

Role of Erythropoietin in 7,12-Dimethylbenz(a)anthracene Induction of Acute Chromosome Aberration and Leukemia in the Rat

(anemia/polycythemia/lysosomes)

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ABSTRACT The incidence of chromosome aberrations in rat bone marrow, examined 6 hr after the administration of 7,12-dimethylbenz(a)anthracene, was significantly enhanced by induction of anemia 0-48 hr before the carcinogen treatment and was suppressed by induction of polycythemia. The suppressive influence of polycythemia was reversed by sheep erythropoietin injected shortly before or after the carcinogen injection; this suppressive effect was proportional to the dose of erythropoietin used. These data suggest that erythropoietin is essential to make bone-marrow cells susceptible to chromosome aberrations with 7,12-dimethylbenz(a)anthracene. The incidence of carcinogen-induced leukemia was also increased by anemia and suppressed by polycythemia induction.

The rat leukemia induced by 7,12-dimethylbenz(a)anthracene (DMBA) (1) and related compounds offers a model for study of the role of hormones in the initiation process of neoplastic cells. This leukemia is predominantly erythroblastic in nature (1, 2). Recent investigations have shown specific chromosomal changes in this leukemia (2-4) and a nonrandom acute effect (5), both significantly involving the largest telocentric chromosome. In the present study, the role of erythropoietic stimuli in induction of both acute chromosome damage and leukemia was investigated. The results demonstrate that a hemopoietic stimulus (the erythropoietin concentration) at the time of DMBA injection is important in leukemia induction and susceptibility of the bone-marrow cells to the acute chromosome-damaging effects of the carcinogen.

MATERIALS AND METHODS

Acute Chromosome Damage in Bone-Marrow Cells. Random-bred, male rats of the Long-Evans strain, 26 days old, were studied. Since the chromosome aberrations in bone-marrow cells 6 hr after injection of DMBA have been quantitatively and qualitatively characterized (5), all observations on the frequency of aberrant metaphase cells were made on chromosome specimens obtained 6 hr after the injection of DMBA. Each rat received a single intravenous injection of a lipid emulsion of DMBA at a dose of 50 mg/kg of body weight. Colchicine (0.3 mg/rat), dissolved in saline, was injected intraperitoneally 1 hr before the rats were killed. The chromosome specimens were prepared from femur marrow by air-drying on a flame (2, 3). Metaphase cells having one or more chromosome aberrations were counted in each case from 165

well-spread metaphases. The aberrations consisted mainly of breaks and gaps. Breaks were defined by chromatid discontinuity, and gaps by an achromatic lesion with continuity. Isochromatid aberrations were counted as one aberration. The frequency of aberrations was expressed as a percentage of aberrant metaphase cells.

Hemopoietic Stimuli. Hemopoiesis was suppressed by polycythemia and stimulated by anemia and exogenous erythropoietin. These stimuli were given at various intervals before and after DMBA injection. The following schedules were chosen to study the influence of the above hemopoietic stimuli on DMBA induction of chromosome aberrations. *A.* DMBA alone. *B.* Anemia + DMBA. *C.* Polycythemia + DMBA. *D.* Polycythemia + exogenous erythropoietin + DMBA. In schedule *B*, animals were divided into six groups of six animals; anemia was induced by removal of 2.0 ml/100 g body weight of whole blood by cardiac puncture in each group 0, 6, 12, 24, 48, and 96 hr before DMBA administration. In schedule *C*, polycythemia was induced by intravenous injection of washed erythrocytes. Erythrocytes, obtained from adult males of the same strain were washed three times in physiological saline by centrifugation, and were finally suspended in saline and adjusted to a concentration of $79 \pm 2\%$ (vol/vol). The final suspension was assumed to be almost free from white blood cells because buffy coat was removed in each washing. For the induction of a maximum degree of polycythemia (60-70% in hematocrit Schedule *C*), 4 ml/100 g body weight of the erythrocyte suspension was injected intravenously. Time-sequence studies were performed by induction of the maximum degree of polycythemia 96-hr before to 2-hr after DMBA injection. Each group contained 6 rats. The effect of exogenous erythropoietin was tested, in schedule *D*, in the rats that were made maximally polycythemic 24-hr before injection of DMBA. Sheep erythropoietin, Steps 1 and 3, purchased from Connaught Medical Research Laboratories, University of Toronto, was dissolved in saline to 10 units/ml and was injected intraperitoneally 1 hr before DMBA in the dose-response studies, and intravenously before and after DMBA in a time-sequence study. Both Step 1 and Step 3 erythropoietin were used in the dose-response experiment, and Step 3 was used exclusively in the time-sequence experiment.

