

HUNDRED DAY LEUKEMIA: PREFERENTIAL INDUCTION IN RAT BY PULSE-DOSES OF 7,8,12-TRIMETHYLBENZ(A)ANTHRACENE*

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This paper is concerned with a method to elicit leukemia rapidly and in high yield in the rat and with the recognition of the disease in living animals. A series of pulse-doses (intravenous injections) of large but tolerable amounts of homogenized 7,8,12-trimethylbenz(a)anthracene was highly effective in calling forth mammary cancer and leukemia. In juvenile rats leukemia occurred preferentially; in young adults, male and female, both mammary cancer and leukemia developed in profusion. Leukemia was detected within 1 month in many rats and within 100 days in all young animals which withstood the toxic effects of reiterated pulse-doses. Predominantly, leukemia of a single sort was induced; it was a stem-cell leukemia often associated with erythroblastosis.

In developing this simple and reproducible method we considered the leukemogenic potency of several hydrocarbons and the physiologic status of the recipient most conducive to the development of neoplasms.

Bachmann and Chemerda (1) synthesized 7,12-DMBA and 7,8,12-TMBA.¹ These compounds are of exceptional interest: (a) they are the most potent polycyclic hydrocarbons in inducing tumors, and (b) they selectively destroy the middle layer of the adrenals of adult rats (2, 3). Schurr (4) devised a method to prepare rather concentrated lipid emulsions of hydrocarbons (0.5–1.0% w/w) suitable for intravenous injections. The toxicity of a single pulse-dose of emulsions of hydrocarbons has been determined. In the rat 7,12-DMBA (LD₅₀ 60 mg/kg) is more toxic (5) than is 7,8,12-TMBA (LD₅₀ 125 mg/kg).

The leukemogenic action of hydrocarbons was discovered by Morton and Mider (6) by repeatedly painting the skin of mice with 3-methylcholanthrene. Induction of leukemia by intravenous injection of hydrocarbons was first accomplished by Huggins and Sugiyama (7). Under stated conditions multiple pulse-doses of an emulsion of

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¹ The abbreviations used in this paper are: DMBA, dimethylbenz(a)anthracene; DMSO, dimethylsulfoxide; TMBA, trimethylbenz(a)anthracene; \pm , standard deviation of mean.

7,12-DMBA induced leukemia in every rat, whereas a single dose elicited it in 6% of the animals; the mean survival time of leukemic animals was 102 ± 22 days.

Huggins and Sugiyama (7) found that biopsy of the liver was valuable in the early diagnosis of leukemia in living rats. In 100 consecutive cases of leukemia, the liver was enlarged (greater than 4% of the body weight) in 76 rats. Whenever leukemia was detected in any site, leukemic foci always were found in the liver by hepatic biopsy. Several kinds of leukemia were elicited by 7,12-DMBA, but the most common category (80% of the cases) was a diffuse hepatic form of a stem-cell leukemia often associated with erythroblastosis.

Methods

The experimental animals were exclusively rats of Long-Evans (L-E) strain (8). Our colony has been maintained by breeding at random *inter se* for more than 10 yr. The animals were housed in metal cages in air-conditioned rooms at $25^{\circ}\text{C} \pm 2$, fed a commercial ration (Rockland Mouse/Rat Diet, Teklad, Inc., Monmouth, Ill.) and given water *ad libitum*. 6 days each week the animals were weighed. Every day the vaginal smear of each female was examined using a Pasteur pipette and saline. Rectal temperature was measured each morning with a thermocouple.

Lipid emulsions containing hydrocarbons² were prepared (3). Cottonseed oil was emulsified in water containing lecithin and a nonionic detergent by the method of Schurr (4); the hydrocarbon was dissolved in dimethyl sulfoxide (DMSO) and diluted with the Schurr emulsion until the final concentrations (w/v) were: hydrocarbon, 0.5%; DMSO, 5%. A reflux condenser was attached to the flask and the mixture was boiled for 1 hr with *slow* stirring.

