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STUDIES ON THE MECHANISM OF RESISTANCE OF SELECTED MURINE TUMORS TO L-ALANOSINE

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Abstract—Sublines of P388 and L1210 leukemia were rendered resistant to L-alanosine [L-2-amino-3-(*N*-hydroxy-*N*-nitrosamino) propionic acid] and designated P388/LAL and L1210/LAL. Assessments were made of certain biochemical and pharmacological determinants of the sensitivity or resistance to L-alanosine of these sensitive and resistant lines. It was observed that the antibiotic strongly inhibited adenylosuccinate synthetase and DNA synthesis only in the parent or sensitive lines; moreover, after a therapeutic dose of the drug, the concentration of L-alanosyl-AICOR (L-alanosyl-5-amino-4-imidazole carboxylic acid ribonucleotide), the putative active anabolite of L-alanosine, was dramatically higher in these parent lines as compared with the resistant variants. Enzymologic studies established that, in P388/LAL, the specific activity of the enzyme SAICAR synthetase (5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide synthetase), which is believed to conjugate L-alanosine with the nascent purine AICOR (5-amino-4-imidazole carboxylic acid ribonucleotide), was depressed significantly; the same was not true for L1210/LAL. In both resistant lines, however, the enzymes of purine salvage were present at levels about 200 per cent higher than those measured in the native strains. These studies establish that resistance to L-alanosine is very likely pluricausal, but that the ability of susceptible cells to synthesize or retain L-alanosyl-AICOR is an element important to the process.

It is a well established fact that parenteral doses of the antitumor antibiotic L-alanosine [L-2-amino-3-(*N*-hydroxy-*N*-nitrosamino)propionic acid] interrupt the biosynthesis of AMP and therefore of ATP, dATP and DNA [1–3]. A substantial body of evidence indicates that this interruption takes place at the level of adenylosuccinate synthetase, the penultimate enzyme involved in the biosynthesis of adenine nucleotides [3, 4]. It also seems clear that L-alanosine itself is not the proximate inhibitor of this enzyme; an anabolite of the drug, resulting from its conjugation with AICOR (5-amino-4-imidazole carboxylic acid ribonucleotide), is the final antimetabolite [3, 5]. The enzyme accomplishing this unique reaction is SAICAR synthetase (5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide synthetase), and the product, L-alanosyl-AICOR (L-alanosyl-5-amino-4-imidazole carboxylic acid ribonucleotide) has been shown to be a powerful inhibitor of adenylosuccinate synthetase [3]; Fig. 1 is a diagram of these interrelationships. This diagram also suggested the possibility that L-alanosyl-AICOR, by analogy to its natural homolog, SAICAR (5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide), ought to be susceptible to decomposition by adenylosuccinate lyase. Thus, it can be appreciated that three enzymes of *de novo* purine biosynthesis are involved in the action of L-alanosine. To assess the relative importance of these enzymes, measurements have been made of their specific activities in several murine tumors known to be responsive to the antibiotic, and also in variants of these same tumors rendered resistant to L-alanosine by repeated subcurative treatments with the drug. Inasmuch as the correlations observed were imperfect, a search has also been mounted for additional biochemical and pharmacological determinants, that might contribute to the refractory state.

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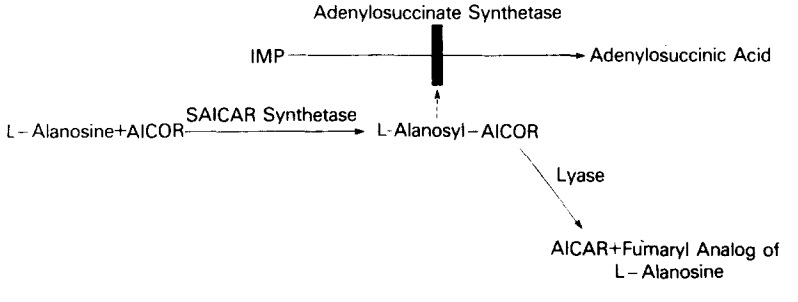

Fig. 1. Interaction of L-alanosine with the enzymes of purine biosynthesis.

MATERIALS AND METHODS

L-Alanosine (NSC 153353) was obtained from the Drug Research and Development branch of the National Cancer Institute, Bethesda, MD. DL-[1-^{14}C] and [3-^{14}C]Alanosine (sp. act. 7.14 and 7.41 mCi/mmole respectively) were supplied by the Stanford Research Institute, Menlo Park, CA. This radioactive alanosine was purified as described earlier [1]. L-[4-^{14}C]Aspartic acid (sp. act. 19.5 mCi/mmole) was obtained from Amersham/Searle, Arlington Heights, IL. [2-^{14}C]Thymidine (sp. radioact. 53.2 mCi/mmole) was purchased from the New England Nuclear Corp., Boston, MA. Adenosine-5′-triphosphate, adenosine-5′-diphosphate, guanosine-5′-triphosphate, inosine-5′-monophosphate and adenylosuccinic acid were purchased from the Sigma Chemical Co., St. Louis, MO. Dithiothreitol (DDT) was the product of CalBiochem, Gaithersburg, MD. AICOR was synthesized by alkaline hydrolysis of 5-amino-4-imidazole carboxamide ribonucleotide [6] which, in turn, was synthesized from the corresponding ribonucleoside by treatment with $POCl_3$ in trimethylphosphate at 5°, followed by hydrolysis in cold water [6]. Most of the microchemical and radiochemical analyses were carried out in Eppendorf 1600 μl polypropylene centrifuge vessels with secure snap-on lids procured from Brinkmann Instruments, Inc., Westbury, NY [7].

Animals. Male BDF_1 and CDF_1 mice, on an *ad lib*. diet of Purina mouse chow, were used during the course of these studies.

Preparation of homogenates. BDF_1 mice bearing ~ 1 cm subcutaneous tumors were killed by cervical dislocation; the tumors were removed, flash frozen, and homogenized (1:3, w/v) in 0.1 M Tris–HCl buffer, pH 7.5, containing 0.5 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM dithiothreitol. Homogenates were centrifuged at 12,000 *g* for 3 min, and the supernatant fractions were used for the analyses described below.

Protein estimation. Protein was estimated using the Bio-Rad kit for protein determination [8]. Thus, 10 μl aliquots of a suitably diluted supernatant solution were added to 1 ml of the dye solution, prepared and diluted according to the manufacturer's directions. The absorption at 595 nm was then measured within 5–60 min. Aliquots of a solution of bovine albumin, Fr. 5 (1 mg/ml), were used as standards.

