Controlled Phenylhydrazine-Induced Reticulocytosis in the Rat

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CONTROLLED PHENYLHYDRAZINE-INDUCED RETICULOCYTOSIS IN THE RAT\textsuperscript{1, 2}

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**ABSTRACT**

The pattern of development of phenylhydrazine-induced rat reticulocytosis was studied over a period of nine days. Intraperitoneal injections of phenylhydrazine (4 mg/100 gm) every other day caused a fall in hematocrit which leveled off at 60% of normal by the fifth day. Increased erythropoiesis was indicated by a three-fold increase in the number of circulating reticulocytes after the first three injections. The immediate response was the release of stored mature reticulocytes from the bone marrow. As the anemia progressed, more and more young reticulocytes appeared until 70 to 85% of the red cells in the peripheral circulation were reticulocytes and 20% of these were juvenile forms.

**INTRODUCTION**

Administration of phenylhydrazine to experimental animals causes an increase in erythropoiesis, as evidenced by the release of reticulocytes into the circulating blood. Phenylhydrazine is an oxidant drug which destroys red cells by its effect on enzymes involved in energy metabolism (Jandl \textit{et al.}, 1960). It results in the denaturation of red cell hemoglobin \textit{in vitro} or \textit{in vivo} with little evidence of toxicity to other tissues, if the dosage is carefully adjusted (Cruz, 1941; Rifkind and Danon, 1965). The drug primarily damages mature erythrocytes inducing an erythropenia which, in turn, leads to an accelerated erythropoiesis (Smith and McKinley, 1968). Controlled bleeding of animals also results in accelerated erythropoiesis, but the reticulocytosis evoked by phenylhydrazine is more rapid and is more easily controlled for experimental purposes (Jacobson \textit{et al.}, 1948). In order to investigate the cytological and biochemical changes that occur during reticulocyte maturation, it was necessary to obtain rats with large numbers of circulating reticulocytes. Studies of phenylhydrazine anemia enabled us to produce rats with predictable percentages of reticulocytes in their blood.

Reticulocytes appear in the circulation as non-nucleated cells that complete their hemoglobin synthesis and differentiate into erythrocytes in approximately two days (Lowenstein, 1959; Plum, 1949). When supravitally stained with dilute solutions of Brilliant Cresyl Blue (Heath and Daland, 1931) or New Methylene Blue (Brecher, 1949), reticulocytes show characteristic granules or a reticular network. Transitional forms of reticum have been described and several investigators have classified reticulocytes by the amount, form, and distribution of stained reticulum (Heilmeyer, 1932; Jandl, 1960; Seip, 1953). Jensen \textit{et al.} (1965) recently demonstrated that the basophilic stippling of reticulocytes is due to aggregation of the ribosomes present in the maturing cells. As reticulocytes mature into erythrocytes, their ribosomes gradually disappear and less stainable reticulum is evident. There is general agreement that reticulocyte maturity is inversely proportional to the amount of stained reticulum, and that the course of a reticulocytosis can be analyzed by the types of reticulocytes that appear in stained blood smears. Reinvestigation of phenylhydrazine-induced

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anemia enabled us to obtain rats with high percentages of young or mature reticulocytes in their circulating blood, and to study the course of reticulocyte release and maturation (Lessler et al., 1967).

METHODS AND MATERIALS

Reticulocytosis was induced in mature male Wistar rats by interperitoneal injection of phenylhydrazine hydrochloride (4 mg/100 gm of rat) on days 1, 3, 5, 7, of a nine-day experimental period. The initial weight of the rats ranged from 300 to 490 grams, and the final weights (day 9) from 280 to 430 grams. The animals were watered and fed Purina lab-chow without added supplements of iron.

The rats were weighed, and blood samples were obtained from the tail vein just before each phenylhydrazine injection and also on the 9th day, 48 hours after the final injection. Duplicate microhematocrits and blood smears were made from each blood sample. Supravital staining of reticulocytes was done by mixing one drop of blood with one drop of 0.5% New Methylene Blue in 0.65% potassium oxalate and incubating for 10 minutes in a moist chamber at room temperature before smearing (Brecher, 1949). The air-dried New-Methylene-Blue-stained smear was counterstained with Wright's stain, and 1000 red cells were counted on each of two duplicate slides with an oil-immersion objective.

Reticulocytes were counted as young (group I, II) or mature (group III, IV) cells, according to the amount and distribution of stained reticulum. Based on the classification of Heilmeyer and Westhauser (1932) and of Seip (1953), group I reticulocytes have a dense coherent reticulum filling the greater part of the cell. Group II reticulocytes have an extended reticulum, which is looser than that in group I because of occasional breaks in the network. Group III reticulocytes have some small bits of reticulum, and group IV cells have only a few blue granules and are essentially erythrocytes.

Counts were made by two independent investigators over a period of two years on a series of 20 rats experimentally treated in groups of three to four with consistent results. These data were averaged, and the standard deviations (Table I) show a relatively small spread, except for the day-seven and day-nine rats, which were severely anemic. One control (untreated) rat was counted with each group; these animals showed little variation from the 2% reticulocytes observed in colony rats whose blood was studied before any treatment had been given.