Hematocrit Value. The hematocrit values of aortic blood obtained at the time of death in the above experiments were measured by a microhematocrit method. The correlation of hematocrit values and percentage of aberrant metaphase cells

Abbreviation: DMBA, 7,12-dimethylbenz(a)anthracene.

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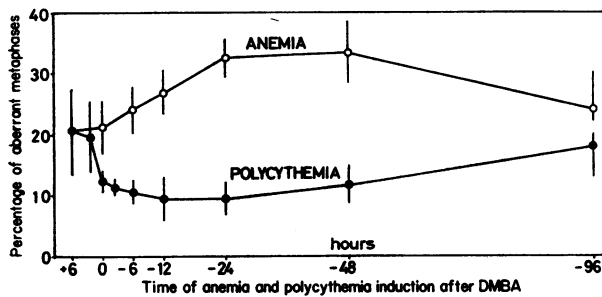


FIG. 1. Variation of the incidence (%) of aberrant metaphase cells in the animals with anemia or polycythemia induced at various intervals before and after administration of DMBA. The animals were examined 6 hr after they received DMBA. The mean hematocrit value and standard deviation in each group at the time of death are given.

was studied in DMBA-injected animals with various degrees of polycythemia and anemia induced by injection of or removal of various dosages of erythrocytes 24 hr before administration of DMBA.

Lysosome Stabilizer and Labilizer. A lysosome stabilizer, cortisone acetate, suspended in saline, was injected intramuscularly (200 $\mu\text{g}/\text{day}$) in the thigh for 3 successive days; DMBA was given on the last day of this treatment. A lysosome labilizer, Vitamin A (Type I), dissolved in sesame oil (200 $\mu\text{g}/\text{day}$) was injected similarly for 3 days before injection of DMBA.

Erythropoietic Stimuli and Leukemia Induction. Male rats, 40 days old, were used in the following experiments to avoid death of animals from frequent cardiac puncture. Four experiments were designed. A. DMBA alone; the animals were treated with five pulse injections of DMBA with intervals of 2 weeks. The dose used for each injection was 27.5–32.5 mg/kg. B. Anemia + DMBA; anemia was induced by cardiac puncture with removal of 1.5–2.0 ml of whole blood per 100 g body weight several hours before each injection of DMBA. C. Polycythemia + DMBA; polycythemia (hematocrit level of 60%) was produced by injection of 2–3 ml/100 g body weight of washed erythrocyte suspension

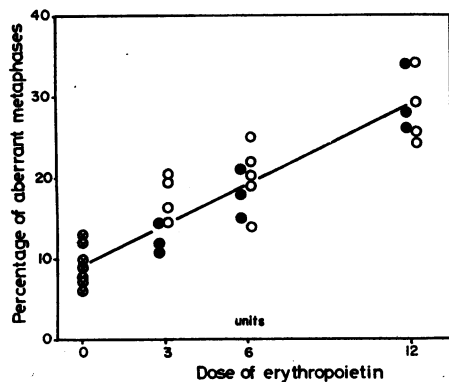


FIG. 2. Relationship between the incidence of DMBA-induced chromosome aberrations and the dose of erythropoietin injected. \odot : Control polycythemic rats; \circ : Step 1 erythropoietin; and \bullet : Step 3 erythropoietin.

TABLE 1. Effects of cortisone acetate and Vitamin A on the yield of DMBA-induced chromosome aberrations

Experiment	Treatment*	Aberrant cells (%)
A	Control	1.9 \pm 0.7 \dagger
	Erythropoietin	1.7 \pm 0.6
	Vitamin A	1.8 \pm 0.8
B	DMBA only	20.4 \pm 7.0
	Cortisone + DMBA	19.8 \pm 5.7
	Vitamin A + DMBA	18.7 \pm 3.4
C	Polycythemia + DMBA	9.5 \pm 2.7
	Cortisone + polycythemia + DMBA	9.8 \pm 4.3
	Vitamin A + polycythemia + DMBA	9.3 \pm 2.1
D	Polycythemia + erythropoietin + DMBA	15.3 \pm 3.6
	Cortisone + polycythemia + erythropoietin + DMBA	18.8 \pm 3.3

* Erythropoietin, 6 units; cortisone, 200 $\mu\text{g}/\text{day}$; Vitamin A, 200 $\mu\text{g}/\text{day}$; DMBA, 50 mg/kg of body weight, see text for other details.

