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Resistance in Leukemic Cells to an Adenine Antagonist, 6-Mercaptopurine. (20663)

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Transformations have been obtained to resistance or dependence in leukemic cells of the mouse by the use of several antimetabolites: folic acid analogs(1,2-4) and the triazolopyrimidine analog of guanine, 8-Azaguanine(5). Such transformations have been shown to be stable, irreversible and heritable, and have been produced readily in several transplantable lines of lymphocytic leukemia (6). Experimental findings favor the assumption that the variant lines produced by the use of antimetabolites arise by mutation and the role of antimetabolite is merely as a selective agent(7).

Preliminary evidence which suggests that purine analogs act as antagonists of nucleic acid synthesis has encouraged investigation of the role of these compounds in the inhibition of cancerous growths. The adenine analog, 6-mercaptopurine, has been shown to act as a purine antagonist in the metabolism of *Lactobacillus casei*(8). It has also been shown to be a unique inhibitor of Sarcoma 180(9) and of certain mammary adenocarcinomas(10). Limited clinical trials of this compound in advanced leukemia of children have been encouraging(11).

Definite, regular and reproducible inhibition of growth of leukemic cells has been obtained in 2 transplantable lines of acute lymphocytic leukemia. No increase in survival time of mice bearing 2 other transplantable acute lymphocytic leukemias could be obtained. In leukemia L 1210(12), particularly, striking increases in survival time were observed and are described here. Following the methods used previously in obtaining transformations to folic acid antagonists(4) and 8-Azaguanine(5), a resistant subline of leukemia L 1210 has been developed using 6-mercaptopurine as the selective agent. Some of the pertinent characteristics of these L 1210 resistant leukemic cells will be considered.

Transplant generations 202 to 251 of the sensitive subline of leukemia L 1210 have been used as control material for the present study. Beginning with the 202nd transfer, a standard dose of leukemic cells (8 x 10^5 cells) in Lockes' solution was inoculated subcutaneously in the right axillary region of 18-20 g DBA/2 strain mice. These mice subsequently received daily injections, beginning at 24 hours, of the adenine analog, 6-mercaptopurine.† The compound was dissolved in 0.1 N NaOH then diluted with sterile saline so that the constant volume injected subcutaneously was 0.025 cc per g of body weight. Daily injections were continued until palpable growths sufficient for transfer were obtained. During the first 3 transfers the total dosage of analog was 262.5 mg/K (37.5 mg/K x 7 days) and subsequently the total dosage was 525 mg/K (75 mg/K x 7). Transfers of leukemic cells were done at 9 to 11 days during the first 4 transfer generations and later at 7 and 8 days as larger subcutane-

* National Institutes of Health, U. S. Public Health Service.

† This compound furnished by Dr. George Hitchings of The Wellcome Research Laboratories, Tuckahoe, N. Y. A-methopterin and 8-Azaguanine, were supplied by Lederle Laboratories Division, American Cyanamid Co.

TABLE I. Development of Resistance in Leukemia L 1210 Following Continuous Treatment with Adenine Analog 6-Mercaptopurine. DBA strain of mice.

Transfer generations	No. mice	Time transplanted, days	Dosage (mg/kg) Daily	Dosage (mg/kg) Total	Tumor wt (mg) at 8 days
L 1210-6-mercaptopurine-resistant					
G- 1 to 3‡	3*	9–11	37.5	262.5	0
4	5	8	75	525	280.4
4	5	8	0		420
5 to 7	3*	8	75	525	†
8	5	8	75	525	81
8	5	8	0		45
9 to 12	3*	8	75	525	†
13	10	8	75	525	200
13	10	8	0		251.8
14 to 39	5*	7–8	75	525	Approx. 500
L 1210 sensitive					
206	8	8	75	525	0
	8	8	0		450.9
210	8	8	75	525	0
	8	8	0		496.3
212	8	8	75	525	0
	8	8	0		590.2

* Each.
† Carried only.
‡ Transfer generation G-1 was derived from transfer generation G-202 of the L 1210 sensitive line; thus, transfer generation G-206 is the control transfer for G-4 of the resistant subline.

ous growths appeared in animals receiving the near-MTD dosage. The difference in response to 6-mercaptopurine in the transfer line grown only in mice receiving the analog and in the control (sensitive) line may be seen in Table I. Very little difference in weights of the subcutaneous tumor grown in mice with and without analog were observed in the line being developed for resistance whereas complete inhibition of growth of leukemic cells, at 8 days, was observed in the sensitive control line at comparable transfer generations.

