Response of a Resistant Variant of Leukemic Cells to an Antagonist of Pteroylglutamic Acid. (18773)

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We have reported the development of resistance in 3 separate sublines of a transplantable lymphoid leukemia following serial transplants in mice treated with the MTD of 3 different 4-amino substituted PGA antagonists (1). The variants produced proved to be stable, irreversible heritable changes(2) and recent information(3) indicates that the PGA antagonist has acted as a selective agent, inhibiting the growth of sensitive leukemic cells and allowing the resistant forms, which arise as spontaneous mutants, to survive. The variant forms proved to be resistant to all 4-amino substituted PGA antagonists tested but were inhibited in growth by several other antileukemic compounds: 8-Azaguanine, alpha-peltatin(2), triethylene-melamine and X-rays (3). Optimal growth of the variant resistant leukemic cells was obtained when these cells were grown in animals receiving the MTD of PGA antagonists.

The present report concerns the following observations on the behavior of the resistant and dependent leukemic cells of one subline, the AM-resistant subline, resistant to 4-amino-N^{10}-methyl PGA (A-methopterin): (1) the hematologic and histologic response of resistant leukemic cells, as compared with sensitive controls, to various dosage levels of A-methopterin from the MTD level to the LD_{50} level, (2) the effect of Citrovorum factor (C.F.)(4) on the reversal of effects of A-methopterin in the resistant and sensitive sublines and (3) a consideration of the mechanism involved in the adaptation of resistant leukemic cells to dependence upon the PGA antagonist for optimal growth.

Methods and results. Transplant generations 96 to 108 inclusive of the AM-resistant subline and of the corresponding sensitive control subline of acute leukemia L 1210 have been used in the present study. The resistant cells have been carried serially in transplant in strain dba mice receiving 3 mg/kg of A-methopterin* intraperitoneally (.01 cc/g body weight) every other day for 4 days beginning at 48 hours after transfer of leukemic cells. Inoculations of a standard dose of leukemic cells (8×10^5 cells) were done subcutaneously into the right axillary region as described previously(1,2), resulting in a profuse localized growth of lymphomatous tissue which was dissected free of subcutaneous tissues and weighed on a micro-torsion balance. Table I shows the characteristic growth responses of resistant and sensitive leukemic cells to A-methopterin.

The morphology and biologic behavior of the sensitive subline of leukemia L 1210 has been closely observed. Terminally, following subcutaneous transfer of this leukemia, numerous leukemic cells are dispersed in lymph nodes, spleen and liver, enlarging these organs and obscuring the architecture. Leukemic cells appear in the blood a few days after transfer giving a moderately high leukocyte count. By reference to Table II it can be seen that the behavior of the resistant leukemic cells grown in mice receiving 3 mg/kg of A-methopterin is similar to the growth behavior of the sensitive cells grown in antagonist-free mice. There results a florid leukemia with massive lymphomatous growth subcutaneously, a striking secondary anemia, a leukocytosis with escape of lymphoblasts into

1. Law, L. W., and Boyle, P. J., Proc. Soc. Exp. Biol. and Med., 1950, v74, 599.
2. Law, L. W., *J. Nat. Cancer Inst.*, 1950, v11, 849.
3. Law, L. W., Unpublished observations.
4. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1948, v176, 165.

* The folic acid antagonist, A-methopterin, and Citrovorum factor were kindly supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

TABLE I. Comparison of Growth Capacities of Resistant (L 1210 R) and Sensitive (L 1210) Leukemic Cells of Acute Lymphoid Leukemia L 1210 to 4-amino-N10-methyl PGA.

	Transfer generations	No. mice	Dosage (mg/kg) Daily	Total	Mean wt tumor in mg (range)	Dependency ratio
L 1210 R	96–108	78	3	12	833.3 (540.6–1375)	3.92
Resistant		29	0	0	212.7 (143.6–279.9)	
L 1210	96–108	30	3	12	6	—
Sensitive		55	0	0	1190 (983.5–1478)	

