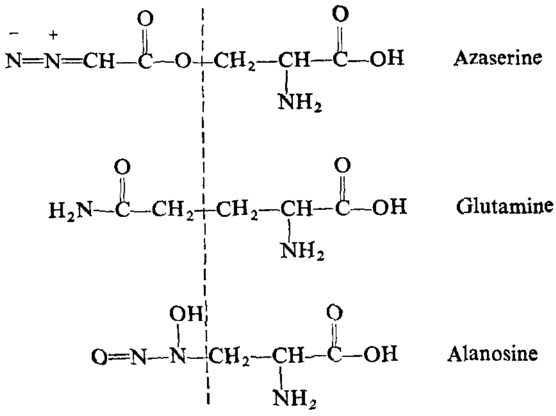

FIG. 1. Structural relationship of alanosine to glutamine and azaserine.

A method similar to that described previously[4] was used to determine rates of synthesis of DNA, RNA, and protein in Ehrlich ascites tumor cells. Cells were removed from mice 5–8 days after i.p. inoculation, washed twice with MEM, and finally suspended to 1% in MEM. Test tubes (18 mm o.d.) served as reaction vessels and were maintained at 37° with gentle agitation. Alanosine was dissolved in dimethyl sulfoxide, and azaserine in saline. Each reaction mixture consisted of 5·0 ml cell suspension, 0·05 ml dimethyl sulfoxide or 0·5 ml saline with or without the drug, and 0·5 ml saline containing 2 μc/ml of thymidine-methyl-^{3}H, uridine-^{3}H, or L-leucine-^{14}C, uniformly labeled, to measure the synthesis of DNA, RNA, and protein, respectively. Two-ml samples were removed at the indicated times and added to 2 ml of cold 10% trichloroacetic acid (TCA). The insoluble cell material was washed 3 times with cold 5% TCA by centrifugation and resuspension, and finally resuspended in 0·5 ml methanol. Two ml of 1·0 M hydroxide of Hyamine (Rohm and Haas) was added to effect solubilization, and the contents of each tube were transferred to 15 ml phosphor solution (PPO-POPOP, Packard Instrument Co.) in toluene. Radioactivity of each sample was measured with a Mark I liquid scintillation spectrometer (Nuclear Chicago Corp.).

Glycine-^{14}C and sodium formate-^{14}C were used to determine the effect of alanosine and azaserine on the *de novo* synthesis of RNA purines. Groups of 3 mice bearing 7- or 8-day-old tumor cells were given each drug i.p. in saline. Control tumor-bearing mice received only saline. After 2 hr, isotopic glycine or formate in saline was injected into all animals. After a further period of 3 hr, all mice were sacrificed and the cells from each group were pooled. Cells were rinsed with cold saline and centrifuged in a Lourdes refrigerated centrifuge at 5000 rpm for 5 min. Supernatant solutions were discarded and the cells were mixed with a small amount of distilled water to lyse the few erythrocytes invariably present. Isotonicity was restored after

45 sec. The isolation of RNA and its hydrolysis to the purine bases were conducted substantially according to the method of Korn;[5] all barium solutions were filtered just prior to use. After hydrolysis to the purine bases, each sample was spotted onto Whatman No. 1 paper; descending chromatography was done with an *n*-butanol: ammonia (6 : 1) solvent, freshly prepared before each experiment. Purines were identified by predicted R_f values and by comparison with migrations of authentic samples. Radioactive sites were located with a radiochromatogram scanner (Actigraph III, Nuclear-Chicago Corp.) and then eluted into 3 ml methanol by agitation in scintillation vials. Fifteen ml phosphor solution was added and the radioactivity of each sample was measured with the liquid scintillation spectrometer.

RESULTS

Table 1 shows the results of two experiments in which various doses of alanosine were given to tumor-bearing mice and the effects on purine incorporation into nucleic acid were assessed. By using glycine as precursor and alanosine at 25 mg/kg, adenine synthesis was markedly reduced and guanine was depressed slightly (experiment A). In experiment B, the inhibition of RNA adenine synthesis was confirmed, and the response over the range tested was dose-dependent. The rate of RNA guanine synthesis was increased at all three dose levels. The data in Table 1 may be compared with those in Table 2, which depict the alterations of both RNA purines conferred by administration of azaserine *in vivo*.

TABLE 1. EFFECT OF ALANOSINE ON THE SYNTHESIS OF RNA PURINES IN EHRLICH ASCITES TUMOR CELLS *in vivo*

Expt.	Precursor (10 μc)	Alanosine (mg/kg)	RNA (cpm/mg)			
			Adenine	Per cent change	Guanine	Per cent change
A	Glycine	0	215		542	
	Glycine	25·0	13	−94	372	−31
B	Formate	0	3977		2867	
	Formate	2·5	2417	−39	3446	+20
	Formate	12·5	633	−84	3100	+8
	Formate	25·0	440	−89	4000	+40

TABLE 2. EFFECT OF AZASERINE ON THE SYNTHESIS OF RNA PURINES FROM FORMATE-^{14}C (10 μc) IN EHRLICH ASCITES TUMOR CELLS *in vivo*

Azaserine, (mg/kg)	RNA (cpm/mg)			
	Adenine	Per cent change	Guanine	Per cent change
0	2567		3031	
2·5	95	−96	655	−78
12·5	28	−99	641	−79
25·0	26	−99	537	−82

To determine if the depression by alanosine of the rate of RNA adenine synthesis was a result of a block in its synthesis or its incorporation after synthesis, the effect of the drug on the incorporation of preformed adenine into RNA purines was investigated; results are shown in Table 3. Isotopic adenine recovered from RNA actually increased as a function of the dose of alanosine administered, while that of guanine decreased slightly. Such a finding is consistent with a hypothesis of inhibition of synthesis of adenine from lower molecular weight precursors. The resulting diminution of the adenine pool would permit a greater extent of incorporation of the exogenous isotopic adenine, and the larger amount of the latter incorporated at higher dose levels would permit lesser amounts to be converted to guanine.

