leukemic agent would stop at this point its "vertical"*(1,6) trend of transmission; it would be only logical to assume that from this second generation the agent will then pass to the next one, and so forth. Thus, a line of mice hitherto essentially free from this disease, would have changed into a line in which leukemia develops spontaneously in successive generations, as in the case of the leukemic Ak inbred line. A new leukemic inbred line of mice would thus have been originated, at will, by inoculation, in the laboratory. Since, however, the mouse leukemia agent, unlike mouse mammary carcinoma, does not pass from parents to their offspring through mothers' milk(7,8,9,4), but is apparently transmitted directly through the embryos(2), like that of chicken lymphomatosis(10), the investigator would have no means at his disposal of stopping the vertical transmission of mouse leukemia once it has been established in a family of mice.

Summary. 1. When centrifugated extracts of Ak mouse leukemia were inoculated into 16 suckling C3H(f) infant mice less than 12 hours old, "spontaneous" leukemia developed in 8 of them at 8½ to 11 months, and in 5 mice at 14 to 18 months of age. 2. When the centrifugated leukemic extracts were inoculated into 20 C3H(f) infant mice 3 to 6 days old, "spontaneous" leukemia developed in 10 of them, but not before they were 18 months of age. 3. Filtered (Seitz) leukemic extracts were inoculated, in 2 experiments, into 7 C3H(f) infant mice 8 days old, and 2 of them developed leukemia at 23 and 27 months respectively. In 5 additional experiments, leukemic filtrates were inoculated into 25 C3H(f) infant mice less than 12 hours old, and 7 of them developed "spontaneous" leukemia at 6½ to 9 months of age. These results suggest that the Ak mouse leukemia agent is filterable. 4. In 5 experiments, suckling infant C3H or C3H(f) mice were inoculated with either centrifugated leukemic extracts, leukemic cell suspensions, or with normal Ak-embryo-cell suspensions. After the inoculated infant mice have grown up and reached sexual maturity, they were mated, Of the 10 inoculated parents, 7 eventually developed "spontaneous" leukemia at 9 to 19 months of age. Of the 27 untreated offspring, 13, or 48%, have thus far developed "spontaneous" leukemia at 14 to 20 months of age. The results of these experiments suggest that the mouse leukemia agent is transmitted from parents to their offspring. The transmission of the agent occurs most probably directly through the embryos(2,4). 5. The incidence of spontaneous leukemia among the untreated males and females of the C3H or C3H(f) colonies of mice in our laboratory has been, during the past several years, less than 0.07%.

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Mechanisms of Amethopterin Resistance in Leukemia. I. Effects of Weak Folic Acid Antagonists on Mouse Leukemias. (19069)

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In the 20% to 50% of children with acute leukemia whose disease initially responds to amethopterin (4-amino-N^{10}-methyl PGA), the eventual failure of therapy is usually due to the development of resistance to this agent by the leukemic cell(1,2). For this reason experimental studies of the mechanism of this resistance have been undertaken in the hope

of finding ways of improving this type of therapy.

In order to conduct such studies, a strain of mouse leukemia resistant to therapy with amethopterin was necessary. A subline resistant to this agent was developed in a previously sensitive lymphoid strain of transplanted leukemia, Ak4(3), by passing the leukemic cells for 4 or more generations through animals treated with amethopterin(4). In the resistant subline, Ak4R(5), there is no difference in the survival time between treated and untreated animals, whereas in the sensitive parent strain, Ak4, the treated animals survive 2 or 3 times as long as the untreated. It is felt that this resistance is a true mutation since there has been no return to sensitivity even after the strain has been passed for more than 30 generations through untreated mice. This line also displays resistance to all other 4-amino derivatives of PGA tested, but not to 2,6-diamino-purine(5,6). The incorporation of radioactive formate into the nucleic acid purines of the viscera of leukemic mice treated with amethopterin is considerably greater with the Ak4R strain of leukemia than with the parent strain(7). Law *et al.*(8) have developed sublines of a lymphoid leukemic tumor resistant to 4-amino-N^{10}-methyl PGA which also display a similar cross resistance to 4-amino-9-methyl PGA, and 4-amino-9,10-dimethyl PGA. One of these strains actually developed dependence and grew better in the mice treated with amethopterin. Weir, Welch, and Heinle(9) showed a definite chemotherapeutic effect from a combination of folic acid free diet, succinyl sulfathiazole, and a crude antagonist of folic acid on a strain of transplanted mouse leukemia in Ak mice. This crude X-methyl folic acid(10) was presumably a mixture of 9-methyl PGA, 7-methyl PGA, and possibly other weak antagonists of folic acid. Although the Ak4R strain is resistant to the 4-amino derivatives of PGA, it was thought possible that it might still be sensitive to antagonists which did not possess an amino group in the 4 position of the pteridine ring.