the peripheral blood, severe infiltration into subcutaneous and internal lymph nodes, spleen and liver and a mean survival time similar to that observed in untreated mice growing the sensitive leukemic cells. In contrast, note the nearly complete inhibition of leukemia in mice bearing the sensitive subline and given 3 mg/kg of A-methopterin. It was of interest, therefore, to observe the effect of increasing dosage levels up to and including the LD_{50} on the growth response of the resistant leukemic cells. It can be seen that at levels of 5.0 and 7.5 mg/kg (total dose of 20 and 30 mg/kg) profuse localized growth and a generalized leukemia result. At the dosage level of 12.0 mg/kg (total dose 48 mg/kg) which represents the LD_{50} for non-leukemic strain dba mice and mice bearing the sensitive transplant, a reduction of localized growth to approximately 50% has occurred but infiltration into lymph nodes and spleen was still obtained. No deaths attributed to drug toxicity occurred in this group. Thus, it is indicated that the resistant cells now show at least a 50-fold increase in tolerance to A-methopterin, since it requires approximately 0.25 mg/kg (total dose, 1.0 mg/kg) of PGA antagonist to inhibit the localized growth of the sensitive cells to a similar weight level.

In addition to the development of a florid leukemia in A-methopterin-treated mice bearing transfers of resistant leukemic cells, it is apparent that the PGA antagonist supplied to these mice is being used directly as a growth factor by the resistant cells or is being converted for use. It has been found in mice bearing the sensitive leukemic cells and given the folic antagonist at a dosage level of 3 mg or 5 mg/kg every other day for 4 days that various symptoms of vitamin deficiency appear. There results a slight anorexia indicated in a lowered average food intake of 2.0 g per mouse per day and a slight loss in weight over a 7-day period (0.3 g or 0.2%). Untreated mice bearing sensitive leukemic cells during this period gain an average of 2.5 g (12.5%) and consume an average of 2.5 g of food per mouse per day. In contrast, leukemic mice bearing the resistant leukemia and given the PGA antagonist at this dosage level show an average gain in body weight of 2.1 g (10.5%) and consume 2.2 g of food. Other signs of PGA deficiency fail to appear in mice growing the resistant and dependent leukemic cells and supplied with A-methopterin at the 3 mg or 5 mg/kg level. The red pulp of the spleen resembles that seen in the normal animal(5) indicating active hematopoiesis. Numerous megakaryocytes and many foci of nucleated cells of the erythroid series are found throughout the pulp. On the other hand, in mice bearing transplants of the sensitive subline, and given the PGA antagonist, there is definite indication of a deficiency state. The red pulp contains relatively fewer cells with very few megakaryocytes and nucleated red cells. There occurs also a reduction in the number of polymorphonuclear leukocytes. The bone marrow of mice bearing sensitive leukemic cells and given chronic doses of A-methopterin reveals the following signs of PGA deficiency: an increase in cellularity mostly of larger immature cells with many in mitoses and a decrease in megakaryocytes, nucleated red cells and cells of the

5. Weir, D. R., Heinle, R. W., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 211.

TABLE II. Comparison in Response to 4-amino-N^{10}-methyl PGA of AM-resistant (L 1210 R) and Sensitive (L 1210) Leukemic Cells.*

Transfer line	Dosage (mg/kg) Daily	Total	Mean tumor wt, mg	Survival days	Peripheral blood counts Hb	wbc	agran.	gran.	blasts	Organ infiltrations† Lymph nodes	Spleen	Liver
L 1210 R	3	12	1362.7	12.4 ± .33	10.1	47760	11940	32299	2821	3+	3+	2+
(resistant)	5	20	1146		12	24930	7719	16000	1211	3+	3+	2+
	7.5	30	1084		12	46835	9835	34190	2810	2+	2+	2+
	12	48	685.7		13.2	6685	1872	4783	30	2+	1+	—
L 1210	0	0	1478	12.7 ± .15	11.2	41150	14568	20921	5761	3+	3+	2+
(sensitive)	3	12	0		13.5	11025	6550	4520	55	—	—	—

* Mean tumor wt and histologic data obtained on 10 mice in each group representing 100th transfer of resistant and sensitive cells in dba mice; survival time determined on 20 mice in each group; blood studies represent means of 5 animals in each group.
† 3+ severe, 2+ moderate, 1+ slight, — no infiltration, of leukemic cells into designated organs as determined by microscopic study.

granulocytic series. These changes are evident particularly at the higher dosage level. The picture appears nearly normal, however, in dba mice given the same dosage of antagonist but bearing transplants of the resistant and dependent cells indicating that the PGA antagonist is used, directly or indirectly, in the profuse growth of the resistant leukemic cells.