TABLE 3. EFFECT OF ALANOSINE ON THE INCORPORATION OF ADENINE (5 μC) INTO RNA PURINES IN EHRLICH ASCITES TUMOR CELLS *in vivo*

Alanosine (mg/kg)	RNA (cpm/mg) Adenine	Per cent change	Guanine	Per cent change
0	3461		637	
2·5	4397	+27	620	−3
12·5	4585	+32	564	−11
25·0	5564	+61	506	−21

The action of azaserine on RNA purine synthesis is readily antagonized by glutamine.[2] To determine if this characteristic is shared by alanosine, glutamine was administered to tumor-bearing mice 30 min prior to injecting alanosine. The total amount of glutamine administered per mouse was adjusted so as to be 100 times that of alanosine on a molar basis. Aspartic acid was also tested as a potential antagonist in the same experiment at the same molar dose as glutamine. Table 4 shows that glutamic acid was without appreciable antagonistic activity on the inhibition of RNA adenine synthesis. The 51 per cent increase in RNA guanine synthesis in the presence of alanosine and excess glutamine was consistent with an increased velocity of an uninhibited sequential enzymic system resulting from an elevated substrate concentration. Aspartate was totally devoid of antagonistic activity.

TABLE 4. EFFECT OF GLUTAMINE AND ASPARTATE ON THE INHIBITION BY ALANOSINE OF RNA PURINE SYNTHESIS FROM FORMATE-^{14}C (5 μC) IN EHRLICH ASCITES TUMOR CELLS *in vivo*

Condition	RNA (cpm/mg) Adenine	Per cent change	Guanine	Per cent change
Control	1515		2500	
Alanosine, 25 mg/kg	163	−89	2346	−6
Alanosine + glutamine, 2540 mg/kg	408	−73	3777	+51
Alanosine + aspartate, 2230 mg/kg	133	−91	1941	−22

Azaserine had no effect on the synthesis of DNA, RNA, or protein *in vitro* when these parameters were assessed by the rate of incorporation of isotopic thymidine, uridine, or L-leucine, respectively. Alanosine was without effect on the synthesis of DNA and RNA under these conditions, and induced only a moderate depression of the rate of protein synthesis (Fig. 2).

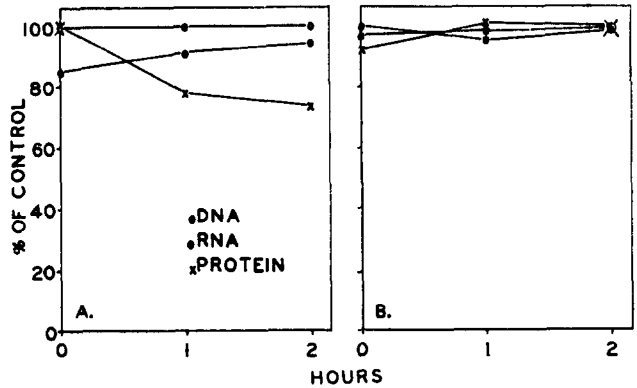

FIG. 2. Effect of (A) alanosine and (B) azaserine on the synthesis of DNA, RNA, and protein in Ehrlich ascites tumor cells *in vitro*, as assessed by the incorporation of isotopic thymidine, uridine, and L-leucine, respectively. Drugs were present at 10^{-3} M.

DISCUSSION

The data concerning the effects of alanosine on experimental viral infections and tumors suggest that some therapeutic benefits may be achieved ultimately with this agent. Of considerable interest is the mechanism by which the relatively selective pharmacological action is achieved. Examination of the two-dimensional structural formula of alanosine reveals that it, like azaserine, is an alanine derivative, and contains a hydroxylamino and a nitroso group instead of the diazo group of azaserine. The preliminary results reported here support a hypothesis of purine synthesis inhibition; however, unlike the action of azaserine, the action of alanosine appears to be one of inhibition of adenine synthesis only with no appreciable effect on guanine synthesis.

Since there is a common pathway for the synthesis of adenine and guanine up to the step at which 5-formamido-4-imidazole-carboxamide ribonucleotide undergoes closure of the 6-membered ring to form inosine monophosphate (IMP), the site of action is likely subsequent to this step. In addition, since preformed adenine is readily incorporated into the RNA of alanosine-treated cells, it appears that a possible locus of action is interference with the conversion of IMP to adenosine monophosphate (AMP). The two steps of primary concern are, consequently, the addition of aspartate to IMP,[6] and removal of fumarate from the adenylosuccinate thus formed to yield AMP.[6, 7] The failure of aspartate to antagonize the inhibition of RNA adenine synthesis under the conditions tested should not rule out the possibility of inhibition of the former reaction, since the potentially alkylating nitroso group of alanosine may confer inhibition of an irreversible nature. Clearly, additional studies of the two reactions above in cell-free systems are needed to clarify the exact site of action and the kinetics of the enzyme depression.

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