

## A Comparison of the Effects of 4-Amino-N<sup>10</sup>-Methyl-Pteroylglutamic Acid and 2,6-Diaminopurine upon Sensitive and Resistant Sublines of a Strain of Mouse Leukemia

By J. H. BURCHENAL, M.D., L. F. WEBBER, B.A., G. M. MEIGS, B.A. AND J. L. BIEDLER, B.A.

THE DEVELOPMENT of resistance to the antagonists of pteroylglutamic acid (PGA) in children with acute leukemia who have originally responded to these drugs is a well recognized phenomenon. A similar situation has been demonstrated in transplanted mouse lymphoid leukemia Ak4<sup>1</sup> where a hitherto sensitive strain, after continued passage through treated mice, became so resistant to 4-amino-N<sup>10</sup>-methyl-PGA<sup>2</sup> that no prolongation of survival time resulted from the usual therapeutic course of the drug. After fifteen passages through treated animals, this strain of leukemia (Ak4R) was then passed through ten generations of untreated animals with no loss of resistance.<sup>3</sup> Similar resistance of a leukemic tumor L 1210 to several 4-amino antagonists of PGA has recently been reported by Law and Boyle.<sup>4</sup>

In an attempt to quantitate this resistance a method reported previously<sup>5</sup> for studying the effects of varying doses of 4-amino-N<sup>10</sup>-methyl-PGA on the total and differential leukocyte count was employed on the sensitive and resistant strains.

### METHOD

The parent strain of transplanted leukemia (Ak4) and the development of the resistant variant (Ak4R) employed in these studies have been described.<sup>5-7</sup> Young male mice of the inbred Akm stock, weighing approximately twenty grams each were used. As in previous studies,<sup>6</sup> the leukemia was transmitted by intraperitoneal inoculation of 0.1 cc. of a saline suspension of leukemic spleen so diluted as to contain 1,000,000 cells.

Two types of experiments were used in this study. In the first, treatment was begun forty-eight hours after the inoculation of the leukemia and all injections of the compound were given intraperitoneally in 0.2 cc. of saline 3 times weekly for 10 doses. The treated mice received 1 or 2 mg./Kg. of 4-amino-N<sup>10</sup>-methyl-PGA in saline, whereas the controls were given only saline. The mice were weighed weekly and the dose adjusted on a weight basis to conform to the average weight of the animals in each cage. As a rule, mice on such therapy remained at a constant weight or gained slightly less than the controls. All mice were kept on a standard diet of Purina Laboratory Chow throughout the experiments.

In these experiments the effect of such courses of therapy on the total leukocyte and differential count of leukemic mice was evaluated. Control leukocyte and differential counts were done before either the leukemia was transmitted or treatment begun and were then repeated every four days. In order that the counts be done on free-flowing blood the mice

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From the Division of Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York City.

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were warmed for ten minutes under an electric lamp. A small cut was made in a tail vein with a razor blade and the studies were done on the blood obtained in this manner. Blood smears were stained with Wright's stain.

The animals that died in the course of treatment were autopsied and the spleen, liver and lymph nodes were examined for gross evidence of leukemia. Microscopic sections were also taken.

In the second group of experiments, mice with advanced leukemia and high leukocyte counts were given a single injection of 3 to 50 mg./Kg. of 4-amino-N<sup>10</sup>-methyl-PGA on about the seventh day of the disease. Total and differential leukocyte counts were obtained twenty-four hours after the single injection of the drug.

In the testing of 2,6-diaminopurine survival time experiments were used where the drug was administered intraperitoneally 3 times weekly beginning forty-eight hours after the