Citrovorum factor (C.F.) has been shown to be effective in preventing the toxicity of PGA antagonist in *Leuconostoc citrovorum* (6) and in mice (7), and in reversing the antileukemic action of these antagonists in mice (8). Since it appears that C.F. on a weight basis, is more effective than PGA in reversing the effects of folic antagonists, it was of interest to study the effects of this factor on the growth of the resistant and sensitive lines of leukemia L 1210 and to determine the blocking effect of C.F. on (a) the anti-leukemic activity of 4-amino-N^{10}-methyl PGA on sensitive leukemic cells and (b) the optimal growth-promoting capacity of this antagonist on resistant leukemic cells.

Citrovorum factor† was injected intraperitoneally in mice at a dosage level of 2 x 10^6 units(4) 24 hours after inoculation subcutaneously of a standard dose of leukemic cells and then 30 minutes prior to injection of 2 mg/kg of 4-amino-N^{10}-methyl PGA. It can be seen from Table III that C.F. did not affect the growth of the resistant leukemic cells, nor was any effect noted on the growth of sensitive leukemic cells. C.F., however, was effective in partially blocking the utilization of the PGA antagonist by the resistant leukemic cells. Only 71% optimal growth of resistant leukemic cells was obtained when C.F. was given prior to 4-amino-N^{10}-methyl PGA. This represents a partial reversal of optimal effect of antagonist equal to 42%. A partial reversal of the anti-leukemic effect of the antagonist

6. Sauberlich, H. E., *Arch. Biochem.*, 1949, v24, 224.

7. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H., *J. Biol. Chem.*, 1950, v185, 399.

8. Burchenal, J. H., Babcock, G. M., Broquist, H. P., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 735.

† The C. F. used in these experiments had an activity for *L. citrovorum* of 3 million units per cc.

TABLE III. Citrovorum Factor (C. F.) and the Effects of 4-amino-N^{10}-methyl PGA on Sensitive and Resistant Leukemic Cells of Leukemia L 1210.*

Experiment	No. mice	Dosage† Daily	Dosage† Total	Relative‡ mean wt	Absolute mean wt, mg (range)	% reversal
			L 1210 resistant			
Amethopterin	42	2	8	1	(758.5–1362.8)	—
Amethopterin + C. F.§	28	2 2×10^6	8 10×10^6	.71 (.59–.87)	(544.1–854.3)	42
C. F.	36	2×10^6	8×10^6	.35 (.31–.42)	(275–580)	—
Controls	36	0	0	.31 (.25–.38)	(195–320.9)	—
			L 1210 sensitive			
Amethopterin	54	2	8	.03 (.0–.09)	(.0–100.8)	—
Amethopterin + C. F.	44	2 2×10^6	8 10×10^6	.28 (.09–.45)	(87–428.3)	29
C. F.	30	2×10^6	8×10^6	.91 (.70–1.12)	(1087–1330.3)	—
Controls	50	0	0	1	(704–1559.4)	—

* Transfer generations 98-102 inclusive. Strain dba mice of both sexes, weighing 18-22 g.
† Dosage of A-methopterin in mg/kg; citrovorum factor dosage in units/kg.
‡ For convenience, optimal growth in both groups considered as 1.0.
§ C. F. inj. intrap. 24 hr prior to first inj. of antagonist, then ½ hr prior to antagonist at 2, 4, 6, and 8 days.

on sensitive leukemic cells was also found (Table III). The reversal of anti-leukemic effect by C.F., as measured by tumor weight, was considerably less than that reported by Burchenal *et al.*(8), who used survival time as a criterion, despite the fact that a considerably higher dosage level of C.F. was used here.

Discussion. It is apparent that we are dealing here with a stable and irreversible, heritable change in leukemic cells such that the requirements for PGA or Citrovorum factor are altered profoundly. Optimal growth of the resistant variants is attained in mice given antagonist at dosage levels far above the MTD, resulting in a florid leukemia with profuse localized growth of lymphomatous tissue, severe infiltration into other organs and leukocytosis with escape of lymphoblasts into the circulation. Evidence that the transformed resistant and dependent leukemic cells are using the PGA antagonist, 4-amino-N^{10}-methyl PGA, either directly or in a converted form is to be found in the lack of signs of vitamin deficiency in mice growing the resistant leukemic cells and provided with antagonist.

