Anemic Stress as a Trigger of Myelogenous Leukemia in Rats Rendered Leukemia-Prone by X-ray

Abstract. *All of the 128 Sprague-Dawley female rats bled two-thirds of the blood volume at 1, 2, or 3 months after irradiation (50, 170, or 350 roentgens) succumbed to leukemia by 16 months after bleeding. Some nonbled irradiated rats developed leukemia possibly as a result of triggering by a radiation-induced anemia. The threshold leukemogenic x-ray dose is probably below 25 roentgens. Elevated levels of basophils, neutrophils containing the "pseudo-Pelger" type nuclear anomaly, and myeloblasts exhibiting Auer rods seen in these animals were generally not found in earlier models of rat myelogenous leukemia.*

A latent marrow injury in the rat, lasting at least 14 months after an acute total body exposure to 170 r of x-ray, has been reported (*1*). The injury was manifested as a subnormal increase in marrow nucleated red blood cells following acute anemia induced by bleedings of one-third or two-thirds of the circulating blood. The absolute increases during 48 hours after bleeding ranged from 0.30 to 1.66, and 1.77 to 5.62×10^9 cells per kilogram of body weight for the irradiated and the nonirradiated rats, respectively, when studied during the first 3 months after irradiation. The increase in total nucleated marrow cells was similar in both irradiated and unirradiated groups (*2*). The marrow defect is interpreted as an inability to divert pluripotential stem cells from the myeloid to the erythroid line of development. These observations suggested that prolonged anemic stress in irradiated animals could result in abnormally severe and uncontrolled granulocytosis, essentially a myelogenous leukemia (ML). This report describes experiments which verify that hypothesis.

Two groups of female Sprague-Dawley rats were used. Group A consisted of rats which, at age 24 weeks, were exposed to 0, 25, 50, 110, 260, or 350 r. Animals in group B were irradiated with doses of 0, 50, 170, or 350 r at age 12 weeks; some of these were then bled 1, 2, or 3 months after irradiation. The irradiation technique was similar to that described earlier (*1*). Bleeding, when done, consisted of an acute removal of one-third of the total blood volume (considered equal to 6.4 percent of body weight) twice in 24 hours, via the jugular vein, that is, a total of two-thirds of the blood volume.

All rats survived the acute effects of the radiation and the venesection. By 8 months after irradiation (that is, 5 to 7 months after bleeding), 6 percent of the nonbled irradiated and 87 percent of the bled irradiated rats had died. Within days before death, all animals showed labored breathing, weight loss (15 to 30 percent), epistaxes, conjunctival hemorrhages, ecchymoses of the regions of the paws and ears, and unkempt appearance, while some also showed blood-stained genital regions and ulcerations of the skin. Leukocytosis (*3*) and moderate to severe anemia (hematocrits of 15 to 30 percent) were also seen. Death in all cases was attributed to complications generally associated with the terminal phase of ML (that is, pneumonia, anemia, and inanition). The low mortality of the nonbled irradiated animals was related to the low leukemic incidence (17 percent) in these animals. All unirradiated animals survived the study period. Only the bled irradiated rats were followed until they expired. All other animals were killed by the tenth month of the study. Figure 1 shows that survival time of the bled irradiated rats was a complex function of the x-ray dose and time when they were bled after irradiation.

Myelogenous leukemia was diagnosed on the basis of at least one of the following: (i) circulating basophil level, at least 3.5 percent of white blood cells, (ii) marrow basophils, at least 3.5 percent of total nucleated cells, (iii) marrow myeloblasts, at least 3.5 percent of total nucleated cells, and (iv) chloroma tumor (*4, 5*). Approximately one-fifth of the leukemic rats had myeloblasts exhibiting Auer rods and a similar fraction showed neutrophilic polymorphs containing the "pseudo-Pelger" type nuclear anomaly (*6*) (see Fig. 2). Table 1 shows that while all bled irradiated rats developed ML, none among the bled unirradiated groups did. Thus, x-irradiation followed by bleeding was 100 percent effective in the production of ML. However, Table 1 also shows that even without phlebotomy, some irradiated rats developed ML. The incidence was directly proportional to the x-ray dose between 25 and 170 r (Fig. 3). Above 170 r, the ML incidence decreased with increased dose. [A similar type of dose-response curve for ML incidence in the RF mouse had been reported (*7*).] The presence of ML among the nonbled ir-