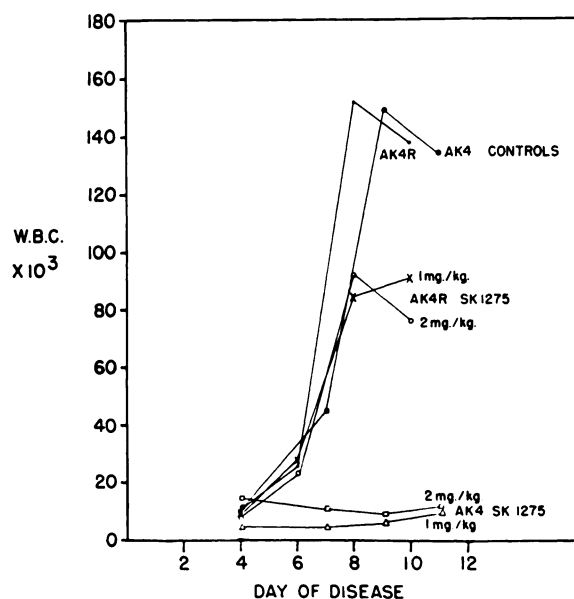


FIG. 1.—Effect of 4-amino-N<sup>10</sup>-methyl-PGA (SK 1275) 1 mg./Kg. and 2 mg./Kg. 3 times weekly on developing Ak4 and Ak4R leukemia.

injection of the leukemia. The effect of this drug was also studied on advanced leukemia when given every four hours for 3 doses on the seventh day of the disease.

#### RESULTS AND DISCUSSION

Mice injected with the Ak4 and Ak4R strains of leukemia developed a morphologically similar picture of leukemia. As can be seen from figure 1, the total leukocyte count begins to rise on or about the sixth day of the disease, and there is a concurrent rise in the percentage of polymorphocytes.<sup>5</sup> These abnormal forms are indistinguishable in the two strains. The only difference that has been noted in untreated mice with the two strains is the tendency of the high total leukocyte count in the Ak4R to level off or fall slightly just before death, whereas in the parent Ak4 line the count generally continues to rise. Although the two leukemias are morphologically similar, a definite biochemical difference must

exist since at ordinary therapeutic dosage there was a marked difference in the response of Ak4 and Ak4R to 4-amino- $N^{10}$ -methyl-PGA. At 1 mg./Kg. or 2 mg./Kg. 3 times weekly there was depression of the leukocyte count (figure 1), prevention of visceral infiltration (figure 2) and prolongation of survival time in strain Ak4; but with the resistant strain, Ak4R, the leukocyte count (figure 1, table 1)

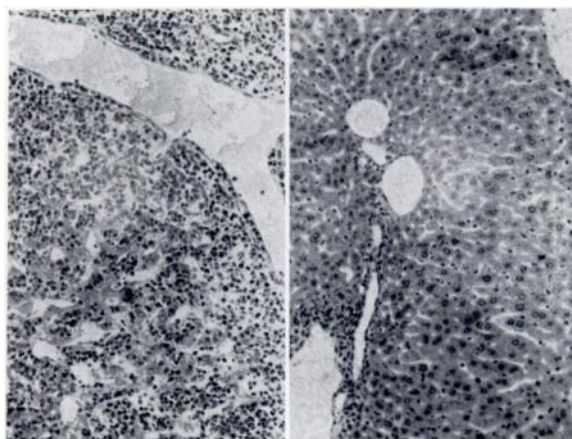


FIG. 2.—Sections of liver from mice on eighth day after inoculation with Ak4 leukemia. Left section from control mouse showing heavy leukemic infiltration. Right section from mouse treated with 4-amino- $N^{10}$ -methyl-PGA 3 mg./Kg. 3 times weekly showing very slight leukemic infiltration.

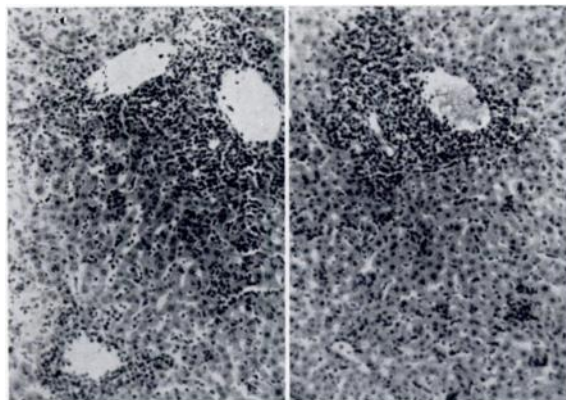


FIG. 3.—Sections of liver from mice on the eighth day after inoculation with Ak4R leukemia. Left control mouse, right mouse treated with 4-amino- $N^{10}$ -methyl-PGA 3 mg./Kg. 3 times weekly. There is a similar degree of leukemic infiltration in each section.

visceral infiltration (figure 3) and survival time were approximately equal in both treated and untreated mice.