The lipid emulsions were injected in a caudal vein; the first injection is designated day 0. Biopsy of the liver was performed under ether anesthesia with aseptic precautions. An incision (1 cm) was made in the midline in the epigastrium; the spleen was delivered by gentle traction and inspected. The liver was not handled; a wedge 50–100 mg was excised cleanly with scissors from the margin; hemostasis was not necessary. Blood was obtained from a vein or by cardiac puncture for hematologic studies by conventional methods.

Most of the experiments were terminated when leukemia was detected or at 100 days.

RESULTS

Spontaneous leukemia is rare in our colony of Long-Evans rats. It was detected in one animal among 6000+ untreated rats maintained as a breeding stock until age 6 months. This leukemia was of thymus-lymph node type.

Leukemogenic Hydrocarbons.—Six hydrocarbons (Table I) were evaluated for leukemogenic potency. A lipid emulsion of each compound was freshly prepared and a set of pulse-doses, 35 mg/kg, administered to young rats. Control mates received intravenous injections of emulsions devoid of hydrocarbons.

The compounds were derivatives of 12-methylbenz(a)anthracene and possessed two or three methyl substituents. The compounds were closely related to 7,12-DMBA which was tested; four of the derivatives had the structure *x,7,12-TMBA*.

² We thank: Paul E. Schurr, The Upjohn Co., Kalamazoo, Mich., for lipid emulsions; John Pataki, Ben May Laboratory for Cancer Research, The University of Chicago, Chicago, Ill., and M. S. Newman, Department of Chemistry, The Ohio State University, Columbus, Ohio, for hydrocarbons.

7,12-DMBA was the most toxic compound. It caused gastric distension, ileitis, and diarrhea on days 1-3. Disturbances of this sort were not observed in rats which received the other hydrocarbons or in control rats.

Two compounds, 7,12-DMBA and 7,8,12-TMBA (Table I), elicited leukemia in high and equal yield. Two compounds, 6,8,12-TMBA and 7,9,12-TMBA, evoked leukemia in low yield.

TABLE I

Leukemia Evoked by Sets of Pulse-Doses of Polycyclic Aromatic Hydrocarbons

Female rats were given five pulse-doses of hydrocarbon, 35 mg/kg; control rats received lipid emulsion devoid of hydrocarbon. The injections were given at biweekly intervals beginning at age 30 days; the experiment was terminated on day 100.

Compound	Number of rats	Rats with leukemia	
		Total number	%
7,12-DMBA	44	35	80
5,7,12-TMBA	10	0	0
6,7,12-TMBA	8	0	0
6,8,12-TMBA	9	2	22
7,8,12-TMBA	10	8	80
7,9,12-TMBA	14	1	7
Control; no hydrocarbon	20	0	0

TABLE II

Number of Pulse-Doses of 7,8,12-Trimethylbenz(a)anthracene Related to Incidence of Leukemia

Female rats of L-E strain were injected with 1-5 pulse-doses of 7,8,12-TMBA, 35 mg/kg, at intervals of 10 days beginning at age 28 days; the experiment was terminated on day 100.

Number of pulse-doses	Number of rats	Rats with leukemia	
		Total number	%
1	10	1	10
3	23	7	30
4	22	18	82
5	20	19	95

Leukemia was not observed in rats injected with 5,7,12-TMBA, 6,7,12-TMBA, or control lipid emulsion. It is remarkable that substitution of the hydrogen atom at positions 5 or 6 in 7,12-DMBA (but not at position 8) by a methyl group eliminated the ability of the molecule to induce leukemia; thus, 7,12-DMBA and 7,8,12-TMBA were strongly leukemogenic, whereas 5,7,12-TMBA and 6,7,12-TMBA were inactive in this regard.

Preferential Induction of Leukemia.—Some rats which received a series of pulse-doses of 7,8,12-TMBA were unusually vulnerable and they died early in the experiment. At necropsy, pneumonitis or a wasting disease was found in

these animals. Rats of L-E strain are advantageous because, in comparison to other strains, they are rather resistant to pneumonitis.³

The wasting disease caused by hydrocarbons in rats is characterized by cachexia and shrinkage of most of the tissues with an accompanying anemia

TABLE III

Tumors Elicited by Pulse-Doses of 7,8,12-Trimethylbenz(a)anthracene in Older and Younger Rats

Rats received four pulse-doses of 7,8,12-TMBA, 35 mg/kg, at biweekly intervals. The injections were started in older rats at age 50 days and in younger groups at age 25 days.