Decarboxylation reagent. The decarboxylation reagent of Milman and Cooney [9] was used. The reagent contained α-ketogutarate (0.005 M) and zinc sulfate (0.03 M) in 0.66 M sodium acetate buffer, pH 5.0; to this solution, 40 I.U./ml of L-glutamate oxaloacetate transaminase was added at the time of use.

Development of sublines of P388 *and* L1210 *leukemia resistant to* L-*alanosine*. Resistant sublines were developed by the intraperitoneal (i.p.) transplantation of 10^5 cells of L1210 or 10^6 cells of P388 into $(BALB/c \times DBA/_2)F_1$ mice (commonly known as CDF_1) that were treated daily with 40 mg L-alanosine/kg. Treatment was continued until florid ascites was evident, at which time tumor cells were collected from two animals and pooled. Inocula of 10^6 or 10^5 cells in the case of P388 or L1210, respectively, were then implanted i.p. in another group of mice, and treatment with L-alanosine was repeated. At every second to fifth transplant generation, cells fromdrug-treated animals (L1210/LAL or P388/LALcells) were implanted in groups of six $(BALB/c \times DBA/_2)F_1$ mice, as were the parental cell lines. These animals were treated with L-alanosine on days 1, 5, and 9 at 600, 400, and 267 mg/kg, i.p. Increase in survival time at the maximally tolerated dose of L-alanosine was determined to indicate whether resistance to the drug had arisen. After resistance to L-alanosine had been clearly demonstrated,* the sublines were serially transplanted for many generations in untreated mice to show whether resistance to L-alanosine was a stable characteristic of L1210/LAL or P388/LAL.

The sensitivity of L1210/LAL to a variety of antitumor agents was also compared with that of L1210. Drugs were administered i.p. on days 1–9 or days 1, 5, and 9 after i.p. implantation of 10^5 leukemia cells in groups of six $(BALB/c \times DBA/_2)F_1$ mice. Drugs were administered at four logarithmically spaced dose levels, and activity was determined by mean survival time relative to untreated controls. Similar comparisons were done with P388 and P388/LAL. The inoculum level for these tumors, however, was 10^6 cells. L-alanosine was tested in all experiments in which other drugs were evaluated.

Transport of L-*alanosine in* P388 *and* L1210 *sensitive and resistant tumors*. Tumor cells for the study of the transport of L-alanosine were prepared by the following techniques: (1) male BDF_1 mice were injected (i.p.) with 10^5 cells of L1210 or L1210/LAL; 5 days later, tumor cells were aspirated from the peritoneum and processed as described below; and (2) male BDF_1 mice were injected with 10^6 cells of P388 or P388/LAL; 5 days later, tumor cells were aspirated from the peritoneum into Dulbecco's PBS medium* with 1% glucose (pH 7.4); they were washed twice in the same medium and the cell density was adjusted to 1.5×10^6 cells/ml. Transport studies were performed at 37° with gentle agitation of the cells; DL-[1-^{14}C]alanosine was added to yield final concentrations ranging from 0.2 to 50.0 mM; at appropriate times, 900 μl aliquots were transferred into microcentrifuge tubes containing 700 μl of Versilube-F50 silicone oil. Transport was terminated by centrifugation of the cells through the oil at 12,000 *g* for 1 min in an Eppendorf centrifuge. The cell pellets were solubilized in 1.5 ml of 0.2 N NaOH at 37° overnight. The pH was adjusted back to neutrality using 5 N acetic acid; scintillation fluid was then added, and radioactivity was measured by liquid scintillation spectrometry.

DNA synthesis in P388 *and* L1210 *tumors sensitive and resistant to* L-*alanosine*. One million cells each

* The term "sensitive" is used to indicate an increment of 30 per cent or more in life span (ILS) relative to untreated control mice; the term "resistant" represents any ILS less than this.

* Dulbecco's PBS medium contains: 8 g sodium chloride, 0.2 g potassium chloride, 1.15 g disodium hydrogen phosphate, 0.2 g potassium dihydrogen phosphate, 0.1 g magnesium chloride, 0.1 g calcium chloride and distilled water added to make it 1 liter.

of P388 and P388/LAL were separately implanted subcutaneously into the right and left flanks, respectively, of twenty BDF_1 mice. After 10 days, a group of ten animals received L-alanosine (500 mg/kg), while another group of ten animals received an equal volume of saline. After 4 hr, all twenty animals were given 5 μCi of [2-^{14}C]thymidine, i.p. One hr after the injection of thymidine, animals were killed and the tumors were removed immediately. A group of twenty animals was treated exactly in the same manner with the L1210 or L1210/LAL cells, except that only 10^5 cells were implanted in this case. The rest of the treatment was the same as in the case of the P388 line.

Tumors were homogenized in 5% trichloroacetic acid (TCA), and the homogenates were centrifuged at 12,000 *g* for 3 min; the pellets were washed thrice with 1.0% TCA, after which it was verified that the supernatant fraction from a 1.0% TCA suspension of the resulting pellet was free of radioactivity. After decanting this supernatant fraction, 1.0 ml of 10% TCA was added to the pellets and mixed well; then the suspensions were heated at 95° for 1 hr in closed Eppendorf tubes. After cooling and centrifugation at 12,000 *g* for 3 min, 100 μl of the supernatant fraction was taken, and its radioactivity was measured by scintillation spectrometry. A suitable aliquot was also taken for the spectrophotometric measurement of DNA [10].

Effect of L-*alanosine on protein synthesis in* P388 *and* L1210 *tumors sensitive and resistant to* L-*alanosine.* Groups of sixteen BDF_1 male mice were injected with 10^6 cells of P388 or 10^5 cells of L1210 into the left flank and 10^6 cells of P388/LAL or 10^5 cells of L1210/LAL into the right flank. Eight days later, the animals were fasted overnight, and divided into two groups of eight animals each. The first group of animals received an i.p. dose of L-alanosine (500 mg/kg); the second group received saline. Four hours later, 4 μCi of L-[2-^{14}C]valine/mouse was administered i.p. After 1 hr, tumors were removed, frozen on solid CO_2, and later homogenized in freshly prepared 10% trichloroacetic acid. The homogenate was centrifuged at 12,000 *g* for 5 min, the supernatant fraction was discarded, and the pellet was washed twice with 1.0% TCA; 250 μl of 40% NaOH was then added to the final pellet and the samples were heated at 95° for 10 min. After dissolving the proteins, 750 μl of water was added to each vessel, and a suitable aliquot was taken for the measurement of radioactivity and protein using the method of Lowry *et al.* [11].