When the leukemias were allowed to reach an advanced stage with high total counts a single dose of 3 mg./Kg. caused a marked fall in the total leukocytes of the peripheral blood within twenty-four hours in Ak4 leukemia, but

similar doses were without effect on the Ak4R strain (figure 4). When the single dose was increased to 50 mg./Kg. or above, however, both strains showed a fall in white blood count within the twenty-four hour period (table 2). Thus it

TABLE 1.—The effect of small doses of 4-amino-*N*<sup>10</sup>-methyl-pteroylglutamic acid given 3 times weekly on Ak4 and Ak4R leukemia

Strain	Dose mg./Kg.	Total White Blood Count					
		Fourth day		Eighth Day		Tenth Day	
		Range	Mean	Range	Mean	Range	Mean
Ak4	control	10.1-14.8	10.6	11.0-107.0	44.8	124.0-143.0	133.2*
Ak4	1 mg./Kg.	3.5- 8.5	5.1	2.5- 5.0	3.5	4.4- 26.8	9.0
Ak4	2 mg./Kg.	9.2-17.8	14.3	7.0- 18.8	11.5	7.3- 22.0	12.4
Ak4R	controls	7.6-13.5	10.2	105.0-279.0	151.0	84.0-252.0	136.7
Ak4R	1 mg./Kg.	5.2-15.7	9.4	50.0-118.0	83.1	58.0-117.0	90.3**
Ak4R	2 mg./Kg.	5.7-12.9	9.1	53.0-124.0	91.5	33.0-117.0	75.6

\* Represents only two mice remaining.

\*\* Represents only three mice remaining.

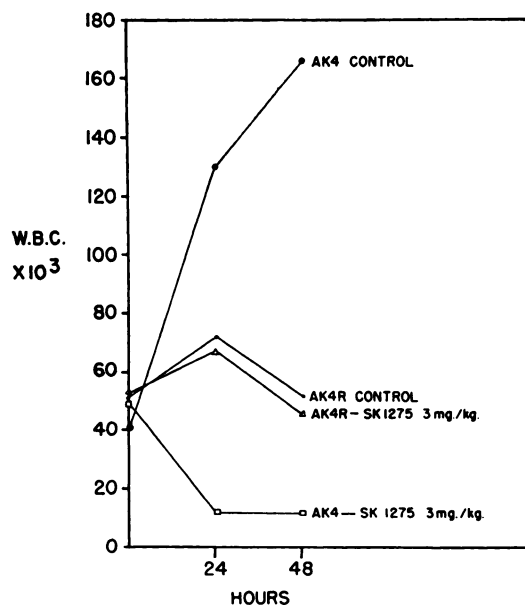


FIG. 4.—Effect of single dose of 4-amino-*N*<sup>10</sup>-methyl-PGA (SK 1275) on advanced Ak4 and Ak4R leukemia.

would appear that the resistance of strain Ak4R was relative and was only for dosage levels in the therapeutic range.

It has been demonstrated that streptomycin resistance may progress to streptomycin dependence in strains of meningococci,<sup>7</sup> tubercle bacilli,<sup>8</sup> Staph. aureus,<sup>9</sup> E. coli,<sup>9</sup> and Pseud. aeruginosa.<sup>9</sup> By analogy it was expected that after

many generations of exposure to 4-amino-N<sup>10</sup>-methyl-PGA the resistant strain would develop dependence to this drug. Despite the fact that leukemia Ak4R has been transferred through treated mice for over fifty generations there has so far been no conclusive evidence that these leukemic cells proliferate more

TABLE 2.—The effect of varying single doses of 4-amino-N<sup>10</sup>-methyl-pteroylglutamic acid on advanced Ak4 and Ak4R leukemia

Strain	Dose mg./Kg.	Total White Blood Count			
		Before Injection		Twenty-four Hrs. After Injection	
		Range	Mean	Range	Mean
Ak4	control	11.9- 99.0	38.2	70.0-250.0	128.9*
Ak4	3 mg./Kg.	10.5-123.0	49.2	5.0- 20.4	11.2
Ak4R	control	18.4- 67.0	50.4	30.9-115.0	70.7
Ak4R	3 mg./Kg.	26.2-108.0	65.7	33.0-150.0	80.1
Ak4R	50 mg./Kg.	11.2-126.0	50.4	6.5- 62.0	17.7

\* Represents the only 5 mice remaining.