	Older group		Younger groups	
	Female	Male	Male	Female
Original number	18	11		14
Early death	0	1		2
Effective number	18	10		12
Mammary cancer	18 (100%)	1 (10%)		6 (50%)
Leukemia	10 (56%)	8 (80%)		10 (83%)

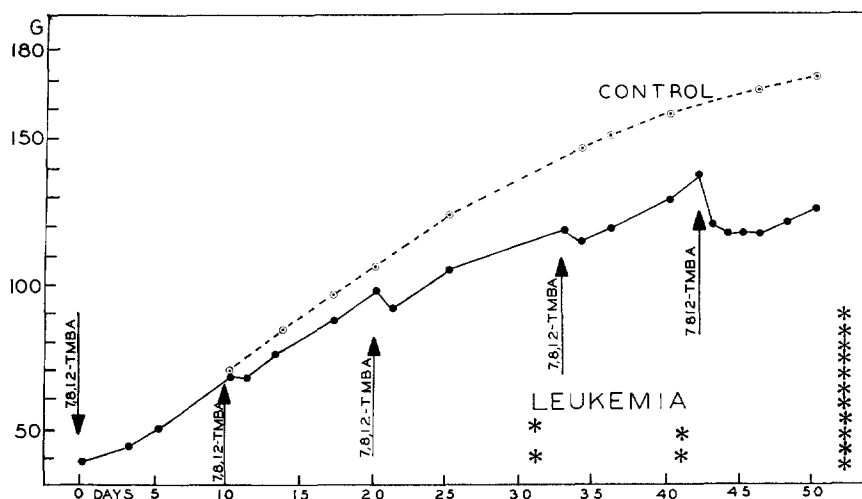


FIG. 1. Curves of body weight of (1) a group of 20 rats which received 5 pulse-doses of 7,8,12-TMBA, 35 mg/kg, beginning at age 25 days and (2) control mates. The day of detection of leukemia in a member of the injected group is denoted by *.

and leukopenia; liver is small and spleen is tiny, whereas the adrenals are big. Rats with leukemia also lose weight and develop anemia, but in the leukemic rats the liver is somewhat or greatly enlarged and there is leukocytosis.

³ Rats of Sprague-Dawley strain which received a set of pulse-doses of 7,8,12-TMBA were very susceptible to pneumonitis, but leukemia developed in half of the rats which survived 100 days.

The incidence of leukemia elicited by 7,8,12-TMBA in L-E rats is directly related to the number of pulse-doses (Table II). In our experiments the minimum number of intravenous injections to cause a high yield of leukemia was *four*. A very high incidence of leukemia (greater than 80% of the effective animals) was found before 100 days in 10 consecutive series, each comprising 20-100 rats.

18 young adult rats which had had one or more estrus cycles were injected with four pulse-doses of 7,8,12-TMBA, 35 mg/kg, at biweekly intervals. Each animal developed mammary cancer (Table III) which was detected on

TABLE IV

Anatomical Findings in Male Rats with Leukemia

Leukemia was elicited in 12 male rats by pulse-doses of 7,8,12-TMBA; necropsy was performed on day 42-78 when leukemia was in advanced stage. 10 controls were untreated and sacrificed at age 128 days.

	Leukemia	Control
	<i>g/100 g</i>	<i>g/100 g</i>
Liver	8.1 ±* 3.4 (4.5-15)	3.0 ± 0.2 (2.6-3.4)
Spleen	0.3 ± 0.1 (0.1-0.7)	0.74 (0.4-1.0)
Thymus	0.06 ± 0.01 (0.03-0.09)	0.22
Testis (1)	0.24 ± 0.01 (0.1-0.3)	1.9 ± 0.1 (1.8-2.0)
Lymph nodes	0.01	0.006

* ±, standard deviation of mean.

days 42-98 (70 ± 16 days). Benign mammary tumors appeared in many animals after day 120; 4 rats developed aural cancers. Myriads of mammary cancers emerged in these animals during the first 9 months requiring repeated excision of sizable tumors to preserve life; in these rats three or more newly appearing mammary tumors were excised each month to preserve life of the animal. Leukemia was observed in 10 members of the group (56%) on days 70-160 (99.5 ± 38 days).

