Autoradiographic Studies of the Action of Leucogenenol on the Blood Cells of The Rat[1] (35191)

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Leucogenenol, a compound isolated from the metabolic products of *Penicillium gilmanii* by Rice (1) and from human and bovine liver by Rice and Shaikh (2) and indicated by degradation studies to be 2-(1, 2-dihydroxy-3-methyl-5-oxo-cyclohexyl)-3,11-dihydroxy-11-(hydroxymethyl)-9-methyl-1-oxa-5-azaspiro [5,5] undeca-2,4-diene-7-one (3), induces a leukocytosis when it is injected intravenously or intraperitoneally into rabbits (4), dogs, mice, or monkeys (5, 6).

Injection of leucogenenol into animals is followed by a relative increase in the number of myeloblasts in the bone marrow and lymphoblasts in the spleen (5, 6), suggesting that leucogenenol stimulates the rate of replication of these cells from their respective precursors. The stimulatory action of leucogenenol on lymphoid cells is also indicated by the finding that leucogenenol decreases the latent period required for antibody production in sublethally X-radiated mice (7). The addition of leucogenenol to a culture of human lymphoblastoid or mouse leukemic cells increases their respiratory quotient and their rate of replication (8), suggesting that leucogenenol acts directly on the blood cells of animals.

It was of interest, therefore, to establish whether or not leucogenenol acted specifically on one or more of the progenitors to the circulating blood cell. Patt and Maloney demonstrated (9) that tritiated thymidine is first incorporated in the blast forms of myeloid and erythroid cells and then is carried through the series of myeloid and erythroid cells to appear in the corresponding circulating cells approximately 24 hr later. It appeared therefore that studies of the effect of leucogenenol on the time and intensity of the labeling of bone marrow blood cells with tritiated thymidine should indicate whether or not leucogenenol acted on one or more types of myeloid and erythroid blood cells. Similarly it might be possible to establish whether or not leucogenenol acted on one or more types of lymphoid cells. Accordingly rats were injected with tritiated thymidine and leucogenenol and the type and number of labeled blood cells in their bone marrow, spleen, and peripheral circulation compared by standard autoradiographic techniques (10–12) with the number of corresponding labeled cells in the bone marrow, spleen, and peripheral circulation of rats that were injected with tritiated thymidine alone.

Materials and Methods. Each of 60 rats (Wistar, approx. 250 g) was injected intraperitoneally with tritiated thymidine[2] (1 μCi/g of body wt). At approximately the same time 30 of the rats were injected intravenously through the tail vein with 0.02 μg of the calcium salt of leucogenenol dissolved in 0.2 ml of pyrogen-free isotonic saline. At intervals of 6, 12, 24, 48, and 96 hr thereafter a sample of blood was obtained from the tail vein of each rat, diluted in the usual manner in a Trenner pipet and the number of leukocytes was determined by means of a hemocytometer. Counts made in duplicate differed by less than 10%. Smears of blood for differential counts and autoradiographs were made at the same time on a minimum of six microscope slides. At the same time intervals of 6, 12, 24, 48, and 96 hr, 6 animals that were not injected with

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[2] Thymidine-methyl-^{3}H, 15 Ci/mmoles, obtained from New England Nuclear Corp., Boston, Mass.

leucogenenol and 6 animals that were injected with leucogenenol were exsanguinated under ether anesthesia by cardiac puncture. At least 12 smears of small samples of bone marrow from the femur of each of the sacrificed animals were made on microscope slides for autoradiographs. A minimum of 12 slides of spleen imprints was made from each animal by touching the freshly cut surface of the spleen to a microscope slide freshly coated with egg albumin. The spleen tissue was fixed in acetate buffered 10% formalin and 5-μ sections were cut and mounted on microscope slides for autoradiographs.[3] The smears of bone marrow were immediately fixed in absolute methanol, dipped in Kodak NTB-3 nuclear track emulsion and allowed to stand at 5° in light-proof boxes for 14 days. The slides were then developed in Kodak D-19, fixed in Kodak thiosulfate solution and washed. The developed slides were stained with Wright's and counterstained with Giemsa. Spleen imprints were fixed in a 1:1 (v/v) mixture of absolute ethanol and ether, dipped in nuclear track emulsion, developed, fixed, and stained in the same manner as the bone marrow smears. Smears of peripheral blood were treated as the bone marrow smears. The sections of spleen mounted on microscope slides were dipped in nuclear track emulsion, stored in light-proof boxes at 5°, and after 14 days, were developed and stained with hemotoxylin and eosin.