The transformation to resistance and dependence has provided a unique and specific modification of a metabolic pathway for biochemical studies. Examples of resistant mutant bacteria requiring specific drugs for growth have been reported(9,10), but it is quite common in bacteria for reversion to occur to the sensitive type. The mechanism of dependence of these transformed leukemic cells on the specific PGA antagonist, 4-amino-N^{10}-methyl PGA or on any of the 4-amino substituted antagonists remains enigmatic. Several theoretical explanations, however, may be considered: (a) the transformed leukemic cells have acquired the ability to convert PGA antagonist to PGA or Citrovorum factor by one of several methods, by deamination of the antagonist at the 4 position of the pteridine ring or by methylation or acetylation of the 4-amino group. This possibility seems unlikely since neither PGA nor C.F. provides for optimal growth of the resistant cells. Furthermore, it has been shown that C.F. actually blocks, partially, the use of the PGA antagonist by the resistant cells. (b) The resistant

9. Miller, C. P., and Bohnhoff, M., *J. Bact.*, 1947, v54, 467.

10. Paine, T. F., and Finland, M., *Science*, 1948, v107, 143.

variants have acquired the ability to synthesize their own PGA or C.F. This possibility seems unlikely for the same reasons given in (a) above, (c) the leukemic cells have an increased ability to detoxify the PGA antagonist. From the studies reported here, it is apparent that toxic effects of antagonist do not appear in mice growing the transformed leukemic cells because the antagonist is metabolized in supporting optimal growth. If this were a case of detoxification of antagonist, there would not be expected a differential in the growth capacities of resistant leukemic cells supplied with or lacking antagonist. That is, detoxification could be an explanation for the resistance but not for the dependence characteristic, or (d) the transformed leukemic cells have acquired the ability to use 4-amino-N^{10}-methyl PGA without conversion to PGA or C.F., employing a different mechanism for the synthesis of nucleic acids than that suggested as occurring normally(11,12). This would seem the most likely explanation available at present, although recently Skipper and Burchenal(13) have presented evidence which indicates that the differences in formate fixation in sensitive and resistant strains of the Ak-4 leukemia is only a relative one. It seems improbable, however, from the evidence presented(14), that the resistant Ak-4 cells which were used in their studies are dependent upon PGA antagonist for optimal growth.

Summary. 1. The effect of a PGA antagonist, 4-amino-N^{10}-methyl PGA on resistant leukemic cells of the mouse has been studied. The development of a florid leukemia with profuse localized growth of lymphomatous tissue, leukocytosis with escape of leukemic cells into the blood and extensive infiltration into various organs occurs at dosage levels as high as the LD_{50}. 2. Symptoms of vitamin deficiency did not appear in mice growing the resistant leukemic cells and supplied with a total dose of antagonist as high as 20 mg./kg, indicating that the resistant leukemic cells are metabolizing the antagonist in some manner. Tolerance to 4-amino-N^{10}-methyl PGA has been increased approximately 50-fold. 3. Citrovorum factor did not influence the growth of resistant leukemic cells but partially blocked the optimal growth-promoting effect of the PGA antagonist. 4. Possible mechanisms of action of 4-amino-N^{10}-methyl PGA on resistant and dependent leukemic cells are considered.

11. Gordon, M., Ravel, J. M., Eakin, R. E., and Shive, W., *J. Am. Chem. Soc.*, 1948, v70, 878.

12. Skipper, H. E., Mitchell, J. H., Jr., and Bennet, L. L., Jr., *Cancer Res.*, 1950, v10, 510.

13. Skipper, H. E., and Burchenal, J. H., *Cancer Res.*, 1951, v11, 229.

14. Burchenal, J. H., Robinson, E., Johnston, S. F., and Kreshida, M. N., *Science*, 1950, v111, 116.

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Non-Specificity of Biotin Activity for Leuconostoc.* (18774)

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Literature evidence(1-5) indicates that micro-organisms possess definite configurational specificity with regard to biotin activity.

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1. Melville, D. B., Dittmer, K., Brown, G. B., and du Vigneaud, V., *Science*, 1943, v98, 497.

2. Pilgrim, F. J., Axelrod, A. E., Winnick, T., and Hofmann, K., *Science*, 1945, v102, 35.

3. Wood, J. L., and duVigneaud, V., *J. Am. Chem. Soc.*, 1945, v67, 210.

4. Rubin, S. H., Drekter, L., and Meyer, E. H., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 352.

5. Krueger, K. K., and Peterson, W. H., *J. Bact.*, 1948, v55, 693.