Actinomycin and Erythropoiesis and the Production of Erythropoietin in Mice

By Geoffrey Keighley and Peter H. Lowy

Actinomycin D selectively inhibits DNA-dependent RNA synthesis. Some of its effects in animals, for instance on the bone marrow, are like those of x-radiation and comparisons of such effects of the two agents have been made. Actinomycin blocks the action of some hormones; the block may not be complete or may vary in different tissues. It can depress bone marrow activity and stop erythropoiesis. Erythropoietin is a hormone (from animals with various forms of oxygen deficiency) which is a powerful stimulant of red cell production. Gurney and Hofstra have measured the additive effects of actinomycin and radiation on the erythropoietic capability of mice injected with erythropoietin, and the effects of single doses of actinomycin. Goldwasser, Gallien-Lartigue and Dukes have studied the action of actinomycin and erythropoietin in vitro.

In this paper we report results obtained in mice on the degree of suppression of erythropoiesis by various doses of actinomycin, on the time course and reversibility of the suppression, and on the failure of actinomycin to prevent increases of erythropoietin concentration in the plasma.

Materials and Methods

We used young adult B6D2F1 female mice. Erythropoiesis was measured in most experiments by the incorporation of Fe59 into the red cells under two standard schedules. In normal unmanipulated mice approximately 0.2 μc. of Fe59 in buffer solution was given intravenously and 18 hours later blood was taken by heart puncture for counting. The activity of an aliquot of the Fe59 was measured, and taking the blood volume to be 6.5 per cent of the body weight at time of bleeding, the fraction which had been incorporated into the red cells was calculated. In normal mice the incorporation averages about 25 per cent. The incorporation is a reflection of the rate at which red cells are maturing and appearing in the circulation in the 18 hours between giving the iron and bleeding the mouse.

Some mice were made polycythemic by a modification of the method of Cotes and Bangham. They were exposed to a reduced air pressure of 345–355 mm Hg in a pumped tank, with sufficient air flowing through to avoid a buildup of humidity or CO2. After 10 days of exposure they were removed to ambient pressure. As a result of their acquired polycythemia, erythropoiesis is repressed, and for the next several days Fe59 incorporation in these mice is very low, typically no more than about 1.0 per cent.

Under these circumstances a small, induced increase of erythropoiesis and incorporation can be measured readily. Three days after removal from low pressure, erythropoietin is...
given; on the fifth day Fe$^{59}$ is injected, and the mice are bled 42 hours later. The incorporation is calculated as before except that the blood volume of the polycythemic mice is taken as 7.5 per cent of body weight.

Similar mice are used to assay the erythropoietin content of substances which can be injected. The Fe$^{59}$ incorporation found is compared with that caused by known quantities of a standard preparation.

Some normal mice were exposed to hypoxia for a short time as a means of inducing a measurable concentration of erythropoietin in their plasma. They were placed in a chamber through which there was an adequate flow at ambient pressure of a mixture of air and nitrogen. A Beckman oxygen analyzer monitored the mixture and kept it at a P${\text{O}}_2$ of 59 mm. Hg. Under this degree of hypoxia erythropoietin appears in the plasma of the mice with a peak at 12 hours.13 Immediately after the 12 hours exposure the mice are bled and their plasma is separated, pooled and frozen. It is tested for erythropoietin content by injecting it into polycythemic mice, prepared according to the modified Cotes and Bangham schedule.

As sources of exogenous erythropoietin we used a pool of plasma from anemic rabbits, a concentrate of erythropoietin made from human urine and one from rabbit plasma.14 They had been assayed by Fe$^{59}$ incorporation in polycythemic mice, and compared with a working standard calibrated in terms of standard A.15

Blood was taken from the orbital sinus for microhematocrits and stained with new methylene blue for counting the reticulocytes in 1000 cells. Actinomycin D was a gift from Richard B. Anderson of the Merck Institute for Therapeutic Research. It was dissolved in saline, kept frozen until used, and injected intraperitoneally.

**RESULTS**

*Normal Mice: Effect of Single Doses of Actinomycin on Fe$^{59}$ Incorporation.* Figure 1 shows the effect of actinomycin on the subsequent course of erythropoiesis in otherwise unmanipulated mice. Single 10 μ doses were given I.P. at zero time to groups of 4-8 mice. Erythropoiesis was estimated during different, later intervals by injecting Fe$^{59}$ at the times shown and bleeding the mice 18 hours later. When Fe$^{59}$ was given after 5 hours (third point on the curve), the subsequent incorporation reflected an already reduced activity; 98 per cent suppression was reached after 48 hours. Incorporation was suppressed for another 3 days, then recovered rapidly. In an experiment (not shown) with only 5 μg. of actinomycin, erythropoiesis slowed at about the same rate but recovery began 24 hours earlier. In 10 control mice without actinomycin mean incorporation was 24.4 ± 2.2 per cent (S.E.).