Groups of male and female weanling rats received four pulse-doses of 7,8,12-TMBA, 35 mg/kg (Table III). Leukemia was observed in 80% of each group. The incidence of mammary cancer in the juvenile males was 10%.

A group of 25 female rats received a series of 5 pulse-doses of 7,8,12-TMBA starting at age 25 days (Fig. 1). There were 5 early deaths which occurred on

days 48–69: wasting disease, 4; pneumonitis, 1. Among 20 effective animals the following neoplasms were observed: breast cancer, 3; leukemia, 19; other tumors, 0. Leukemia was detected by hepatic biopsy on days 31–95 (53.1 ± 15 days). Leukemia was classified: stem cell with or without erythroblastosis (Figs. 5, 6), 18 cases; myelogenous, 1. The neoplastic myelogenous cells contained peroxidase granules.

The most striking anatomical findings (Table IV) in rats with erythroblastic leukemia were found in the liver. In an advanced stage the liver was huge, dusky rose-red in color, mottled, slightly granular, and friable. The hepatic

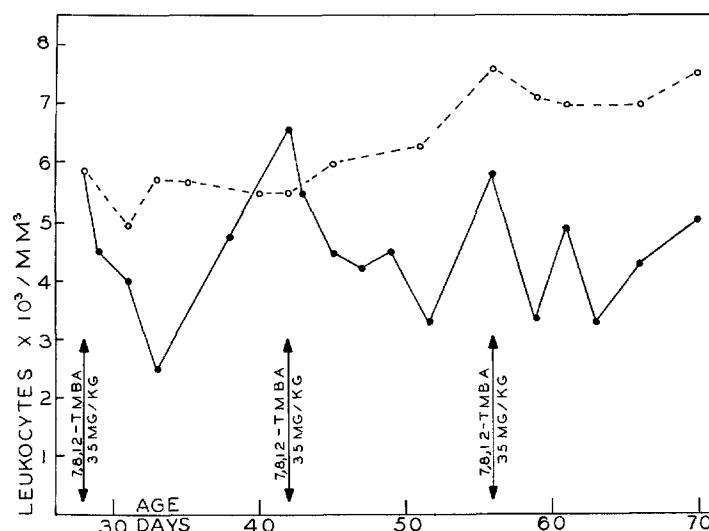


FIG. 2. Leukocyte counts in cardiac blood of female rats injected with pulse-doses of 7,8,12-TMBA, 35 mg/kg, and of uninjected mates. There were 5 rats in each group; mean values are given. ●—●, injected. ○- - -○, controls.

edge was rounded. Thymus, lymph nodes, spleen, and testis were much reduced in size. In advanced cases, clumps of leukemic cells were found invariably in the sinusoids of the middle layer of the adrenal. In 17 cases, the leukocyte count in cardiac blood was 5,900–82,000/mm³ ($24,200 \pm 7,800/\text{mm}^3$).

Rats which develop leukemia after a set of pulse-doses of 7,8,12-TMBA are somewhat smaller than uninjected controls are (Fig. 1). There was no decline in body weight after pulse-dose one, but a retardation of growth was evident after subsequent injections. In early leukemia the rats appear healthy and active, but smaller than uninjected mates of the same sex.

After each pulse-dose of 7,8,12-TMBA there was a characteristic pattern of decrease in the number of leukocytes (Fig. 2) of the circulating blood. The leukocyte count was at its lowest level (about 40% of the preinjection value)

on days 3–5 with recovery to the initial preinjection value on days 7–10. We considered an interval of 14 days between the pulse-doses to be optimal for rapid induction of leukemia with the smallest number of early deaths from toxicity.

Characteristics of Leukemia in Living Rats.—Two novel signs of leukemia were found in this study: (a) depression of pituitary function and (b) decrease of body temperature. These characteristics are useful in following the course of leukemia. Both effects are nonspecific and they indicate far advanced leukemia.