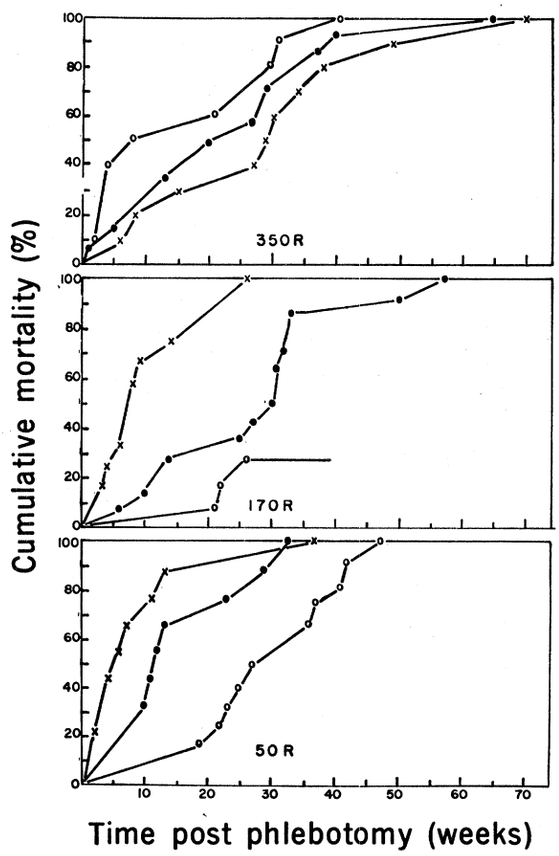

Fig. 1. The effect of x-ray dose and time of bleeding on cumulative mortality (percent). In all cases, two-thirds of the blood volume was removed. The number of rats used for each curve is shown in Table 1. Note that times for 50 percent cumulative mortality (CM_{50}) were 5.0, 7.5, and 29 weeks for rats given 50, 170, and 350 r, respectively, when all were bled 1 month after irradiation. The study of the 170-r animals bled 3 months after irradiation was discontinued 39 weeks after bleeding. All rats had ML. x——x, Bled 1 month after x-irradiation; ●——●, bled 2 months after x-irradiation; ○——○, bled 3 months after x-irradiation.

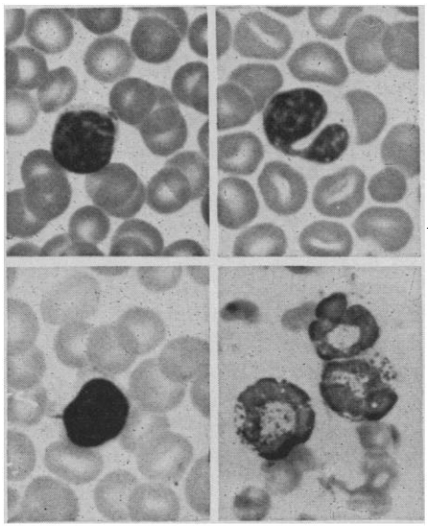

Fig. 2. (Top left and bottom left) Myeloblasts with Auer rods. (Top right) Neutrophilic polymorph with "pseudo-Pelger" type nuclear anomaly. (Bottom right) Three basophils.

radiated animals need not indicate a development of ML in the absence of anemia. Radiation-induced anemia having a severity roughly proportional to the x-ray dose occurs within 1 month after irradiation (*8*). If the anemia were very near the severity required for ML induction, the increased incidence of ML seen as the x-ray dose is raised could reflect the greater probability of individual rats becoming sufficiently anemic to result in their developing the disease. (The explanation for the declining portion of the curve is considered below.)