*Polycythemic Mice: Actinomycin in Graded Doses.* When 38 mice, made polycythemic by exposure to reduced pressure as described above, were injected S.Q. with 0.25 A units of erythropoietin on day 3 of the schedule, erythropoiesis was so stimulated that the incorporation of Fe$^{59}$ injected on day 5 rose to a mean of 23.5 ± 1.1 per cent, while the incorporation in 25 polycythemic, saline-injected, control mice was only 0.99 ± 0.1 per cent. When groups of polycythemic mice (Fig. 2) were injected with the same dose of 0.25 A units of erythropoietin followed immediately by actinomycin, the stimulating effect of the erythropoietin was suppressed to a degree proportional to the dose of actinomycin. A dose of only 2.0 μg. was sufficient to reduce erythropoiesis to the base line level.

*Polycythemic Mice: Effectiveness of Actinomycin as a Function of its Time of Administration.* In Figure 3 the mice had been exposed to reduced pressure
Fig. 1.—Changes of Fe59 incorporation in normal mice as a function of time after a single dose of actinomycin. AM = 10 μg. of actinomycin at zero hours. Time is the number of hours between the actinomycin and the injection of Fe59. Four to 8 mice per point, mean and standard error of the mean.

According to schedule, and were given a stimulating dose of 0.25 units of erythropoietin at the usual time on day 3. They were also given a dose of actinomycin, 4.0 μg., which is more than enough to suppress erythropoiesis when given along with erythropoietin (Fig. 2), but the actinomycin was given at various times before or after the erythropoietin. Zero time on Figure 3 is the time on day 3 of the schedule when erythropoietin was given; its effect was measured in the standard way by giving Fe59 on day 5 and bleeding the mice 42 hours later. Actinomycin given up to 24 hours before, or 8 hours after, time zero completely prevented the effect of erythropoietin on Fe59 incorporation. At 14 hours after, suppression was almost complete; 24 or more hours after there was no suppression.

Normal Mice: Repeated Doses of Actinomycin. Four groups of normal mice were injected with 2.5 or 5.0 or 7.5 or 10.0 μg. of actinomycin, on the same day once a week, for 10–17 weeks (Table 1). Each week, randomly from 1–7 days after the actinomycin, blood samples were taken from the orbital sinus.

Since it is not possible to make repeated measurements of Fe59 incorporation on a mouse because of the build-up of Fe59 and the size of the blood samples needed for counting, we measured erythropoiesis in these mice by counting reticulocytes and taking the hematocrit. The amount of blood taken each week has no effect on the reticulocyte count or hematocrit in control mice bled in the...
Fig. 2.—Effect of graded doses of actinomycin on Fe$^{59}$ incorporation. Polycythemic mice were stimulated by a single injection of 0.25 A units of erythropoietin, followed immediately by the various doses of actinomycin. Four to 15 mice per point, mean and standard error of the mean.

same way for several weeks. Table 1 is a composite of all these results. Each week following the actinomycin there was a drop in reticulocytes to day 4, followed by a rise. This pattern was repeated week after week. At all doses the counts on day 4 were significantly different from the counts on other days ($p < 0.01$). Hematocrits in mice at doses of 2.5 and 5.0 $\mu$g showed no weekly trend; the values on day 4 (in the table) are not significantly different from the values on any other day. At the end of the experiment, 17 weeks, the hematocrits of these mice were not significantly different from the values at the start. A few mice were sacrificed and bone marrow smears showed changes in numbers of erythroid cells consistent with the changes in reticulocytes.

Normal Mice: Effect of Actinomycin on the Appearance of Erythropoietin in the Plasma of Mice Exposed to Hypoxia. Mice were exposed to a $pO_2$ of 59 mm. for 12 hours. Some (Table 2) had been injected immediately or some hours before with doses of actinomycin which are adequate (Figs. 1–3) to suppress erythropoiesis. Other mice were exposed to hypoxia without any treatment with actinomycin. All were bled immediately and the plasmas assayed for erythropoietin content by measuring their ability to cause increased
ACTINOMYCIN AND ERYTHROPOIESIS

Fig. 3.—Polycythemic mice. Relation between the time when actinomycin is given and its effectiveness against a dose of erythropoietin. EP = 0.25 A units of erythropoietin at zero hours. Hours = time when the actinomycin was injected before or after the injection of erythropoietin. Four to 9 mice per point, mean and standard error of the mean.