Decrease of pituitary function: This effect was found out by studying the

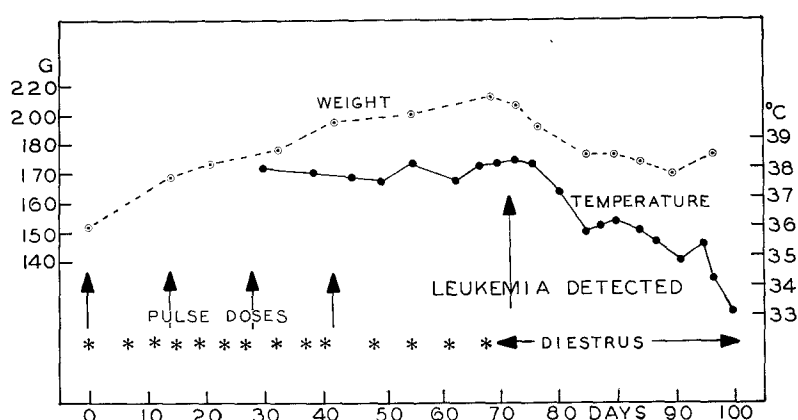


FIG. 3. Curves of body weight and rectal temperature of a rat which received the first of 4 pulse-doses of 7,8,12-TMBA, 35 mg/kg, at age 50 days. Leukemia was detected by hepatic biopsy on day 72; thereafter a decline in body weight and temperature ensued and estrus cycles ceased. Estrus is denoted by *.

estrus cycle. In our untreated control rats, first estrus occurred at age 46–68 days (52.3 ± 6.4 days). The onset of puberty was delayed in juvenile rats injected with the leukemogenic hydrocarbons. A group of 24 rats received 3 pulse-doses of 7,8,12-TMBA, 35 mg/kg, at biweekly intervals starting at age 25 days; first estrus occurred at age 38–125 days (77.3 ± 28).

In adult rats, four pulse-doses of 7,8,12-TMBA caused little or no irregularity in the periodicity of the estrus cycle. When leukemia was manifest the cycles stopped (Fig. 3). Estrus was absent for prolonged periods (15–38 days) before death occurred from leukemia. The cause of diestrus in leukemia is not a primary defect in ovary but a deficiency of gonadotrophin production. In five rats with advanced leukemia, subcutaneous injections of equine gonadotrophin were given for 3 days and estrus was restored.

Decline of body temperature: The rectal temperature was measured with a

thermocouple in groups of normal control male and female rats, age 60–81 days. The findings were similar in males and females; the range of temperature was 37.6–38.4°C ($38.0 \pm 0.2^\circ\text{C}$).

We observed a progressive decline in body temperature (Fig. 3) beginning some 2–4 wk before death from leukemia.

Hepatic biopsy: Frequently small foci of proliferating cells are found in liver in untreated rats in our colony; many of these small dense cells are normal erythroblasts but mitosis has not been seen in the clumps.

In leukemia the findings are quite different. In the stem-cell leukemias clumps of large cells with an intensely basophilic nucleus *and* cytoplasm were found in the hepatic sinusoids. Mitoses were always present in these tiny foci of early leukemia (Fig. 4). In some cases the stem-cell leukemia was not associated with hemopoiesis (Fig. 5), but usually there was an associated proliferation (Fig. 6) of abnormal erythroblasts and normoblasts with multinucleated cells in abundance.

In histologic sections of liver stained with hematoxylin-eosin the clumps of deep blue leukemic cells form a distinctive and striking contrast to the pink acidophilic cytoplasm of liver cells.

It is characteristic of the leukemic cells to progress, often at an explosive rate, until the sinusoidal endothelium of the liver is completely replaced, causing hepatic insufficiency. This violent advance of leukemia in successive biopsies of the liver differentiates the malignant process from benign extramedullary hemopoiesis.

In contrast to the diffuse hepatic type of erythroblastic leukemia which is sinusoidal in site, other types of leukemia induced by hydrocarbons located and proliferated in the periportal areas.