\dagger Standard deviation of mean ($n = 6$ in all experiments).

several hours before injection of DMBA. D. Anemia + DMBA + polycythemia; in addition to the treatment in experiment B, 2 ml/100 g body weight of washed erythrocyte suspension was injected 48 hr after each injection of carcinogen to restore a moderately high hematocrit level. It has been shown (5) that few chromosome aberrations are induced 48 hr after the carcinogen injection. Thus, this procedure should eliminate the influence of anemia on the growth of DMBA-affected cells. Leukemia was detected by loss of body weight and by liver palpation; the diagnosis was later confirmed by hematological and pathological findings at the time of death.

RESULTS

Influence of anemia and polycythemia on DMBA-induced aberrations

The percentage of aberrant metaphase cells 6 hr after the carcinogen injection in six normal rats was 20.4 ± 7 . The incidence of aberrant cells was considerably modified by

TABLE 2. Incidence of leukemia in normal, anemic, and polycythemic rats

Group	Hematologic status	No. of rats	No. with leukemia	Difference from Group A
A	Normal	44	28	—
B	Anemia	39	33	0.02 < P < 0.05
C	Polycythemia	14	1	$P < 0.001$
D	Anemia + DMBA + polycythemia	10	9	

All rats were injected with five pulse-doses of 50 mg/kg of DMBA at intervals of 14 days; leukemia was detected within 4 months after the first injection.

either anemia or polycythemia induced before the carcinogen injection (Fig. 1). Anemia enhanced the yield of chromosome aberrations. A significant rise in the percentage of aberrant metaphase cells was observed when the anemia was induced 6 hr before injection of DMBA. The enhancement was maximum when the anemia was induced 24–48 hr before the carcinogen was administered. The effect was reduced after 96 hr. Polycythemia, on the contrary, suppressed the incidence of chromosome aberrations when it was induced 0–48 hr before the carcinogen injection. The suppressive effect of the polycythemia induced 96 hr before, or 2 hr after, the carcinogen injection was not significant. These studies show that the susceptibility of bone-marrow cells to the chromosome-damaging action of DMBA is dependent on erythropoietic stimulus.

Influence of exogenous erythropoietin on DMBA-induced chromosome aberrations

The percentage of aberrant metaphase bone-marrow cells in six polycythemic rats, 6 hr after carcinogen administration, was 9.5 ± 2.7 . The incidence of aberrant cells in maximally polycythemic rats injected intraperitoneally with 3, 6, and 12 units of erythropoietin 1 hr before the carcinogen injection is shown in Fig. 2. A clear dose-response relationship was observed at the dosages used. In another experiment, the injection of exogenous erythropoietin in relation to the time of the carcinogen injection was studied. In this experiment, erythropoietin (6 units) was administered intravenously to four groups of polycythemic animals; 2 hr before and 0, 2, and 4 hr after the administration of carcinogen to each group. The incidence of aberrant metaphase cells was considerably enhanced when erythropoietin was administered to the rats between 2 hr before and 2 hr after the injection of carcinogen. A significant enhancement was detected even in the animals in which erythropoietin acted during the last 2 hr of DMBA action (Fig. 3). These data indicate that erythropoietin is important in the induction of chromosome aberrations in bone-marrow cells of the rat, and that the change in the susceptibility of the bone-marrow cells induced by erythropoietin takes place very quickly.

The incidence of aberrant metaphase cells showed an inverse correlation with hematocrit values (Fig. 4). However, in the polycythemic rats that had received various doses of erythropoietin, the incidence of chromosome aberrations was

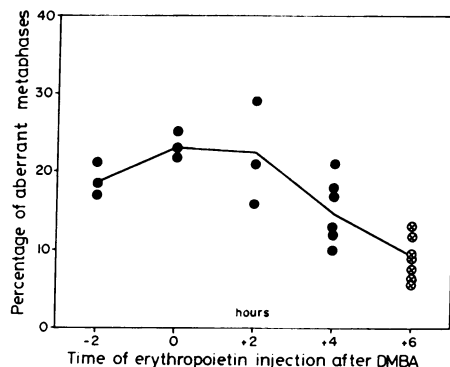


FIG. 3. The incidence of DMBA-induced chromosome aberrations in polycythemic animals given erythropoietin, 6 units of Step 3, at various intervals before and after DMBA. Difference between +4-hr group and the control group (shown in 6-hr position) is significant ($0.01 < P < 0.05$).