Method. Mice of the inbred Akm stock were placed on the folic acid free and riboflavin free diet† containing 1% sulfasuxidine and a weak folic acid antagonist (either crude X-methyl folic or 9-methyl PGA†) for periods of time ranging from 7-13 days before inoculation of the leukemias and were maintained on the diet throughout the course of the experiments. The mice were inoculated intraperitoneally with 0.1 cc of a saline suspension of either Ak4 or Ak4R spleen so diluted that each mouse received 1,000,000 cells. Mice which were not on the special diet, but had been maintained on Purina Laboratory

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TABLE I. Survival Time Results.

Exp.		Leukemia AK4 No. mice	Leukemia AK4 Avg surv. time (days)	Leukemia AK4 Inc. surv. time (%)	Leukemia AK4R No. mice	Leukemia AK4R Avg surv. time (days)	Leukemia AK4R Inc. surv. time (%)
I	Control diet				9	13.4	—
	+ ameth @ 2 mg/kg				10	13.2	–1
	Basal diet (no sulfasuxidine)				10	13.4	0
	Basal diet				10	13.9	4
	+ 1% CA				10	16.9	26
	+ 1% 9-methyl-PGA				8	21.9	63
II	Control diet	10	8.2	—	10	10.9	—
	Basal diet + 1% CA	10	13.2	61	9	15.9	46
	+ PGA @ 15 mg/kg				10	12.4	14
III	Control diet	10	9	—	10	11	—
	+ ameth @ 2 mg/kg	8	23.1	157	10	11.6	5
	Basal diet + 1% CA	10	17.8	98	10	18.6	69
	+ PGA @ 30 mg/kg	10	11.6	29	9	13.6	24
	+ riboflavin @ 5 mg/kg	10	17	89	10	17.3	57
IV	Control diet				9	11.8	—
	+ ameth @ 2 mg/kg				9	11.9	1
	Basal diet + .1% 9-methyl-PGA				10	20.2	71
	+ .25% N^{10}-methyl-PGA				10	14	19
	+ 1% CA				10	18.4	56
	+ CF @ 15 mg/kg				10	13.5	14
V	Control diet				10	11.4	—
	+ ameth @ 2 mg/kg				10	12.7	11
	Basal diet + .3% 9-methyl PGA				10	18.6	63
	+ .9% " "				7	23.7	108
VI	Control diet	10	9.6	—			
	Basal diet + 1% CA	9	16.2	69			
	+ CF @ .1 mg/kg	9	12.2	27			
	+ PGA @ 7.5 mg/kg	8	7.8	–19			
VII	Control diet	7	8	—	9	13.3	—
	+ ameth @ 2 mg/kg	9	24.1	201	9	13.6	2
	+ PGA @ 7.5 mg/kg	8	17.4	118	10	13.3	0
	+ CF @ .2 mg/kg	9	15.9	99	9	13.8	4
	Basal diet + 1% CA	7	22	175	10	21.1	59
	+ CF @ .2 mg/kg	10	11.9	49	10	15.5	17
	.4 mg/kg	10	9.4	18	10	15.8	19
VIII	Control diet				19	9.4	—
	+ ameth @ 2 mg/kg				10	10.1	7
	Basal diet + 2.5% 9-methyl PGA				10	17.9	90
	+ .00025% ameth				6	9.7	3
	+ .0005% "				7	9.7	3
IX	Control diet	10	8.8	—			
	+ ameth @ 2 mg/kg	10	14.6	66			
	Basal diet + .000125% ameth	9	14.7	67			

Basal diet = folic acid free and riboflavin free diet plus 1% sulfasuxidine.