Measurement of L-*alanosine and its catabolic products in sensitive and resistant tumors.* To groups of ten BDF_1 mice, 10^6 cells of P388 or 10^5 cells of L1210 were implanted subcutaneously into the right flank and 10^6 cells of P388/LAL or 10^5 cells of L1210/LAL were implanted subcutaneously into the left flank. After 10 days, the animals received DL-[1-^{14}C] alanosine (10 μCi/mouse) plus L-alanosine (500 mg/kg). Four hours later, the animals were killed, the tumors were removed and homogenized in 1 N acetic acid (1:3, w/v), and the homogenates were centrifuged at 12,000 *g* for 12 min. Five microliters of the resulting supernatant fraction was then electrophoresed using 0.05 M soduim phosphate buffer, pH 7.2, at 25 V/cm. All samples were overspotted with a mixture of L-alanosine, "α-decarboxy-L-alanosine" [1-amino-2-(*N*-hydroxy,*N*-nitrosamino) ethane] and "α-hydroxy-L-alanosine" [2-hydroxy,3-(*N*-hydroxy, *N*-nitrosamino) propionic acid] [12]. The spots of L-alanosine and its derivatives were identified by u.v. absorption and excised for the measurement of their radioactivity [12].

Estimation of SAICAR synthetase. SAICAR synthetase was estimated as described by Tyagi *et al.* [6].

Adenylosuccinate synthetase. In a total volume of 20 μl were admixed: 5 μl of L-[4-^{14}C]aspartic acid (6.37 nmoles, 125 nCi), 5 μl of GTP–$MgCl_2$ (150 nmoles each), 5 μl of IMP (150 nmoles); or 5 μl of Tris–HCl buffer, pH 8.0 (250 nmoles); 5 μl of supernatant fraction was added last to initiate the reaction. After 30 min at 37°, the reaction was stopped by adding 50 μl of decarboxylation mixture and incubating at 37° for 3 hr or at room temperature overnight [9]. Residual radioactivity was then measured by scintillation spectrometry.

Adenylosuccinate lyase. Adenylosuccinate lyase was measured as described earlier [13].

Concentration of L-*alanosyl-AICOR in* P388 *and* L1210 *sensitive and resistant tumors.* Measurements of the concentration of L-alanosyl-AICOR were carried out as described earlier [3]. Mice bearing P388 and L1210 sensitive and resistant tumors were treated with 50 μCi of DL-[1-^{14}C]alanosine/mouse, along with 500 mg/kg L-alanosine. After 2 hr, the animals were killed, and the tumors were removed, frozen, homogenized in 1 N acetic acid, and centrifuged for 12 min at 12,000 *g*. A 0.8 ml aliquot of the resulting supernatant fraction was loaded on a 0.8 × 15.0 cm column of Hamilton HA-X4 resin equilibrated with 0.015 M lithium–citrate–chloride buffer, pH 2.65 [14], and operated at a flow rate of 0.67 ml/min. After loading the sample, the column was developed with the equilibrating buffer for 90 min at 37°, followed by 0.0375, 0.075, 0.15 and 0.225 M lithium–citrate–chloride buffers (pH 2.72) applied for periods of 60, 60, 75 and 60 min respectively [14]. L-Alanosyl-AICOR eluted from this column as a single symmetrical peak at 260 min. Radioactivity of peak fractions was counted in a Beckman LS-230 liquid scintillation spectrometer and the concentration of L-alanosyl-AICOR was calculated using the specific activity of the final injectate.

Adenosine kinase. Five microliters of [8-^{14}C] adenosine (7 nmoles, 455 nCi), 5 μl of 0.05 M Hepes buffer* (pH 6.8), 5 μl each of ATP and $MgCl_2$ (20 nmoles and 6 nmoles, respectively, in 0.05 M Hepes buffer, pH 6.8), and, to initiate the reaction, 5 μl of enzyme extract were added into Eppendorf test tubes. Control vessels contained only Hepes buffer instead of ATP and $MgCl_2$. The reactants were incubated for 15 min at 37°, at which point 20 μl of 2 N HCl containing 0.01 M AMP was added to arrest the reaction. Aliquots (5 μl) of the resultant reaction mixtures were then spotted on Whatman 3M paper. Ascending chromatography was performed using isopropanol–EDTA (saturated sol-

* HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

ution)–toluene–14 M NH_4OH (320:44:40:4, by vol.) as solvent for 16 hr [15]. Ultraviolet-absorbing spots of AMP at the origin were excised, and their radioactivity was measured by scintillation spectrometry.

Hypoxanthine and adenine phosphoribosyl transferases. For measuring the activity of hypoxanthine phosphoribosyltransferase (HPRTase) the following were pipetted in Eppendorf tubes: 10 μl of [8-^{14}C]hypoxanthine (6 nmoles, 332 nCi), 10 μl of phosphoribosylpyrophosphate (PRPP) and $MgCl_2$ (60 and 180 nmoles, respectively, in 0.2 M Tris buffer, pH 8.0), and 10 μl of enzyme extract to initiate the reaction. In control vessels, PRPP was replaced by 10 μl of 0.2 M Tris buffer, pH 8.0 The reaction was started by a brief centrifugation, and incubation at 37° was carried out for 30 min, whereupon the reaction was stopped by adding 20 μl of 2 N HCl. After a 3-min centrifugation at 12,000 *g*, 5 μl of the supernatant fraction was spotted on Whatman 3M paper and overspotted with 5 μl of a mixture of IMP and hypoxanthine (0.01 M each). Ascending chromatograms were developed using ammonium acetate (1 M, pH 7.0)–ethanol (30:70, v/v) as solvent for 16 hr. The IMP spots were excised, and their radioactivity was measured. For calculation of enzyme activity, radioactivity in the vessels lacking PRPP was subtracted from that in the vessels receiving PRPP.

In the case of adenine phosphoribosyltransferase (APRTase), [8-^{14}C]hypoxanthine was replaced by 10 μl of [8-^{14}C]adenine (6.0 nmoles, 315 nCi); the chromatograms were overspotted with adenine and AMP, developed, and processed as described above.

Inosine phosphorylase. For the measurement of inosine phosphorylase, 10 μl of [8-^{14}C]hypoxanthine (8 nmoles, 442 nCi), 10 μl of ribose-1-phosphate (300 nmoles in 0.1 M, Tris–HCl buffer, pH 7.0), and 10 μl of the enzyme extract were incubated at 37° for 15 min. Control vessels received 10 μl of 0.1 M Tris buffer, pH 7.0, in place of ribose-1-phosphate. The reaction was stopped by adding 20 μl of 2 N HCl. For the separation of hypoxanthine and inosine, 5 μl aliquots of these reaction mixtures were spotted on Polygram CEL 300 PEI plates and overspotted with 5 μl of a mixture of inosine and hypoxanthine (0.01 M each). The plates were developed in distilled water for 2 hr [16], and the spots of inosine were excised for scintillation spectrometry.