These findings suggest that leukemogenesis involved a two-step process; first, the x-ray rendered the leukemia-prone condition, and second, the anemia triggered the actual disease. The two-step concept of leukemogenesis is not new (*9*) but in the present model a leukemia-prone animal is specifically defined as one which will invariably develop ML when triggered by an adequate anemia. Further, leukemia is considered present as soon as trigger is completed.

Conspicuous shifting of the mortality curves along the time axis in Fig. 1 is seen with different combinations of bleeding time and x-ray dose which, by definition, are factors involved with the leukemia-prone state. Since all rats were bled a similar amount, these fluctuations must reflect changes in the level of leukemia-proneness, suggesting that the severity of the biological damage which constitutes the leukemia-prone state must also determine the severity of the ML state. Thus, high proneness must be directly related to early deaths, that is, short survival duration. Survival durations based on the time of 50 percent cumulative mortality (CM_{50}) suggest that at 1 month after irradiation, the level of proneness was relatively

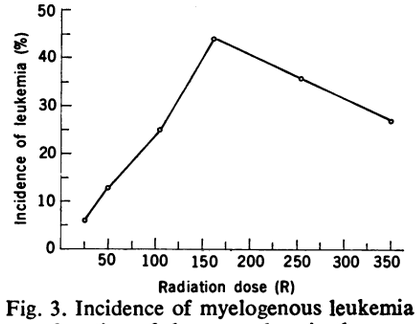

Fig. 3. Incidence of myelogenous leukemia as a function of the x-ray dose in the nonbled irradiated groups. Findings in groups A and B were combined. The number of leukemic rats found per number of rats examined were as follows: 0 r, 0/79; 25 r, 2/35; 50 r, 6/48; 110 r, 8/32; 170 r, 8/18; 260 r, 10/28; and 350 r, 10/38.

higher in the animals that received 50 and 170 r than in those that received 350 r (CM_{50}'s were 5, 7.5, and 29 weeks, respectively). In subsequent months, the proneness to leukemia of the animals that received a low dose decreased, while that of the animals that received a high dose increased.

The levels of leukemia-proneness present at 1 month after irradiation could explain the declining ML incidence seen with doses higher than 170 r (Fig. 3). X-ray–induced anemia occurred at the time (1 month after irradiation) when proneness was relatively high in the low dose animals. This would fit the notion that below 170 r

Table 1. Circulating white blood cells (WBC), basophils in blood (percent of white blood cells), basophils and myeloblasts in marrow (percent of total nucleated cells), number of rats with chloroma, and number of leukemic rats (L) found per number of rats studied (E) (*13*).

Dose (r)	WBC ($\times 10^{-3}$)		Blood basophils*		Marrow basophils*		Marrow myeloblasts*		Rats with chloroma	L/E
	Range	Median	$\bar{X} \pm$ S.E.	Rats (*N*)	$\bar{X} \pm$ S.E.	Rats (*N*)	$\bar{X} \pm$ S.E.	Rats (*N*)		
Rats bled 1 month after exposure										
0	11 to 25	16	0.9 ± 0.2	12	1.3 ± 0.4	11	0.7 ± 0.1	11	0	0/12
50	18 to 80	43	12.8 ± 2.6	12	7.9 ± 2.0	8	8.2 ± 0.7	8	2	15/15
170	2 to 52	37	9.2 ± 0.6	12	7.0 ± 1.3	14	9.0 ± 0.9	14	2	18/18
350	13 to 48	38	8.7 ± 0.6	13	8.0 ± 1.1	10	6.6 ± 0.5	10	3	17/17
Rats bled 2 months after exposure										
0	8 to 33	15	1.3 ± 0.2	10	1.8 ± 0.3	10	0.8 ± 1.0	10	0	0/10
50	15 to 65	37	13.9 ± 0.4	10	7.0 ± 0.2	6	7.4 ± 1.0	6	1	13/13
170	3 to 53	35	8.8 ± 1.1	10	7.3 ± 1.0	6	10.2 ± 0.1	6	5	14/14
350	5 to 70	38	11.6 ± 1.0	11	11.0 ± 1.9	7	12.0 ± 1.8	7	4	16/16
Rats bled 3 months after exposure										
0	8 to 26	10	0.7 ± 0.3	9					0	0/9
50	24 to 57	40	11.4 ± 1.4	11	7.0 ± 0.5	6	6.3 ± 0.6	6	3	13/13
170	25 to 52	31	7.8 ± 0.8	10	6.3 ± 0.9	7	13.2 ± 0.2	7	4	12/12
350	4 to 29	9	10.4 ± 1.8	7	11.0 ± 1.8	6	12.5 ± 2.1	6	3	10/10
Nonbled groups										
0			0.9 ± 0.1	79	1.3 ± 0.1	30	0.9 ± 0.1	30	0	0/79
25 to 350†					1.2 ± 0.1	155	1.1 ± 0.1	155	0	0/155
25 to 350†			8.0 ± 0.4	44	4.8 ± 0.2	44	5.2 ± 0.2	44	9	44/44