DISCUSSION

Novel findings in the present work are (a) the uniformity of the type of leukemia which was elicited, and (b) the rapidity with which the disease became manifest.

One of the keys to the rapid induction of leukemia in high yield is the selection of an appropriate experimental animal. Rats of L-E strain are especially suitable: for, there is a remarkably low incidence of spontaneous leukemia in untreated controls; the yield of leukemia in the injected animals approaches 100%; the stock is hardy and unusually tolerant to the drastic effects of reiterated doses of the strongly leukemogenic hydrocarbons, 7,12-DMBA and 7,8,12-TMBA.

A series of pulse-doses of the potent hydrocarbons is required to elicit leukemia in high yield, whereas a solitary dose is ineffective in this regard. Uematsu and Huggins (5) found that a pulse-dose of 7,12-DMBA or 7,8,12-TMBA causes a profound decrease in synthesis of DNA in rat lymphoid tissue. It is

known (9) that 7,12-DMBA destroys only those cells which synthesize DNA; germinal cells which proliferate by meiosis (they do not synthesize DNA) are unaffected by heavy doses of 7,12-DMBA. The leukemia-producing effect of *sets* of pulse-doses of 7,8,12-TMBA is associated with repeated major depressions of DNA synthesis in hemopoietic cells.

A stem-cell erythroblastic leukemia developed rapidly in nearly all L-E rats which received a series of 4 or more pulse-doses of 7,8,12-TMBA, 35 mg/kg, at biweekly intervals. It is a simple and serviceable method. The diffuse hepatic leukemia has an unique characteristic—it grows rampantly in the sinusoids of the liver, destroying their endothelial lining. The explosive course of erythroblastic leukemia results from the exuberant growth of the leukemic cells *plus* hepatic insufficiency.

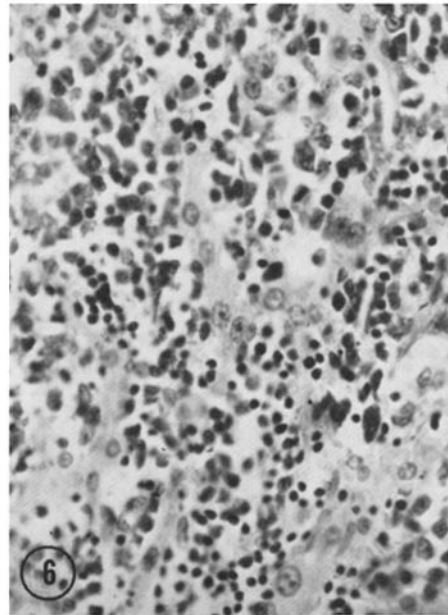
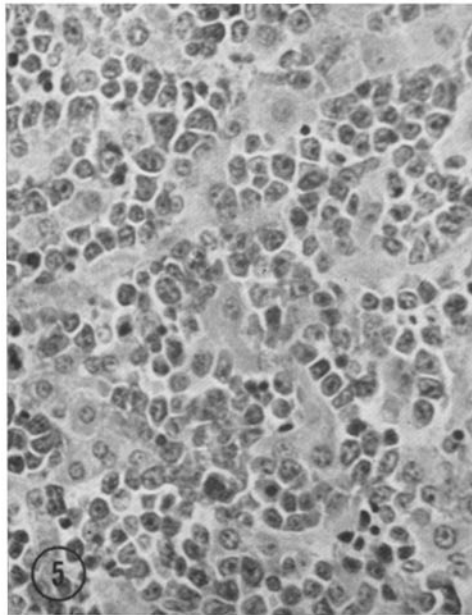
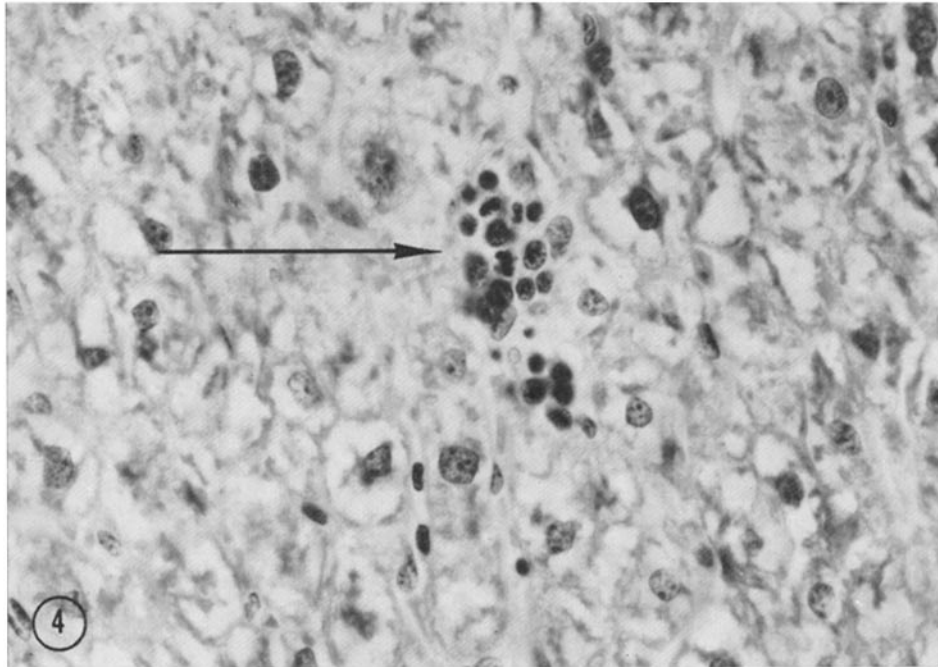
SUMMARY