RESULTS

The pattern of development of the phenylhydrazine-induced anemia was evident from the fall in hematocrit from a value of 49, in untreated rats, to 30 (Table 1). Hematocrit values fell 36% after 2 injections (4 days) as the percentage

<table>
<thead>
<tr>
<th>Day</th>
<th>Hematocrit</th>
<th>Total</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49±1.8</td>
<td>2.2±0.7</td>
<td>0.0±0.0</td>
<td>0.2±0.5</td>
<td>0.4±0.4</td>
<td>1.6±0.6</td>
</tr>
<tr>
<td>3</td>
<td>38±2.5</td>
<td>7.4±0.8</td>
<td>0.7±0.5</td>
<td>0.9±0.7</td>
<td>2.2±1.4</td>
<td>3.6±1.2</td>
</tr>
<tr>
<td>5</td>
<td>31±2.5</td>
<td>22.5±5.6</td>
<td>2.2±1.6</td>
<td>3.9±2.1</td>
<td>6.8±2.6</td>
<td>9.2±2.8</td>
</tr>
<tr>
<td>7</td>
<td>30±3.9</td>
<td>52.0±12.6</td>
<td>6.5±4.3</td>
<td>11.1±6.3</td>
<td>16.2±6.7</td>
<td>18.2±9.5</td>
</tr>
<tr>
<td>9</td>
<td>29±4.8</td>
<td>70.1±9.1</td>
<td>6.0±4.4</td>
<td>12.9±7.8</td>
<td>22.9±4.4</td>
<td>28.3±9.5</td>
</tr>
</tbody>
</table>

Initial weights ranged from 300 to 490 grams.

Standard deviation of the mean.
of reticulocytes increased to approximately 30% of the total circulating red-cell population (fig. 1). The decrease in hematocrit tended to level off at about 30, by the fifth day, but the percentage of reticulocytes, which rose from 2 to 20% in five days, more than doubled by the seventh day (after 3 injections). On the ninth day (after 4 injections) the hematocrit was 29, but 70 to 80% of the red cells in the peripheral blood were reticulocytes. Juvenile reticulocytes (group I, II) increased with each injection until they made up approximately 20% of the red-cell population in the anemic rats on days seven and nine (Table 1).

During the course of the developing anemia, the average weight of the animals tended to fall, with the greatest decrease seen after the second injection (fifth day). There was an inverse relationship between percent of reticulocytes and weight loss. The greatest weight loss was apparent on the fifth and seventh days and occurred after the animals had developed a severe reticulocytosis (Fig. 1). The first

![Figure 1](image-url). Relationship of hematocrit and body weight to percent reticulocytosis in Wistar rats injected with phenylhydrazine (40 mg/kg) on days 1, 3, 5, and 7.
phenylhydrazine injection resulted in an increase in reticulocytes from 2 to 7%. This was due mainly to the release of mature reticulocytes. Successive injections of phenylhydrazine resulted in an increase in the percentage of young reticulocytes in the peripheral blood until they made up approximately 33% of the circulating reticulocyte population after the third and fourth injections (Table 1).

DISCUSSION

Under normal laboratory conditions, healthy Wistar rats have 2 to 3% reticulocytes in their peripheral blood and these are almost exclusively mature forms (group III, IV). Reticulocytes in the circulation mature into erythrocytes in approximately two days. A single injection of phenylhydrazine destroys many of the red cells as well as some of the mature reticulocytes (Jandl et al., 1960; Cruz, 1941). The destruction of red cells causes an anemia which stimulates the release of the mature reticulocytes normally stored in the bone marrow. Although erythropoiesis was increased, as indicated by the small rise in immature forms, the anemia caused by a single injection of phenylhydrazine was not severe enough for the early release of large numbers of young reticulocytes. Each successive injection of phenylhydrazine, however, challenged the animals to increase erythropoiesis further in order to replace the destroyed red cells. This they did, as indicated by the increase in the number of young reticulocytes released after the second, third, and fourth injections.

There is a gradient of increasing susceptibility of red cells to the denaturative effects of phenylhydrazine and related compounds which is directly proportional to cell age (Cruz, 1941). Thus, mature erythrocytes are more susceptible than are reticulocytes or the reticulocyte precursor cells. Injection of phenylhydrazine every other day resulted in a progressive anemia because of the selective destruction of mature red cells which resulted in an erythropoietic stimulation of the bone marrow. After each injection the bone marrow had two days to increase erythropoiesis and release reticulocytes. The reticulocytes in the circulation also matured into erythrocytes during that period. Thus, it was possible to destroy additional erythrocytes and the reticulocytes that had matured into erythrocytes after each of the subsequent injections with further stimulation of bone marrow erythropoiesis. Controlled reticulocytosis by alternate-day injections of phenylhydrazine works best with mature male rats (300–400 gm). Similar injections given to young rats (125–150 gm) causes a severe anemia and many of the animals die after one or two injections. Mature male rats survive all four injections and have larger numbers of circulating reticulocytes (70 to 85%) than can be obtained by sequential bleeding (Jacobson et al., 1948). Although reticulocytosis can be induced in animals by other doses of phenylhydrazine, alternate day injections of 4 mg/100 gm make it possible to routinely prepare anemic rats with 70% or more of their circulating red cells as reticulocytes.

LITERATURE CITED