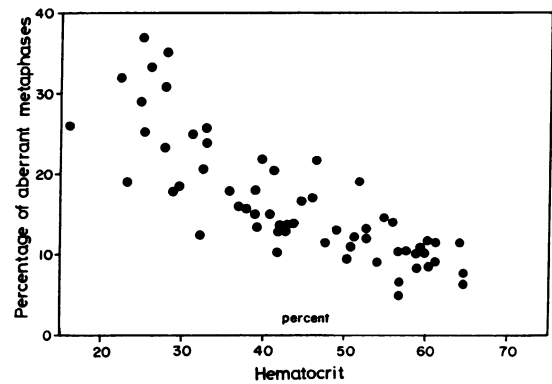


FIG. 4. Relationship between the incidence of DMBA-induced chromosome aberrations and the hematocrit value of the animals.

dependent on the amount of erythropoietin injected, rather than on the hematocrit values (Fig. 3); in these rats the hematocrit values ranged between 60 and 70%. Thus, the erythropoietin concentration seems to be essential in enhancing the susceptibility of the bone-marrow cells to the chromosome-damaging action of DMBA.

Influence of a lysosome stabilizer and a labilizer on DMBA-induced chromosome aberrations

Since a role of instability of lysosome membranes in the production of chromosome aberrations is known (6), and some hormones have been known to change the stability of lysosome membranes (7), the effects of cortisone acetate, a known stabilizer, and Vitamin A, a known labilizer, on the incidence of chromosome aberrations were studied. The results (Table 1) indicate that cortisone acetate, at a dose that caused a marked loss of body weight in every animal, had no significant influence on the incidence of DMBA-induced chromosome aberrations. Vitamin A, alone, also did not produce chromosome aberrations and did not enhance the effect of erythropoietin. The results do not support a mechanism of erythropoietin enhancement of chromosome aberrations by a direct effect of this agent on liposomal membranes.

Influence of anemia and polycythemia on DMBA-induced leukemia

The incidence of leukemia was significantly increased in group B, in which the animals were made anemic shortly before the injection of carcinogen (Table 2). Only one rat among the 14 tested developed leukemia in group C, in which polycythemia was induced before each carcinogen treatment. In group D, where polycythemia was induced 2 days after each carcinogen injection and anemia had been induced previously, 9 out of 10 rats developed leukemia within the observation period of 120 days. These results suggest that erythropoietic stimulus at the time of the carcinogen action, but not thereafter, has a definite influence on the yield of DMBA-induced leukemia.

DISCUSSION

These data indicate that bone-marrow cells in the rat become extremely susceptible to the chromosome-damaging action of DMBA under the influence of erythropoietin. In addition, the induction of DMBA-induced leukemia was accelerated by preliminary induction of anemia and suppressed by polycythemia. The parallelism between the incidence of chromo-

some damage and leukemia shown in these experiments suggests the possibility that erythropoietin augments DMBA-induced leukemogenesis by enhancing the damage to the chromosomes of the target cells.

Erythropoietin is a hormone that induces differentiation and mitosis in erythropoietic stem cells in the bone marrow (8). Since it has been proposed that lysosome labilization mediates chromosome damage (6), and some hormones such as glucocorticoids act as lysosome stabilizers (7), the influence of cortisone acetate, a known stabilizer, and Vitamin A, a known labilizer were tested. The results show that erythropoietin itself causes no chromosome damage, and that the erythropoietin effect was not influenced by the stabilizer or the labilizer. Therefore, such a mechanism does not seem to operate in augmentation of chromosome damage by erythropoietin. It is of interest that the enhancement of chromosome aberrations after erythropoietin administration starts within 2 hr before the induction of DNA synthesis (8).

The earliest known effect of erythropoietin on the bone marrow is induction of RNA synthesis (8, 9); only 15 min are needed for this effect to start (9). It has been generally agreed that hormones that affect growth and differentiation enhance the template activity of cistrons that code for the proteins necessary for cell growth and differentiation. This process entails the derepression of cistrons in specific chromosomal regions, as evidenced by puff-formation in insect chromosomes in response to a molting hormone, ecdysone. Therefore, it is possible that erythropoietin derepresses some cistrons and makes them susceptible to the effects of DMBA. The view is consistent with the view that proliferating cells are more susceptible to chromosome damage and carcinogenesis, because such cistrons are fully transcribed in these cells. Further studies are necessary to test the above possibility, as well as

the possibility that such stimuli specifically suppress repair of chromosome damage.

A similar role of erythropoietin in the induction of erythro-leukemia with Rauscher virus has been reported (10). This study also shows the role of this hormone in early leukemogenesis. The present data that induction of leukemia is strikingly influenced by the concentration of erythropoietin present suggest that the target cells of DMBA in the bone marrow are erythropoietin-responsive cells, namely, early and maturing erythroblasts. A similar deduction was made for virus-induced erythro-leukemia (10).

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