The distribution of labeled and unlabeled blood cells of the bone marrow, spleen imprints, and peripheral blood was determined by counting 100 cells of each type and tabulating them as: (a) unlabeled; (b) labeled with up to 5 grains above background; (c) labeled with 5 to 10 grains above background; and (d) labeled with over 10 grains above background. Differential counts were made on 1000 cells.

Results and Discussion. Table I shows the percentage of labeled blood cells in the bone marrow of rats at intervals following the injection of tritiated thymidine and leucogenenol. As shown, 6 hr after injection there is a significantly lower percentage of labeled myeloblasts in the bone marrow of the rats that were injected with tritiated thymidine and leucogenenol than in the rats that were injected with tritiated thymidine alone. However, at the same time there is a significant increase in the percentage of labeled myelocytes in the bone marrow of animals that received leucogenenol, suggesting that a number of labeled myeloblasts in the animals that recieved leucogenenol were transformed into promyelocytes and then into myelocytes in a 6-hr period. In contrast there was no countable number of labeled myelocytes in animals that were not injected with leucogenenol.

Twelve hr after animals were injected with both leucogenenol and tritiated thymidine they showed approximately twice the percentage (Table I) and approximately 5 times the relative number (Table II) of labeled myeloblasts in their bone marrow as the animals that were not injected with leucogenenol. There was also a significant increase in the percentage (Table I) and the relative number (Table II) of myelocytes and metamyelocytes in the bone marrow of animals injected wih leucogenenol as compared to the uninjected controls. The percentage of labeled neutrophils in the bone marrow (Table I) increased approximately threefold and the number of labeled neutrophils in the circulation (Table III) increased approximately twofold in the animals injected with leucogenenol as compared to the controls.

It has been demonstrated that approximately 15 min after tritiated thymidine is injected into an animal the primitive proliferating pool of cells in the bone marrow shows the most intense label and this label is then transferred to the myeloblast and in turn to sequential cells in the myeloid series (9, 13). It would appear, therefore, that the increased number of neutrophils found in the circulation 12 hr after the injection of leucogenenol (Table III) results from an increase in the rate at which the tritiated thymidine is transferred through the cells of the myeloid series. This suggests that leucogenenol increases the rate of transformation of myeloid cells. The

[3] Prepared by Baker Histology Laboratories, Great Falls, Va.

TABLE I. Percentage of Labeled Cells in the Bone Marrow of the Rat at Intervals Following the Injection of Leucogenenol[a] and Tritiated Thymidine.[b] Values for the control animals that were not injected with leucogenenol are given in parentheses.