The 6-mercaptopurine-resistant subline has now been carried through 39 consecutive subcutaneous transfers in DBA/2 mice receiving the near-MTD of this analog, 525 mg/K (75 mg/K x 7). Sizeable tumor growths of nearly 500 mg are always obtained in the presence or absence of the analog, indicating the development of resistance. The dependence phenomenon characteristic of other transfer lines has not been observed although only one other variant subline (also resistant) has been developed in this laboratory using this compound. That the variant cells described here exhibit resistance to 6-mercaptopurine is shown also in experiments using leukemic death, following transfers intraperitoneally, of the standard dose of leukemic cells, as the criterion. The mean survival time of mice bearing the sensitive subline of L 1210 was 7.9 ± 0.06 days whereas an increase of survival of 87.3%, to 14.8 ± 0.18 days was obtained using 6-mercaptopurine within the total dosage range of 250-1200 mg/K. In contrast, identical survival times were obtained in mice bearing the 6-mercaptopurine-resistant cells, with and without the analog (Table II).

Significant and reproducible increases in survival time in DBA/2 or CDF_1 mice bearing the sensitive, control leukemia L 1210 were obtained at dosage levels of 250 mg/K (50 mg/K x 5 days) to 1000 mg/K (125 mg/K x 8 days). Slight losses in body weight, never exceeding 1.5 g, were obtained only at dosage levels of 600-1000 mg/K. Within the dosage range of 250 to 600 mg/K, regular increases in body weight were obtained. No deaths attributable to the drug were found at the highest dosage level used. The effects obtained at the higher dosage levels were within the same range as those obtained at the 300-600 mg/K level.

The possibility was considered that resistance developed to one purine-antagonist might extend to other purine antagonists and possibly folic acid antagonists. Of the compounds used, the triazolopyrimidine analog, 8-Azaguanine has been shown to have leukemia-inhibiting properties in certain leukemias, especially in the lymphocytic leukemia L 1210 used in this study. Thioguanine also has been shown to effectively and regularly increase survival time of L 1210 leukemic mice whereas 8-Azaxanthine (resulting from deamination of 8-Azaguanine) and 2,6-diaminopurine are ineffective. The comparative effects of these compounds, as well as of the folic antagonist, A-methopterin (4-amino-N^{10} methyl PGA) are shown in Table II. Resistance to 6-mercaptopurine is accompanied by resistance to all other purine and pyrimidine

TABLE II. Effects of Purine Analogs and a Folic Antagonist on Survival Time of Mice Bearing (A) Sensitive Leukemia L 1210 and (B) 6-Mercaptopurine-Resistant Leukemia L 1210. DBA or CDF_1 strain of mice.*

Compound	No. separate exp.	Total No. mice	Dosage (mg/kg) Individual	Dosage (mg/kg) Total	Mean survival time, days (range)	% increase in survival
			(A)—L 1210-sensitive†			
Controls	18	145	None		7.9 (7– 9)‡	—
6-Mercaptopurine	15	106	50 –125	250–1000	14.8 (10–22)§	87.3
8-Azaguanine	6	56	75	450– 600	12.4 (10–20)	60.0
8-Azaxanthine	5	48	75 –113	450– 800	8.1 (7–10)	—
2,6-Diaminopurine	3	24	75	450– 600	8.0 (7–10)	—
Thioguanine	4	26	7.5– 10.0	20– 30	13.7 (12–15)	73.5
4-Amino-N^{10} methyl PGA	4	35	3	24	18.1 (14–23)	129.1
"	9	80	3	12	16.7 (13–20)	111.4
			(B)—L 1210-6-mercaptopurine-resistant			
Controls	5	42	None		9.9 (9–11)	—
6-Mercaptopurine	5	42	75	525	10.0 (9–11)	—
8-Azaguanine	3	24	75	600	9.6 (9–11)	—
8-Azaxanthine	4	27	75	600	9.4 (9–13)	—
2,6-Diaminopurine	3	24	75	600	9.8 (9–11)	—
Thioguanine	4	32	7.5	30	9.8 (8–11)	—
4-Amino-N^{10} methyl PGA	2	20	3	24	28.8 (22–38)	190.9
"	2	18	3	12	19.3 (18–22)	95.0

* CDF_1 mice = ♀ BALB/c × DBA/2 F_1 hybrids. No differences in survival times were noted between DBA and CDF_1 mice, nor between sexes.

† Comparable transfer generations were used for these data; for L 1210 sensitive, transfers 202 to 251 and for the transformed, 6-mercaptopurine-resistant line, transfers 10 (210) to 39 (239).

‡ Stand. errors are given in the text.