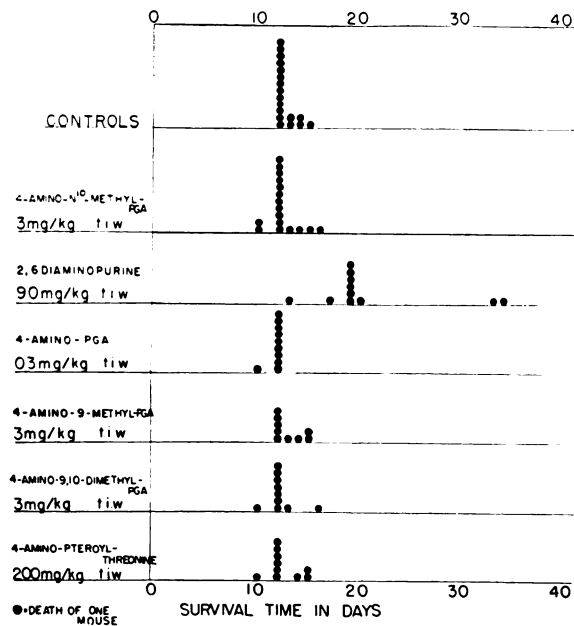


FIG. 5.—Leukemia Ak4R (resistant to therapy with 4-amino-N<sup>10</sup>-methyl-PGA).

rapidly in the treated than in the control animals. Law and Boyle,<sup>4</sup> however, using an acute lymphoid leukemia L 1210, were able after only twelve generations to demonstrate on the ninth day after inoculation a two-fold increase in the size of the tumors treated with A-methopterin over those in the control mice. This latter observation would appear to indicate the induction of at least a partial drug dependence.

It is of considerable interest that in contrast to the relative resistance of

Ak4R to 4-amino-N<sup>10</sup>-methyl-PGA both strains appear equally sensitive to 2,6-diaminopurine, an antagonist of adenine.<sup>10</sup> Three doses of 50 mg./Kg. of this drug given at 4 hourly intervals caused an approximately equal fall in leukocytes in advanced leukemia of both strains, but half this dose had little leukotoxic action.

There was no prolongation of survival time in the Ak4R strain when treated with any of the 4-amino derivatives of PGA which had proven effective against the parent strain Ak4.<sup>6</sup> 2, 6-Diaminopurine, however, caused a significant prolongation of survival time against this anti-folic resistant strain (figure 5). This drug has previously been shown to be effective against Ak4 leukemia.<sup>6, 11, 12</sup>

Many other examples of resistance appearing after exposure to various agents have been reported in viruses,<sup>13</sup> bacteria,<sup>14-18</sup> plasmodia,<sup>19-21</sup> trypanosomes<sup>22-25</sup> and house flies.<sup>26-28</sup> We believe that the resistance to 4-amino-N<sup>10</sup>-methyl-PGA demonstrated in strain Ak4R can be attributed to a random mutation whose growth has been selectively favored by a form of therapy inhibitory to the less resistant strains.<sup>18</sup> The fact that there has been no loss of resistance after over twenty passages through untreated mice is evidence that this was a true mutation.

The degree of resistance to 4-amino-N<sup>10</sup>-methyl-PGA would indicate a significant difference between the metabolism of the cells of the Ak4 and the Ak4R strains. Studies on these biochemical differences are now in progress.

#### SUMMARY

1. Further studies contrasting the effect of varying doses of 4-amino-N<sup>10</sup>-methyl-PGA on a resistant variant (Ak4R) of transplanted mouse leukemia Ak4 have been reported.

2. The usual therapeutic dosage of 4-amino-N<sup>10</sup>-methyl-PGA prevented the increase in total leukocytes, delayed the visceral infiltration, and increased survival time in the Ak4 strain but was without effect in strain Ak4R.

3. In the Ak4 leukemic mice with high leukocyte counts a marked drop within twenty-four hours could be demonstrated after a single dose of 3 mg./Kg. of this drug. With the Ak4R strain there was sufficient resistance so that a dose of at least 25 mg./Kg. was needed to show the marked leukopenia in twenty-four hours.

4. The Ak4R strain was as sensitive as the Ak4 to the leukotoxic effects of 2,6-diaminopurine. This drug also was effective in prolonging the survival time of mice with Ak4R leukemia.

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