Chow, were also inoculated with the same suspension of leukemic cells at the same time to serve as controls. One set of 10 mice, treated with amethopterin, on the regular diet was usually included in each experiment. Intraperitoneal injections of this agent were started 48 hours after transmission of the leukemia at a dosage level of 2 mg/kg, and continued 3 times weekly to a limit of 10 doses. In order to ascertain whether the therapeutic effect of the crude antagonist could be prevented by folic acid (PGA) or citrovorum factor (CF), or riboflavin, these compounds were included in some of the experiments following the same schedule and route as employed with amethopterin. All mice were autopsied at death and if gross manifestations of leukemia were not present, microscopic sections

were taken.

Results and discussion. The survival time results obtained with leukemia Ak4 and Ak4R when the mice were maintained on the folic acid free diet supplemented with either the crude antagonist or 9-methyl PGA are presented in Table I. Exp. I in this table shows the lack of effect of the purified diet with or without the addition of the sulfasuxidine when no antagonist was given. There was a definite prolongation of the survival time of mice inoculated with leukemias Ak4 and Ak4R when placed on a combination of the folic acid free diet, sulfasuxidine, and either the crude antagonist of folic acid or 9-methyl PGA. Similar therapeutic effects against strain Ak4R could not be obtained when amethopterin was substituted for the crude antagonist or 9-methyl PGA in this regime. The antileukemic action of the combination of diet, sulfonamide, and crude antagonist could be prevented by giving injections of PGA or CF, but not by riboflavin.

Although the 4-amino derivatives have been shown by Nichol and Welch(14) to prevent the conversion of PGA to CF, the fact that in the mouse and rat a competitive relationship over a wide range has been demonstrated for amethopterin and CF(11-14), but not for amethopterin and PGA, when metabolite and antimetabolite were given simultaneously (15-17), suggests that amethopterin may perhaps be more correctly termed a CF antagonist. The much greater potency of CF than PGA on a dry weight basis in preventing the antileukemic effects of amethopterin also suggests that these therapeutic effects are due to the amethopterin-CF antagonism(18,19).

Ak4R leukemia has been shown to be equally resistant to 4-amino PGA, 4-amino-N^{10}-methyl PGA, 4-amino-9-methyl PGA, 4-amino-9,10-dimethyl PGA, and 4-amino-pteroyl aspartic acid(5).

Up to the present time we have been unable to demonstrate any greater content of PGA or CF in Ak4R than in Ak4 spleens, any increased ability of Ak4R spleen suspension to detoxify amethopterin on incubation at 37°C for four hours, or any decreased ability of these same spleen suspensions to adsorb or absorb amethopterin(20). In the light of the above mentioned observations, it would seem that the most likely mechanisms to account for the sensitivity to 9-methyl PGA and resistance to 4-amino-N^{10}-methyl PGA and 4-amino-9-methyl PGA are qualitative changes in the enzyme systems in the leukemic cell for which amethopterin and PGA and amethopterin and CF compete, acting to decrease their relative affinity for amethopterin, or possibly the establishment of an alternate metabolic pathway which by-passes citrovorum factor.

Summary. (1) A definite increase in survival time of mice inoculated with the Ak4 and Ak4R strains of transplantable mouse leukemia was noted when the animals were kept on a purified diet containing no folic acid and no riboflavin, to which had been added sulfasuxidine and 9-methyl PGA. (2) This increase in survival time could be prevented by the administration of citrovorum factor or folic acid, but not of riboflavin.

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