Adenosine phosphorylase. Five microliters of [8-^{14}C]adenosine (7 nmoles, 455 nCi), 5 μl of 0.2 M Hepes buffer, pH 7.0, 5 μl of 0.05 M sodium phosphate solution, pH 7.0, and 5 μl of enzyme extract were mixed in Eppendorf test tubes. Control vessels received 5 μl of Hepes buffer in place of phosphate. The vessels were incubated at 37° for 15 min, and the reaction was stopped by adding 20 μl of 2 N HCl; 5 μl aliquots of these reaction mixtures were spotted on Whatman 3M chromatography paper and overspotted with 5 μl of a mixture of adenine and adenosine (0.01 M each). The chromatographs were developed in an ascending manner using ammonium acetate (1 M, pH 7.0)–alcohol (30:70, v/v) as solvent for 16 hr. The u.v. spots of adenine were excised and their radioactivity was measured by scintillation spectrometry.

Decomposition of L-*alanosyl-AICOR by adenylosuccinate lyase.* In a total volume of 800 μl were admixed: 200 μl of L-alanosyl-AICOR (20 nmoles, sp. act. 7.14 nCi/nmole) and 600 μl of lyase, partially purified from baker's yeast [sp. act. 540 nmoles·hr^{-1}·(mg protein)$^{-1}$]. Controls received enzyme pre-

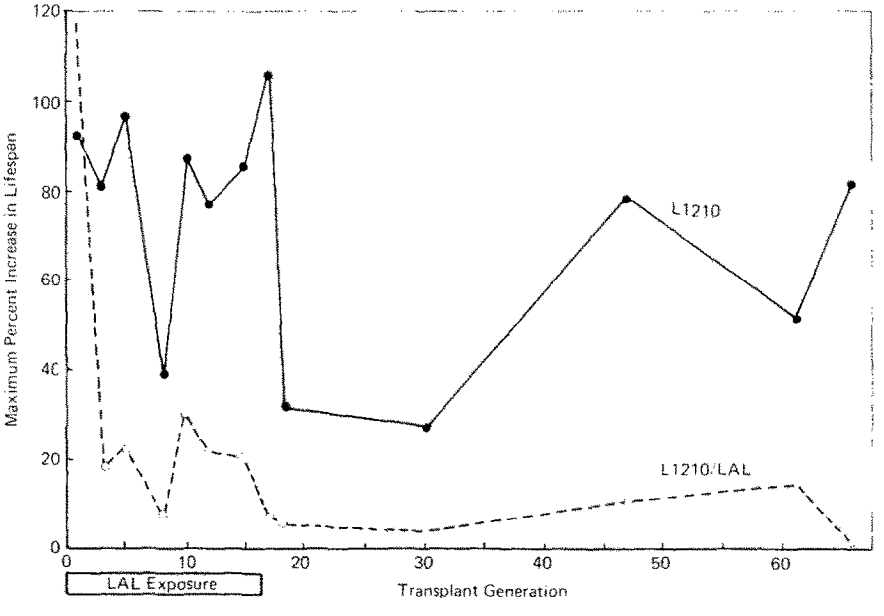

Fig. 2. Development and stability of a subline of L1210 leukemia cells resistant to L-alanosine. Each point represents the maximum increase in lifespan relative to untreated control mice bearing i.p. L1210/LAL or L1210. Tumors were evaluated in parallel at three dose levels of L-alanosine: 600, 400 and 267 mg/kg, i.p. given on days 1, 5, and 9. L1210/LAL was transplanted for 16 generations in mice whcih were treated i.p. with 40 mg L-alanosine/kg daily. L1210/LAL was carried in untreated mice thereafter.

viously heated at 95° for 30 min, and the reactants were incubated at 37° for 4 hr. In another set of experiments, the following were admixed: 20 μl of L-alanosyl-AICOR (20 nmoles, sp. act. 7.14 nCi/nmole) and 100 μl of freshly prepared mouse muscle extract [sp. act. 720.25 nmoles· hr^{-1}· (mg protein)$^{-1}$]; controls received enzyme heated at 95° for 30 min. Incubations were carried out at 37° for 4 hr. The reaction was stopped by the addition of 200 μl of 0.015 M lithium–citrate–chloride buffer, pH 2.65. The volume was made up to 1.0 ml with H_2O, and an aliquot of 800 μl was loaded on a 0.8 × 15 cm column of Hamilton HA-X4 resin, pre-equilibrated with 0.015 M lithium–citrate–chloride buffer, pH 2.65. The elution was performed as described earlier [14]. L-Alanosyl-AICOR and the presumptive fumaryl analog of L-alanosine [3-(*N*-nitroso-*N*-hydroxy) propenoic acid] eluted from this column at 260 and 135 min respectively. Radioactivity in all fractions was measured using a Beckman LS230 liquid scintillation counter.

The same procedure was followed to determine the rates of decomposition of L-alanosyl-AICOR by adenylosuccinate lyase from P388, P388/LAL, L1210 and L1210/LAL tumors, except that the incubations in this case were carried out for 30 min, during which time the reaction remained linear.

RESULTS

Therapeutic evaluation of L-alanosine-resistant sublines of L1210 *and* P388 *leukemia cells*. Resistance to L-alanosine occurred readily in leukemic cells exposed to the antibiotic on a daily basis *in vivo*. As shown in Fig. 2, the maximum response of L1210 leukemia cells to L-alanosine was rather variable. The greatest increase in lifespan (ILS) observed in these experiments was 106 per cent; in three other studies (not shown) the maximum ILS achieved was only 30–40 per cent. After one transplant generation, during which treatment with L-alanosine was continued, this tumor remained fully sensitive to the drug; however, by the third transplant generation, resistance was evident with a maximum ILS of < 25 per cent. Resistance was confirmed on subsequent transplant generations. Treatment with L-alanosine was terminated after sixteen transplant generations, but resistance proved to be stable for at least fifty additional transplant generations in the absence of drug exposure.*

An L-alanosine-resistant subline of P388 leukemia cells developed even more rapidly than L1210/LAL. Considerable resistance was evident after only one passage in drug-treated mice (Fig. 3); complete resistance was observed after the third and subsequent transplant generations. As with L1210/LAL,

* The variable response of L1210 leukemia to L-alanosine is most likely due to the variability of animals and tumor populations and is in agreement with the results obtained with other agents. The maximum response of L1210 to L-alanosine in the thirteen trials shown in Fig. 2 averages 73 ± 26 (S.D.) per cent ILS; thus, the coefficient of variation (cov) in maximum ILS is 36 per cent. In comparison, in our large analog comparison program in the L1210 model, the maximum ILS to cis-platinum was 56 ± 22 per cent ILS (cov = 39 per cent, 77 dose responses); to dimethyl triazino imidazole carboxamide, 52 ± 19 per cent ILS (cov = 37 per cent, 19 dose responses); to 6-mercaptopurine, 50 ± 16 per cent ILS (cov = 32 per cent, 153 dose responses).