* Not included are 21 rats with myeloblast values exceeding 25 percent (the median value of this group was 34 percent). No basophil values exceeded 25 percent. † Doses used were 25, 50, 110, 170, 260, and 350 r. (The numbers of leukemic rats found per number of rats examined, for individual doses, are plotted in Fig. 3.) All nonbled irradiated animals found leukemic were grouped together for average values of basophils and myeloblasts.

ML incidence was limited by the relatively mild induced anemia rather than by low proneness. However, in the high dose animals leukemia-proneness would be relatively low at this time. The declining ML incidence at doses above 170 r could then reflect the decreased probability that proneness present was of a sufficient level that the disease could be triggered, in spite of the increased severity of the anemia resulting from the higher x-ray doses used. Thus, the limiting factor in ML incidence at the higher dose range was the inadequacy of the leukemia-proneness.

In sum, x-rays (25 to 350 r) can render all rats leukemia-prone (*10*). Myelogenous leukemia develops only in the presence of an adequate triggering anemia. Threshold anemic severity for triggering is inversely related to leukemia-proneness. Proneness to leukemia varies according to dose and time after irradiation (up to at least 3 months). Anemia resulting from the removal of two-thirds of the blood volume was adequate to trigger ML in 100 percent of the rats exposed, from 50 to 350 r. Because the 5.7 percent ML incidence among the nonbled animals that received 25 r was probably only a fraction of the rats rendered leukemia-prone, the threshold x-ray dose for leukemogensis, that is, for leukemia-proneness, must be considerably lower than 25 r.

The applicability of this two-step model of leukemogenesis in other species remains to be established. However, the close resemblance of ML seen in rats (*11*, *12*) and the RF mouse (*13*, *14*) to the forms of the disease found in humans has been noted. In addition, the leukemogenic effect of ionizing radiation in both man and animals is well established (*15*).

Finally, should the present two-step leukemogenic mechanism prove valid for other species as well, two additional observations may be made. First, the relative persistence of the radiation-induced leukemia-proneness would suggest the possibility that the leukemogenic effect of ionizing radiation is additive. This would be of concern for those interested in radiological health standards, particularly since this study indicates that the leukemogenic threshold dose is probably below 25 r. Second, the number of leukemia-prone individuals in a given population must far exceed the number showing frank ML.