	Time after injection (hr)[d]									
	6		12		24		48		96	
Myeloblast	7 ± 4	(13 ± 2)[c]	58 ± 12	(32 ± 10)[c]	13 ± 1	(25 ± 2)[c]	2 ± 1	(4 ± 1)[c]	1 ± 1	(2 ± 1)
Promyelocyte	1 ± 1	(1 ± 1)	25 ± 2	(22 ± 8)	32 ± 3	(26 ± 5)[c]	1 ± 1	(4 ± 2)	1 ± 1	(3 ± 2)
Myelocyte	3 ± 2	(0)[c]	16 ± 2	(8 ± 1)[c]	13 ± 3	(14 ± 4)	1 ± 1	(5 ± 3)	1 ± 1	(1 ± 1)
Metamyelocyte	0	(0)	8 ± 1	(6 ± 1)[c]	8 ± 3	(11 ± 2)	3 ± 1	(4 ± 1)	1 ± 1	(1 ± 1)
Band neutrophil	2 ± 2	(3 ± 1)	29 ± 6	(8 ± 2)[c]	12 ± 1	(14 ± 2)	5 ± 3	(4 ± 2)	1 ± 1	(1 ± 1)
Segmented neutrophil	2 ± 2	(2 ± 1)	25 ± 8	(6 ± 2)[c]	2 ± 1	(14 ± 2)[c]	0	(1 ± 1)	1 ± 1	(1 ± 1)
Lymphoblast	7 ± 4	(7 ± 1)	12 ± 1	(17 ± 3)[c]	26 ± 4	(28 ± 3)	17 ± 9	(36 ± 3)[c]	31 ± 1	(30 ± 7)
Lymphocyte	2 ± 1	(4 ± 1)	10 ± 1	(12 ± 2)	21 ± 2	(21 ± 4)	13 ± 8	(26 ± 3)[c]	24 ± 2	(25 ± 5)
Rubriblast	2 ± 1	(2 ± 1)	60 ± 1	(61 ± 3)	18 ± 4	(14 ± 1)	1 ± 1	(2 ± 1)	1 ± 1	(1 ± 1)
Prorubricyte	1 ± 1	(2 ± 1)	49 ± 3	(38 ± 6)[c]	15 ± 6	(10 ± 1)[c]	1 ± 1	(2 ± 1)	1 ± 1	(1 ± 1)
Rubricyte	1 ± 1	(0)	49 ± 4	(45 ± 3)	10 ± 3	(6 ± 1)[c]	1 ± 1	(1 ± 1)	1 ± 1	(1 ± 1)
Metarubricyte	1 ± 1	(0)	36 ± 3	(35 ± 3)	7 ± 3	(6 ± 1)	1 ± 1	(1 ± 1)	1 ± 1	(1 ± 1)
Nucleated red blood cells	1 ± 1	(0)	26 ± 1	(20 ± 2)[c]	8 ± 4	(4 ± 2)	1 ± 1	(1 ± 1)	1 ± 1	(1 ± 1)

[a] Injected intravenously with 0.02 μg of the calcium salt of leucogenenol dissolved in 0.2 ml of pyrogen-free isotonic saline.
[b] Injected intraperitoneally with tritiated thymidine (1 μCi/g of body wt).
[c] $p = <0.05$ for the difference.
[d] Standard deviations calculated on results obtained from 6 animals.

TABLE II. Number of Labeled Cells per 10,000 Cells in the Bone Marrow of Rats Injected with Leucogenenol[a] and Tritiated Thymidine.[b] Values for animals that were not injected with leucogenenol are given in parentheses.

	Time after injection (hr)[d]							
	6		12		24		48	
Myeloblast	12 ± 7	(14 ± 2)	186 ± 38	(35 ± 11)[c]	20 ± 1	(27 ± 2)[c]	1 ± 1	(4 ± 1)[c]
Promyelocyte	1 ± 1	(0)	38 ± 3	(7 ± 2)[c]	58 ± 4	(8 ± 2)[c]	2 ± 1	(1 ± 1)
Myelocyte	1 ± 1	(0)	13 ± 2	(5 ± 1)[c]	9 ± 2	(8 ± 2)	1 ± 1	(3 ± 2)
Metamyelocyte	0	(0)	4 ± 1	(9 ± 2)[c]	2 ± 1	(17 ± 3)[c]	6 ± 1	(6 ± 1)
Rubriblast	6 ± 3	(4 ± 2)	66 ± 1	(110 ± 5)[c]	32 ± 6	(25 ± 2)[c]	0	(4 ± 2)[c]
Prorubricyte	4 ± 4	(5 ± 2)	15 ± 1	(95 ± 7)[c]	3 ± 1	(25 ± 3)[c]	1 ± 1	(5 ± 2)[c]
Rubricyte	9 ± 2	(0)[c]	175 ± 14	(130 ± 20)[c]	57 ± 17	(52 ± 9)	4 ± 4	(9 ± 8)
Metarubricyte	6 ± 1	(0)[c]	177 ± 23	(392 ± 22)[c]	72 ± 30	(44 ± 7)	7 ± 7	(7 ± 7)
Nucleated red blood cells	1 ± 1	(0)[c]	31 ± 1	(22 ± 2)[c]	12 ± 6	(4 ± 2)	7 ± 7	(1 ± 1)

[a] Injected intravenously with 0.02 μg of the calcium salt of leucogenenol dissolved in 0.2 ml of pyrogen-free isotonic saline.
[b] Injected intraperitoneally with tritiated thymidine (1 μCi/g of body wt).
[c] $p = <0.05$ for the difference.
[d] Standard deviations calculated on results obtained from 6 animals.