Fe\textsuperscript{59} incorporation in polycythemic assay mice. Erythropoietin is expressed as A units per ml. In no case did actinomycin prevent the appearance of erythropoietin. The mean concentration in the plasmas of all the actinomycin-treated mice taken together is not significantly different from that in the controls exposed without actinomycin.

DISCUSSION

Previous observations have shown the selective toxicity of actinomycin toward bone marrow.\textsuperscript{3} The intraperitoneal LD\textsubscript{50} has been reported as 15 μg. for a mouse.\textsuperscript{3} When we injected 10 normal mice with 25 μg. doses, 6 mice died within 7 days and 4 lived to 100 days. The range between these doses and the minimal fully effective dose of 2–4 μg. in polycythemic mice (Fig. 2) is not large. Actinomycin was effective against all the exogenous sources of erythropoietin which we tested. It suppressed completely not only 0.25 A units contained in rabbit plasma, but also the erythropoietin in two dry, soluble, purified preparations, one from human urine at a dose of 0.4 units and one from rabbits...
Table 1.—Effect of Repeated Weekly Doses of Actinomycin on Reticulocyte Count and Hematocrit of Normal Mice

<table>
<thead>
<tr>
<th>Weekly Dose (μg)</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>1.7 ± 0.3</td>
<td>—</td>
<td>0.3 ± 0.1</td>
<td>3.2 ± 0.5</td>
<td>3.1 ± 0.7</td>
</tr>
<tr>
<td>5.0</td>
<td>2.3 ± 0.2</td>
<td>—</td>
<td>0.2 ± 0.01</td>
<td>4.0 ± 0.7</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>7.5</td>
<td>2.6 ± 1.1</td>
<td>1.6 ± 0.2</td>
<td>0.1 ± 0.01</td>
<td>—</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>10.0</td>
<td>5.7 ± 1.0</td>
<td>3.0 ± 1.2</td>
<td>0.0</td>
<td>—</td>
<td>2.5 ± 0.6</td>
</tr>
</tbody>
</table>

Hematocrits (%)

<table>
<thead>
<tr>
<th>Weekly Dose (μg)</th>
<th>2.5</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>46.1 ± 0.7</td>
<td>44.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>44.1 ± 1.0</td>
<td>42.4 ± 0.6</td>
</tr>
</tbody>
</table>

Two control mice. 16 reticulocyte counts in 40 days, mean 2.87 ± 0.2.
Four control mice. 56 hematocrit counts in 14 weeks, mean 44.3 ± 0.3.

*Mean ± standard error of the mean, 4–8 measurements.
†Mean ± standard error of the mean, 6–8 measurements.

Table 2.—Effect of Actinomycin on the Appearance of Erythropoietin in the Plasma of Mice Exposed to Hypoxia

<table>
<thead>
<tr>
<th>No. of Mice</th>
<th>Dose of Actinomycin (μg.)</th>
<th>Time Between Actinomycin and Hypoxia (Hours)</th>
<th>Length of Hypoxia (Hours)</th>
<th>Erythropoietin Per ml. of Plasma A Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>7.5</td>
<td>0</td>
<td>12</td>
<td>0.29 ± 0.04†</td>
</tr>
<tr>
<td>4</td>
<td>30.0</td>
<td>0</td>
<td>12</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>10.0</td>
<td>0</td>
<td>12</td>
<td>1.17 ± 0.53</td>
</tr>
<tr>
<td>4</td>
<td>10.0</td>
<td>12</td>
<td>12</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>10.0</td>
<td>52</td>
<td>12</td>
<td>0.38 ± 0.02</td>
</tr>
</tbody>
</table>

Mean of all the above mice given actinomycin

| 20          | 7.5–30.0                  | 0–52                                        | 12                        | 0.49 ± 0.12                            |
| 20          | 0.0                       | —                                           | 12                        | 0.53 ± 0.06                            |

control mice without actinomycin
difference 0.04 = N.S.†

control mice with actinomycin, without hypoxia

| 6           | 10.0                      | (12 hours at normal pressure)               |                           | 0.0                                    |

*Mean ± standard error of the mean.
†N.S. = not significant.

at a dose of 11.1 units. The size of the dose we have found to be effective in mice, and the time relations (Fig. 3) are similar to those found by Goldwasser, Gallien-Lartigue and Dukes in an in vitro erythropoietic system. Only in those mice injected repeatedly with more than 10 μg. of actinomycin did we see any significant changes in white cell counts, with a drop in total WBC and in lymphocytes and an increase of segmented forms.