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References and Notes

1. J. K. Gong, T. J. MacVittie, J. E. Vertalino, *Radiat. Res.* **37**, 467 (1969).
2. J. K. Gong, in preparation.
3. Although several animals had white blood cell levels as high as 80,000 per cubic millimeter of blood, the majority had values ranging from 35,000 to 65,000, and several had values between 2,000 and 4,000. Thus, the majority of the animals had values similar in magnitude to that reported by Moloney *et al.* (*11*) in their transfer chloroleukemia study. The magnitude of leukocytosis in the rat would apear to be quite different from that in chronic ML in man [200,000 white blood cells per cubic millimeter is common—see W. Dameshek and F. Gunz, *Leukemia* (Grune & Stratton, New York, 1964)]. However, calculations show no real difference in granulocytic response. For example, the circulating white blood cells in man number approximately 8000 per cubic millimeter, of which 70 percent, or 5600, are granulocytes. In chronic ML, as many as 95 percent of the 200,000 circulating leukocytes may be granulocytes, an absolute value of 190,000. This would be a 32-fold increase over the normal level. On the other hand, the rat, a lymphoid animal, has a normal white blood cell count on the order of 10,000 per cubic millimeter, of which 25 percent, or 2500, are granulocytes. A white blood cell increase in this animal to 80,000 per cubic millimeter, of which 95 percent are granulocytes, would yield a total granulocytic count of 76,000 cells per cubic millimeter, a 29-fold increase. Thus, in chronic ML, both the human and rat show a similar level of granulocytosis.
4. Marrow imprints and smears were obtained from the femora at autopsy. Blood smears were studied at 2 to 6 months after bleeding, or, in the case of the nonbled animals, at 6 to 10 months after irradiation. All samples were stained with Wright's-Giemsa stains.
5. The green tumor was found in the lungs, liver, spleen, nasopharynx, marrow, and subcutaneous regions, and seeds of the tumor, 2 to 3 mm or less in length, were found in the retroperitoneal spaces. Smears, imprints, and sections of the seeds and marrow infiltrated with chloroma showed large, round cells having pale, vesicular, delicate nuclei.
6. Elevated basophil levels, and the presence of myeloblasts exhibiting Auer rods and polymorphonuclear neutrophilic granulocytes having the "pseudo-Pelger" type nuclear anomaly were not observed in previous models of ML rats (*11*). One case of a leukemic rat demonstrating a Pelger-Hüet nuclear anomaly has been reported [E. Hlavayova and F. Svec, *Acta Haematol.* **19**, 295 (1958)].
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10. That leukemia-proneness was not naturally present was shown when none of the bled unirradiated rats developed the disease. This would be consistent with the lack of any reported cases of spontaneously developed ML in this strain [W. C. Moloney *et al.* (*11*) and R. E. Zipf, L. Chiles, N. Miller, B. J. Katchman, *J. Nat. Cancer Inst.* **22**, 669 (1958)]. In contrast with this, the untreated RF mouse is known to develop ML spontaneously, especially during advanced age [A. C. Upton, F. F. Wolff, J. Furth, A. W. Kimball, *Cancer Res.* **18**, 842 (1958)]. It is possible that this mouse strain is naturally leukemia-prone, and the incidence of ML is a reflection of the risk of the animal becoming sufficiently anemic to trigger the disease, the probability of an anemic episode in a given animal being expected to increase with age.
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16. I thank Mary Byers for technical assistance, and Professors S. A. Ellison and C. A. Glomski for suport and encouragement. Supported by PHS grant FR-5330 and AEC grant AT(30-1)3728.

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Silkworm Bombyx mori L.: Nature of Diapause Factor

Abstract. *The diapause factor which is responsible for the induction of embryonic diapause in the silkworm* (Bombyx mori *L.*) *was isolated from the heads of female moths. It was extracted with 80 percent ethanol or 80 percent methanol and was heat-stable and not dialyzable. Since the active material was precipitated with ammonium sulfate and lost when incubated with proteolytic enzymes, it is highly probable that the diapause factor is a protein or a complex molecule containing peptide bonds. Molecular sieve techniques revealed two species of the diapause factor. The molecular weight of the smaller species lies between 5,000 and 10,000.*

"Diapause factor" (*1*) or "diapause hormone" (*2*), which originates in the subesophageal ganglion, determines embryonic diapause in the silkworm (*Bombyx mori* L.). The factor is released from the ganglion and exerts its effect on the ovary during the pupal stage of the female; diapause then occurs about 24 hours after the eggs have been laid.

In 1957, Hasegawa (*3*) reported the extraction of "diapause hormone" from the complex of brain and subesophageal ganglion in silkworm pupae. He extracted the material with 80 percent methanol, concentrated the extract, washed the concentrate with ether, and finally extracted the active material from the aqueous phase with chloroform. He did not, however, comment on the chemical nature of the factor. In the abstract of their paper on the mode of action of the diapause hormone, Hasegawa and Yamashita (*4*) mentioned that this factor might be "a kind of lipid." No further report on the chemical properties of the factor has appeared.