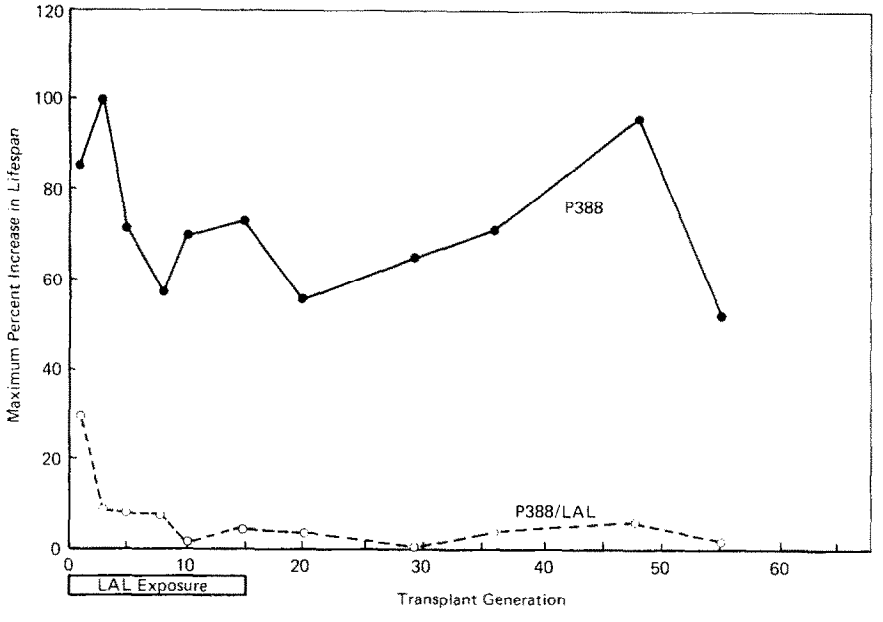

Fig. 3. Development and stability of a subline of P388 leukemia cells resistant to L-alanosine. Each point represents the maximum increase in lifespan produced by L-alanosine relative to untreated control mice bearing i.p. P388 or P388/LAL. Tumors were evaluated in parallel at three dose levels of L-alanosine: 600, 400, and 267 mg/kg, i.p., given on days 1, 5, and 9. P388/LAL was transplanted for fifteen generations in mice which were treated i.p. with 40 mg L-alanosine/kg daily. P388/LAL was carried in untreated mice thereafter.

Table 1. Effects of treatment with L-alanosine on DNA synthesis in L-alanosine sensitive and resistant tumors*

Tumor	[2-^{14}C]Thymidine incorporation (nCi/mg DNA)		
	Treatment		
	Saline†	L-Alanosine†	% Inhibition
L1210	6.95 ± 0.65	1.50 ± 0.17‡	78.50
L1210/LAL	5.80 ± 0.40	4.35 ± 0.70	25.00
P388	8.75 ± 0.60	1.90 ± 0.25‡	78.30
P388/LAL	7.82 ± 0.80	6.10 ± 0.50	22.00

* BDF_1 male mice bearing 10-day-old subcutaneous nodules of the tumors indicated were fasted overnight and treated with either L-alanosine (500 mg/kg) or saline. Four hours later, the animals were given 5 μCi/mouse of [2-^{14}C]thymidine. After 1 hr, the tumors were dissected, frozen, and processed for the estimation of DNA content and radioactivity as detailed in Materials and Methods.

† Values are means ± S.D.

‡ $P < 0.005$.

resistance to L-alanosine in the subline of P388 leukemia was a stable phenotype for at least forty transplant generations in the absence of drug exposure.

In an attempt to gain insight into the mechanism of resistance of L-alanosine of these sublines, we evaluated the sensitivity of L1210/LAL and P388/LAL to a spectrum of antitumor agents. Each subline retained a sensitivity similar to that of the respective parental cell line to the L-glutamine antagonists, azaserine, diazooxonorleucine, and AT-125, as well as to the purine antimetabolites, inosinedialdehyde, 2-aminothiadiazole, 6-selenoguanosine and 6-mercaptopurine. The following drugs were tested and found to have similar activities in P388 and P388/LAL: methotrexate, 5-fluorouracil, cytosine arabinoside, melphalan, cyclophosphamide, methyl-CCNU, adriamycin, and vincristine. Thus, the L-alanosine-resistant sublines appeared to be resistant only to L-alanosine among a variety of antimetabolites and other agents tested.

Biochemical characterization of resistance to L-alanosine. As noted earlier it has been reported that L-alanosine exerts its antitumor effect by restricting the concentration of ATP and ultimately depressing the synthesis of DNA [1–3]. To characterize the state of resistance to L-alanosine biochemically we first studied the effect of the drug on the synthesis of DNA in P388 and L1210 tumors sensitive and resistant to it. A single dose of L-alanosine (500 mg/kg) given to mice bearing subcutaneous nodules of these transplantable tumors inhibited the synthesis of DNA by approximately 80 per cent in the sensitive lines of both tumors but engendered only approximately 25 per cent inhibition in the resistant lines (Table 1).

Influence of L-alanosine on protein synthesis. In contrast to its notable ability to inhibit DNA synthesis in susceptible cells, and despite its structural similarity to L-aspartic acid, L-alanosine produced only negligible inhibition of protein synthesis (< 5 per cent) in each of the four cell lines examined (Table 2).