TABLE III. Concentration of Labeled Neutrophils and Lymphocytes in the Peripheral Blood of Rats at Intervals Following the Injection of Leucogenenol[a] and Tritiated Thymidine.[b]

Values for animals that were not injected with leucogenenol are given in parentheses.

Time after injection (hr)	No. of labeled cells/mm³[d]	
	Neutrophils	Lymphocytes
12	282 ± 50 (132 ± 70)[c]	282 ± 50 (230 ± 80)[c]
24	470 ± 96 (153 ± 50)[c]	1740 ± 650 (387 ± 62)[c]
48	371 ± 37 (140 ± 14)[c]	1190 ± 140 (600 ± 120)[c]

[a] Injected intravenously with 0.02 μg of the calcium salt of leucogenenol dissolved in 0.2 ml of pyrogen-free isotonic saline.

[b] Injected intraperitoneally with tritiated thymidine (1 μCi/g of body wt).

[c] $p = <0.05$ for the difference.

[d] Standard deviations calculated on results obtained from 6 animals.

fact that the number of each type of labeled myeloid cell is not increased at the expense of its precursor (Tables I and II) indicates that leucogenenol increases the rate of transformation of each type of cell in the myeloid series.

Forty-eight hr after injection animals that received leucogenenol showed essentially the same percentage (Table I) and relative number (Table II) of labeled cells in their bone marrow as the uninjected controls. However, at this time the number of labeled neutrophils in the circulation of the animals injected with leucogenenol (Table III) show approximately a threefold increase over that of the uninjected controls. This would also indicate that the tritiated thymidine progressed more rapidly through the myeloid series of cells in the animals injected with leucogenenol.

Leucogenenol does not appear to have any striking action on the erythroid cells. It is suggested that the number of red blood cells is so directly or indirectly controlled by oxygen requirements that any stimulating action of leucogenenol on the erythroid series of cells would not be readily apparent in the present experiment. The significant increase in the percentage of prorubricytes that are labeled 12 and 24 hr after injection with leucogenenol does suggest the possibility that leucogenenol increases the rate of transformation of at least one type of nucleated erythroid cell.

Spleen imprints (Table IV) show a significant increase in the percentage of large and small lymphocytes that are labeled 6 and 12 hr following the injection of leucogenenol. The number of labeled lymphocytes in the peripheral blood (Table III) is increased ap-

TABLE IV. Percentage of Labeled Large and Small Lymphocytes in Spleen Imprints at Intervals Following the Injection of Leucogenenol[a] and Tritiated Thymidine.[b]

Values for animals that were not injected with leucogenenol are given in parentheses.

	Time after injection (hr)[d]				
	6	12	24	48	96
Large lymphocytes	23 ± 4 (13 ± 1)[c]	46 ± 3 (33 ± 2)[c]	25 ± 2 (22 ± 1)[c]	5 ± 2 (28 ± 6)[c]	2 ± 1 (7 ± 2)[c]
Small lymphocytes	10 ± 1 (5 ± 1)[c]	16 ± 2 (12 ± 1)[c]	17 ± 2 (15 ± 1)	3 ± 2 (39 ± 3)[c]	2 ± 1 (7 ± 1)[c]

[a] Injected intravenously with 0.02 μg of the calcium salt of leucogenenol dissolved in 0.2 ml of pyrogen-free isotonic saline.

[b] Injected intraperitoneally with tritiated thymidine (1 μCi/g of body wt).

[c] $p = <0.05$ for the difference.

[d] Standard deviations calculated on results obtained from 6 animals.

proximately fivefold and twofold 24 and 48 hr, respectively, after injection with leucogenenol. Examination of the autoradiographs of spleen sections showed a general increase in the number of cells labeled in the sections from animals injected with leucogenenol.

The increased number of labeled large and small lymphocytes in the spleen accompanied by the increased number of labeled lymphocytes in the ciruclation suggest that leucogenenol stimulates the increased production of large and small lymphocytes from their respective precursors.

Summary. Comparison by autoradiographic means of the labeling of myeloid, nucleated erythroid, and lymphoid blood cells in animals injected with tritiated thymidine and animals injected with tritiated thymidine and leucogenenol indicated that leucogenenol increases the incorporation of DNA in the primitive proliferating pool of cells and increases the rate of transformation of myeloid and lymphoid blood cells. There are indications that the rate of transformation of nucleated erythroid cells is also increased by leucogenenol.

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