§ Mean age at death from leukemia of mice receiving total dosage of 6-mercaptopurine at the 250 mg/kg level was 13.2 days; 300-450 mg/kg—15.3 days; 525-600 mg/kg—15.3 days and 750-1000 mg/kg—14.2 days. See text.

antagonists studied. This is unlike the resistance phenomenon in *L. casei* in which resistance to this compound was not accompanied by resistance to other adenine antagonists, particularly 2,6-diaminopurine, 8-Azadenine, and purine and 8-Azaguanine, a guanine antagonist(13).

Dosage levels of 6-mercaptopurine as high as 600 mg/K were without effect on survival time of DBA/2 of CDF_1 mice bearing an 8-Azaguanine-resistant line of L 1210, indicating again cross-resistance, whereas the analog provided for near-optimal growth, as determined either by weight of localized lymphomatous tissue or survival time of an 8-Azaguanine dependent line of this leukemia(14).

It has been shown in tests employing *L. casei* that 6-mercaptopurine inhibition of growth is not reversible with folic acid (pteroylglutamic acid)(13). Further evidence that this analog is not an "anti-folic" is shown in the sensitivity of the L 1210-6MR subline to 4-amino-N^{10} methyl PGA. A more striking inhibition of leukemic cell growth by this folic acid antagonist is seen in mice bearing intraperitoneal transplants of the 6-mercaptopurine-resistant subline than in mice bearing the control L 1210-S subline, particularly at near-MTD levels (3 mg/K x 8 days). An increase in survival time of nearly 200% from 9.9 ± 0.10 days to 28.8 ± 0.90 days was obtained whereas an increase from 7.9 ± 0.06 to 18.1 ± 0.69 was shown among controls. It remains to be determined whether this increased sensitivity to a folic antagonist results from a significantly increased requirement for folic acid which is found to be characteristic of a strain of *L. casei* resistant to 6-mercaptopurine(13). It is interesting to note that 2 other transformations in leukemia L 1210, resistant to 8-Azaguanine and dependent upon 8-Azaguanine, are also accompanied by even more striking sensitivity to the folic analog, 4-amino-N^{10} methyl PGA, resulting in increases in survival of leukemic mice over the controls to 400% and more(14). On the other hand,

resistant and dependent leukemic cells developed by the use of 4-amino-N^{10} methyl PGA have not shown an increased sensitivity to the purine antagonists.

Extensive attempts to reverse the anti-leukemic activity of 6-mercaptopurine in the L 1210 sensitive line by natural purines have been relatively unsuccessful. Guanine (25 and 50 mg/K x 4) adenine (50, 75, 100, and 150 mg/K x 4), xanthine (100 and 300 mg/K x 4) and hypoxanthine (100, 300, and 500 mg/K x 4) were given intraperitoneally every other day beginning at 24 hours following inoculations of leukemic cells and one hour prior to injections of 50 mg/K of 6-mercaptopurine. In 2 cases significant reversals were seen: 50% reversal, as measured by leukemic death was obtained by adenine at a dosage level of 75 mg/K x 4 and 50% reversal by hypoxanthine (300 mg/K x 4). However, these effects were not consistent from experiment to experiment and are in constrast to results with *L. casei* in which growth inhibition is prevented competitively and apparently with ease by any of these 4 purine bases(13).

No morphologic changes have been observed in the sensitive line of leukemia following exposure to this adenine analog or in the resistant line developed by continuous exposure *in vivo*.

Summary. 1. The adenine analog 6-mercaptopurine has been shown to inhibit leukemic-cell growth of the acute lymphocytic leukemia L 1210 in a definite, regular and reproducible manner. Lower dosage levels of this compound appear to be as effective as higher (near-MTD) levels as measured by the criterion of leukemic death. 2. A resistant subline of leukemia L 1210 has been developed by consecutive passage of leukemic cells in DBA/2 strain mice receiving daily injections of 6-mercaptopurine. Resistant leukemic cells grow optimally either in the presence or absence of the antagonist. 3. Resistance to 6-mercaptopurine is accompanied by resistance to all other purine analogs tested: 8-Azaguanine, 8-Azaxanthine, 2,6-Diaminopurine, and thioguanine, some of which are moderate anti-leukemic agents in the sensitive line of this transplantable leukemia. 4. An increased sensitivity to the folic acid antagonist, 4-amino-N^{10} methyl PGA (A-methopterin) is a characteristic of the resistant variant as well as of 2 other variant lines developed through the use of 8-Azaguanine. 5. Attempts to reverse the anti-leukemic activity of 6-mercaptopurine by the physiologic purines adenine, guanine, xanthine, and hypoxanthine have been relatively unsuccessful.

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