Transport of L-alanosine in sensitive and resistant tumors. The differential inhibition of DNA synthesis just discussed might have its origin in a depressed rate of drug transport in the resistant variants of the tumors studied. To examine this point, single cell suspensions were prepared from ascitic tumors and exposed to radioactive alanosine *in vitro*. It was found that the transport of the amino acid antibiotic was a saturable process which proceeded in a linear fashion for at least 10 min under the conditions used. No prominent differences, however, were observed in the Michaelis constants or the V_{max} values for the

Table 2. Effects of L-alanosine treatment on protein synthesis in L-alanosine sensitive and resistant tumors*

Tumor	[2-^{14}C]Valine incorporation (nCi/mg protein)		
	Treatment		
	Saline†	L-Alanosine†	% Inhibition
L1210	2.1 ± 0.1	2.0 ± 0.9	4.7
L1210/LAL	2.1 ± 0.1	2.1 ± 0.1	None
P388	1.9 ± 0.1	1.8 ± 0.1	5.3
P388/LAL	1.9 ± 0.1	1.9 ± 0.1	None

* BDF_1 mice bearing 10-day-old P388 and L1210 sensitive and resistant tumor nodules were given either L-alanosine (500 mg/kg) or an equal volume of saline. After 4 hr, all the animals were given 4 μCi/animal of L-[2-^{14}C]valine. One hour later, the tumors were dissected out, frozen, and analyzed for radioactivity and protein content as detailed in Materials and Methods.

† Values are means ± S.D.

transport of the drug in sensitive versus resistant lines, the $K_m$values being 6.67 and 11.10 mM for P388 and L1210 cells, respectively, and 10.00 and 12.50 mM for their respective resistant variants. Correspondingly, the maximum velocities obtained under the conditions used were 36.3 and 37.4 nmoles·min^{-1}·(million cells)$^{-1}$ in the case of P388 and L1210 cells, respectively, and 35.0 and 29.3 nmoles·min^{-1}·(million cells)$^{-1}$ for their respective resistant variants. Assuming that body water accounts for 70 per cent of body weight, it can be calculated that the instantaneous molarity of L-alanosine achieved after a dose of 500 mg/kg would be ~ 5 mM, a value lower than the K_m values reported above. It follows that the transport of L-alanosine under these conditions would proceed at less than one-half of its V_{max}.

Concentration of L-alanosine and its catabolic products in sensitive and resistant tumors. Consonant with the foregoing findings *in vitro* were the results of measurements made of the concentration of L-alanosine achieved in tumors *in vivo* after therapeutic doses of the drug.

As Table 3 documents, no significant differences were observed in the level achieved of unchanged L-alanosine or of its principal catabolic products in the sensitive as compared with the resistant tumor pairs. Thus, a barrier to transport of the drug does not appear to have been operative either in L1210/LAL or in P388/LAL.

Influence of L-alanosine on the levels of L-alanosine metabolizing enzymes. Confronted with these negative pharmacological results, attention was next directed to an examination of enzymological variables that might be relevant to the state of resistance to L-alanosine. Two enzymes are known to be involved in the action of this antibiotic: SAICAR synthetase, which anabolizes the drug (Fig. 1), and adenylosuccinic acid synthetase, which is inhibited strongly by the resultant metabolite (L-alanosyl-AICOR). In addition, it seemed reasonable to speculate that adenylosuccinic acid lyase (which decomposes both adenylosuccinic acid and SAICAR) would accept L-alanosyl-AICOR as an alternative substrate on the basis of its structural homology. Figure 4 documents for the first time that this, in fact, is the case: the adenylosuccinic acid lyases from muscle and yeast both catalyzed the decomposition of enzymatically synthesized L-alanosyl-AICOR at appreciable rates. Extracts of the neoplastic cell lines used in the present study also decomposed L-alanosyl-AICOR at appreciable rates; no significant difference, however, was observed in the rate of this process catalyzed by the crude lyase from sensitive versus resistant cells, the values being 38.54 ± 8.82 and 42.46 ± 4.03 nmoles·hr^{-1}·(mg protein)$^{-1}$ in the case of P388 and L1210 tumors, and 43.88 ± 7.61 and 39.51 ± 7.23 nmoles·hr^{-1}·(mg protein)$^{-1}$ in the case of their respective resistant variants. Although the nature of the product resulting from these decompositions was not established rigorously, it was observed to be a molecule with a rather strong net negative charge at pH 2.65 and to co-elute in close proximity to its presumed homolog, fumaric acid—135 v 139 min under the chromatographic conditions used. In addition, adenylosuccinic acid (25 mM) inhibited the generation of this product.*

In view of these results and of previously established determinants of the matabolism of L-alanosine, attention was next directed to an examination of the specific activities of all three of the aforementioned enzymes in the parent and resistant line. Table 4 shows the results of these studies. It was observed that levels of adenylosuccinate synthetase and adenylosuccinate lyase did not differ in the L-alanosine- sensitive and -resistant tumors. The activity of SAICAR synthetase, however, was significantly lower in one of the resistant lines, P388. Conversely, no comparable depression was observed in the resistant L1210 lines. In parallel studies, it was

* For determining the effect of adenylosuccinic acid on the decomposition of L-alanosyl-AICOR by lyase, the following were mixed in a total volume of 800 μl in Eppendorf vessels: 200 μl of L-alanosyl-AICOR (50 μmoles), 500 μl of fresh muscle lyase [sp. act. 689.2 nmoles·hr^{-1}·(mg protein)$^{-1}$] and 100 μl of 0.1 M, Tris–HCl buffer (pH 7.4). In another vessel Tris buffer was replaced by 100 μl of adenylosuccinic acid (20 μmoles, pH 7.4). The reactants were incubated at 37° for 2 hr; controls received enzyme heated at 95° for 30 min. The reaction was terminated by the addition of 200 μl of 0.015 M lithium–citrate–chloride buffer (pH 2.65), and analysis of the decomposition product of L-alanosyl-AICOR was performed as detailed in Materials and Methods. Under the experimental conditions used, adenylosuccinic acid inhibited the generation of the presumptive analog of fumaric acid by 66 per cent.

Table 3. Concentration of L-alanosine and its derivatives in P388 and L1210 tumors sensitive and resistant to the drug*

Compound	P388†	P388/LAL†	L1210†	L1210/LAL†
L-Alanosine	388.6 ± 247.2	217.8 ± 142.3	361.0 ± 86.0	262.1 ± 125.0
α-Decarboxy-L-alanosine	99.6 ± 67.2	127.5 ± 88.5	149.4 ± 35.9	140.1 ± 29.1
α-Hydroxy-L-alanosine	56.5 ± 49.1	56.0 ± 40.0	49.2 ± 39.1	43.0 ± 20.5

* Sensitive and resistant tumors were transplanted into opposite flanks. Six days later, the animals were fasted overnight and treated with alanosine (10 μCi of DL-[1-^{14}C]alanosine + 500 mg/kg of L-alanosine). Four hours later, groups of five mice were killed. Tumors were homogenized in 1 N acetic acid (1:3) and centrifuged at 12,000 *g* for 12 min; 5 μl of supernatant fraction was electrophoresed using 0.05 M sodium phosphate buffer, pH 7.2. All samples were overspotted with L-alanosine, "α-decarboxy-L-alanosine", and "α-hydroxy-L-alanosine". The spots were then cut and counted for radioactivity.