The differentiation and maturation of red cells takes about 60 hours in mice. The change of suppression versus time of Figure 1, with Fe³⁺ incorporation declining at first, remaining low, then recovering, is the result expected from complete inhibition by actinomycin of new erythropoiesis in a population of cells which have some cells already committed to development prior to the dose of actinomycin, if the average time of release into the circulation is about 60 hours and if development of the cells once started is soon inaccessible to the action of actinomycin. The sensitivity to actinomycin in relation to the time
when it is given is consistent with the hypothesis that erythropoietin initiates erythropoiesis and that this part of the process is completed in almost all cells in a few hours and can be inhibited by actinomycin and therefore needs DNA-dependent RNA synthesis. Once this early stage is completed (Fig. 3), the cells are no longer so sensitive to actinomycin; after 24 hours there is little evidence that it has any effect on cells already started. The evidence that actinomycin has no effect on the later stages of maturation of the developing red cells shows that its influence is not through some general metabolic interference, but on a specific process initiated by erythropoietin.

Recovery of the erythropoietic system from the effects of actinomycin is quick and effective (Fig. 1 and Table 1). We did not see in any of our experiments the secondary depression 6 days after actinomycin reported by Gurney and Hofstra, but the conditions of our experiments are not identical. Because mice, injected repeatedly with doses which cause complete but temporary suppression of erythropoiesis, can maintain their hematocrits, therefore during the recovery phases there must be a compensatory above-normal production of red cells. There is evidence of this in the greater than normal Fe incorporation after 120 hours in Figure 1, and in the reticulocyte counts on the seventh days in Table 1. The erythropoietic regulatory system responds to some signal which is adequate to maintain the hematocrit. Reticulocyte counts and Fe incorporation are more sensitive indicators of the corrective changes than the hematocrit. This regulatory system clearly maintains its integrity and adequacy in the presence of repeated doses of actinomycin as great as one-third to one-fifth the LD₅₀. The maintenance of the ability to respond and recover is further evidence that the suppressive effects of actinomycin are on specific processes, not a general metabolic interference.

Nor does actinomycin interfere with erythropoiesis by blocking the production of erythropoietin, as can be seen from Table 2. Even doses which would ultimately kill the mouse do not affect the appearance of erythropoietin in the plasma of mice stimulated by hypoxia. Actinomycin does not prevent the production of erythropoietin or alternatively cause the appearance of a modified, ineffective form.

Erythropoietin is probably a glycoprotein. Since its appearance in the circulating blood is not affected by doses of actinomycin which repress erythropoiesis, the following possibilities can be considered: (1) Erythropoietin arises in sites much less sensitive to actinomycin than those of erythropoiesis; (2) it is synthesized under the influence of a long-lasting messenger RNA; and (3) it is the result of a modification of a precursor substance by enzymes. Endogenous erythropoietin production should be studied during cyclic depressions and recoveries following doses of actinomycin, and the method of using repeated doses holds some promise as a way of inducing synchronized development of erythropoietic cells.

**Summary**

In B6D2F₁ female mice a single dose of 10 µg. of actinomycin will suppress normal erythropoiesis. In polycytemic mice 2 µg. is enough to prevent the stimulatory effect of 0.25 A units of erythropoietin. The curves of suppression
and recovery versus time support the hypothesis that erythropoietin acts for a short time in an early stage of erythropoiesis; after this early stage developing cells are no longer so sensitive to actinomycin. Recovery, even from repeated doses, is rapid and adequate. Amounts of actinomycin which are adequate to abolish erythropoiesis do not prevent the new appearance of erythropoietin in the plasma of hypoxic mice.

**SUMMARIO IN INTERLINGUA**

In muses feminin B6D2F1, un sol dose de 10 µg de actinomycina supprime le erythropoiese normal. In muses polycyttemic, 2 µg suffice a prevenir le effecto stimulatori de 0.25 unitates A de erythropoietina. Le curvas del suppression e del restablimento como function del tempore supporta le hypothese que erythropoietina age durante un breve periodo de tempore in le precoce phases del erythropoiese. Post iste precoce phases, cellulias sub disveloppamento es minus sensibile pro actinomycina. Le restablimento mesmo ab repetite doses es rapide e adequate. Quantitates de actinomycina que es adequate pro abolir erythropoiese non preveni le reapparition de erythropoietina in le plasma de muses hypoxic.

**REFERENCES**


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