A series of pulse-doses of 7,8,12-trimethylbenz(a)anthracene-induced leukemia rapidly and consistently in very high yield in rats of Long-Evans (L-E) strain. The predominant type was a diffuse hepatic leukemia of erythroblastic stem cells.

Progressive hypothermia and a decline in pituitary function are newly recognized signs of advanced leukemia in rat.

BIBLIOGRAPHY

1. Bachmann, W. E., and J. M. Chernerda. 1938. The synthesis of 9,10-dimethyl-1,2-benzanthracene, 9,10-diethyl-1,2-benzanthracene and 5,9,10-trimethyl-1,2-benzanthracene. *J. Amer. Chem. Soc.* **60**:1023.
2. Huggins, C., and S. Morii. 1961. Selective adrenal necrosis and apoplexy induced by 7,12-dimethylbenz(a)anthracene. *J. Exp. Med.* **114**:741.
3. Huggins, C., S. Morii, and J. Pataki. 1969. Selective destruction of adrenal cortex by pulse-doses of derivatives of 12-methylbenz(a)-anthracene. *Proc. Nat. Acad. Sci. U. S. A.* **62**:704.
4. Schurr, P. E. 1969. Composition and preparation of experimental intravenous fat emulsions. *Cancer Res.* **29**:258.
5. Uematsu, K., and C. Huggins. 1968. Induction of leukemia and ovarian tumors in mice by pulse-doses of polycyclic aromatic hydrocarbons. *Mol. Pharmacol.* **4**:427.
6. Morton, J. J., and G. B. Mider. 1941. Some effects of carcinogenic agents on mice subject to spontaneous leukoses. *Cancer Res.* **1**:95.
7. Huggins, C. B., and T. Sugiyama. 1966. Induction of leukemia in rat by pulse-doses of 7,12-dimethylbenz(a)anthracene. *Proc. Nat. Acad. Sci. U. S. A.* **55**:74.
8. Long, J. A., and H. McL. Evans. 1922. The oestrous cycle in the rat and its associated phenomena. *Memoirs of the University of California.* **6**.
9. Ford, E., and C. Huggins. 1963. Selective destruction in testis induced by 7,12-dimethylbenz(a)anthracene. *J. Exp. Med.* **118**:27.



Photomicrographs of paraffin sections of liver stained with hematoxylin-eosin.
FIG. 4. Arrow points to a small clump of leukemia cells. $\times 250$.
FIG. 5. Advanced hepatic stem-cell leukemia. $\times 200$.
FIG. 6. Advanced hepatic stem-cell leukemia with erythroblastic differentiation—
advanced stage. $\times 200$.