† Values are nmoles ± S.D./g tissue.

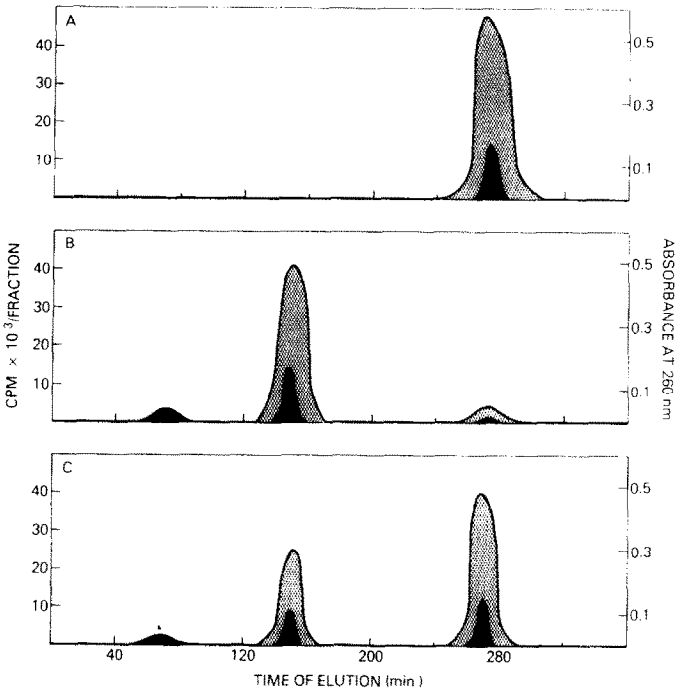

Fig. 4. Enzymatic decomposition of L-alanosyl-AICOR. Panel A represents the elution profile of authentic enzymatically synthesized L-alanosyl-AICOR; panels B and C represent the decomposition of L-alanosyl-AICOR by yeast lyase and muscle lyase respectively. The peaks at 260 and 135 min represent L-alanosyl-AICOR and the presumptive fumaryl analog of L-alanosine respectively. For the conduct of the assays, in a total volume of 800 μl were mixed: 200 μl of L-alanosyl-AICOR (20 nmoles, sp. act. 7.14 nCi/nmole) and either 600 μl of yeast lyase [sp. act. 540 nmoles·hr $^{-1}$·(mg protein)$^{-1}$] or 100 μl of fresh muscle extract [sp. act. 720.25 nmoles·hr^{-1}·(mg protein) $^{-1}$]. Controls received the enzyme heated at 95° for 30 min, and the reaction mixtures were incubated at 37° for 4 hr. The reaction was stopped by the addition of 200 μl of 0.015 M lithium–citrate–chloride buffer, pH 2.65. The volume was made up to 1.0 ml with H_2O, and, after centrifugation, an aliquot of 800 μl was loaded on a 0.8 cm × 15 cm column of Hamilton HA-X4 resin, pre-equilibrated with 0.015 M lithium–citrate–chloride buffer, pH 2.65. The elution was performed as described in Materials and Methods.

Table 4. Effects of treatment with L-alanosine on L-alanosine metabolizing enzymes in tumors sensitive and resistant to the drug*

		Specific activity [nmoles·hr^{-1}·(mg protein) $^{-1}$ ± S.D.]		
Tumor	Treatment	SAICAR synthetase	Adenylosuccinate lyase	Adenylosuccinate synthetase
P388	Saline	10.11 ± 1.20	273.27 ± 18.54	16.14 ± 2.05
	L-alanosine	10.47 ± 2.01	262.94 ± 24.02	4.98 ± 0.92‡
P388/LAL	Saline	6.63 ± 1.02†	302.86 ± 19.21	10.27 ± 1.22
	L-Alanosine	6.01 ± 0.82†	297.05 ± 15.44	8.24 ± 1.27
L1210	Saline	6.58 ± 1.27	320.79 ± 29.24	7.90 ± 1.94
	L-alanosine	4.72 ± 1.22	349.23 ± 11.72	1.86 ± 0.34‡
L1210/LAL	Saline	6.31 ± 1.32	296.44 ± 19.24	8.32 ± 1.29
	L-alanosine	5.48 ± 1.10	310.67 ± 14.22	6.42 ± 0.98

* BDF_1 male mice were implanted with 1×10^6 P388 cells/mouse or 1×10^5 L1210 cells/mouse as described in Materials and Methods. Ten days later, animals were fasted overnight and injected i.p. with 500 mg/kg of L-alanosine. The animals in the control group received the same volume of saline. After 4 hr, the tumors were removed, frozen, and later homogenized in 0.1 M Tris–HCl buffer, pH 7.4, containing 0.5 mM EDTA and 1.0 mM DTT. The samples were analyzed for the activity of various enzymes as described in Materials and Methods.

† Significance compared to sensitive group, $P < 0.005$.

‡ Significance compared to control group, $P < 0.005$.

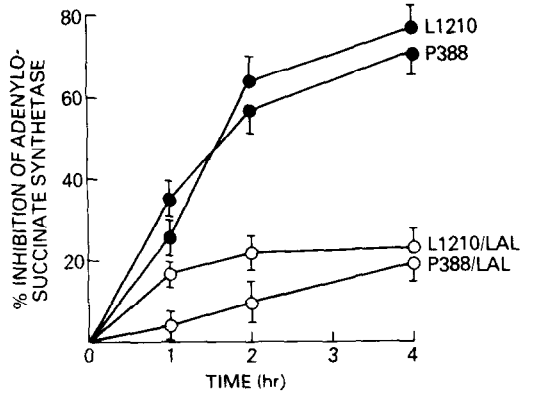

Fig. 5. Effect of L-alanosine on adenylosuccinate synthetase in sensitive and resistant cells. BDF_1 mice bearing L-alanosine sensitive and resistant tumors were given a single i.p. dose of L-alanosine (500 mg/kg). Animals in the control group received the same volume of saline. At designated time periods, the tumors were removed, frozen and homogenized. Adenylosuccinate synthetase activity was then measured as described in Materials and Methods.

observed that parental administration of L-alanosine exerted no effect on SAICAR synthetase or on adenylosuccinate lyase in either the sensitive or resistant tumors. Adenylosuccinate synthetase was inhibited in both the sensitive and resistant lines, but to markedly different degrees. Thus, as Fig. 5 shows, inhibition reached approximately 80 per cent at 4 hr in L1210 and P388, but only ~ 25 per cent in either of the resistant lines.

Concentration of L-alanosyl-AICOR in sensitive and resistant tumors. It has been reported earlier that inhibition of adenylosuccinic acid synthetase *in vivo* is exerted not by L-alanosine but by the antimetabolite L-alanosyl-AICOR [3]. It was therefore reasonable to measure the concentration of this nucleotide reached in the sensitive and resistant tumors after a therapeutic dose of L-alanosine. As shown in Table 5, the concentration of L-alanosyl-AICOR was measured as 84 and 90 μM in the case of the sensitive P388 and L1210 lines, respectively,

Table 5. Concentrations of L-alanosyl-AICOR in P388 and L1210 tumors sensitive and resistant to L-alanosine*

Tumor	Concentration of L-alanosyl-AICOR (μM ± S.D.)
P388	83.69 ± 9.12
P388/LAL	24.56 ± 5.78†
L1210	89.75 ± 12.04
L1210/LAL	26.77 ± 6.79†

* BDF_1 mice bearing 10-day-old tumors were treated with 50 μCi/animal of DL-[1-^{14}C]alanosine and L-alanosine (500 mg/kg). Two hours later, the animals were killed and tumors were removed, homogenized in 1 N acetic acid, and centrifuged at 12,000 *g* for 3 min. The concentrations of L-alanosyl-AICOR were measured as described in the text.
† Significantly different from native lines, $P < 0.005$.

whereas a concentration of only 25 and 27 μM was present in P388 and L1210 resistant tumors respectively.

Effect of treatment with L-alanosine on enzymes of purine salvage. By catalyzing the reutilization of bases, the enzymes of purine salvage might modulate sensitivity to L-alanosine to an important degree. Additionally, the state of adenine deprivation produced by L-alanosine could conceivably induce the enzymes of adenine salvage. For this reason, we next examined the effect of L-alanosine on these processes. Table 6 documents that, while adenosine kinase, adenosine phosphorylase, inosine phosphorylase, adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase were unaffected by a therapeutic dose of L-alanosine, the basal levels of adenosine kinase, APRTase, and HPRTase were approximately 200 per cent higher in P388/LAL and L1210/LAL than in the parent lines.

DISCUSSION

It is believed that L-alanosine exerts its antitumor activity by inhibiting adenylosuccinate synthetase—the penultimate enzyme required for the biosynthesis of AMP [1, 3]. An anabolite of the drug, L-alanosyl-AICOR, is thought to be respon-

Table 6. Influence of L-alanosine on purine salvage enzymes in P388 and L1210 tumors*

Treatment	Animals	Specific activity [nmoles·hr^{-1}·(mg protein)$^{-1}$ ± S.D.] Adenosine kinase	Adenosine phosphorylase	Inosine phosphorylase	APRTase	HPRTase
Saline	P388/LAL	43.80 ± 2.35†	12.20 ± 3.67†	100.92 ± 27.01‡	11.68 ± 2.70†	16.34 ± 2.0†
	P388	25.48 ± 3.41	6.49 ± 1.00	68.87 ± 8.06	6.23 ± 3.53	9.68 ± 2.79
L-Alanosine	P388/LAL	44.27 ± 2.58†	13.37 ± 5.49‡	95.93 ± 14.00†	11.70 ± 3.01†	15.98 ± 3.15
	P388	24.48 ± 2.90	6.48 ± 1.57	63.99 ± 12.50	6.13 ± 1.39	9.62 ± 2.91
Saline	L1210/LAL	32.36 ± 1.41†	10.68 ± 3.01‡	106.14 ± 16.01†	12.05 ± 2.70‡	14.70 ± 3.20
	L1210	18.04 ± 2.40	5.98 ± 1.06	70.74 ± 9.24	9.20 ± 1.95	10.59 ± 2.92
L-Alanosine	L1210/LAL	32.32 ± 4.83‡	8.06 ± 2.69	81.34 ± 9.03†	11.90 ± 3.21	15.23 ± 4.19‡
	L1210	16.79 ± 1.94	5.27 ± 0.92	63.73 ± 5.73	8.95 ± 2.56	10.22 ± 2.80

* BDF_1 male mice bearing 10-day-old P388 and L1210 tumors, sensitive and resistant to L-alanosine, were treated with 500 mg/kg of L-alanosine i.p. The animals in the control group received the same volume of saline. After 4 hr, the tumors were removed and homogenized in 0.1 M Tris–HCl buffer, pH 7.4, containing 0.5 mM EDTA and 1.0 mM DTT. The activity of various enzymes was measured as described in the text.
† Significantly different from sensitive group, $P < 0.005$.
‡ Significantly different from sensitive group, $P < 0.05$.

sible for this activity *in vivo* [3]. In the present investigation into the mechanism of resistance of select murine tumors to L-alanosine, it has been observed that refractory lines accumulate concentrations of L-alanosyl-AICOR significantly lower than their sensitive counterparts after a therapeutic dose of the drug. This finding correlates well with results on the influence of L-alanosine on the synthesis of DNA and on the activity of adenylosuccinate synthetase in these lines (Table 1 and Fig. 5).

SAICAR synthetase, the enzyme responsible for the generation of L-alanosyl-AICOR, was depressed in P388/LAL, suggesting that resistance in this line might be due to a depression in the specific activity of this enzyme; perplexingly, no comparable depression was demonstrable in L1210/LAL. Although an augmented activity of adenylosuccinate lyase might explain the observed depression in the concentration of L-alanosyl-AICOR reached in L1210/LAL in the face of apparently normal levels of SAICAR synthetase, measurements of the lyase, in fact, fell within the normal range. Thus, it remains to be determined why this variant fails to accumulate the anabolite to a concentration comparable to that found in the parent tumor. One explanation, not tested in the present study, is that the SAICAR synthetase from L1210/LAL has a selectively diminished affinity for L-alanosine and so cannot use it efficiently as an aberrant substrate.

As Table 6 demonstrates, the activity of the enzymes of purine salvage also seems to play an important role in the resistance of these murine tumor lines to L-alanosine: the ~ 200 per cent elevation in their activity could be expected to counteract the depression of adenine nucleotides provoked by the drug.

In conclusion, although other undefined factors are doubtless operative, two mechanisms have been found to be dominant in the state of resistance to L-alanosine: a significantly diminished ability to accumulate L-alanosyl-AICOR and a significantly enhanced ability to reutilize preformed purines. As L-alanosine enters clinical trials, it will remain for further studies to establish the relative importance of these mechanisms in human tumors.

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