Hematological and Morphological Analysis of the Erythropoietic Regenerative Response in Phenylhydrazine-induced Hemolytic Anemia in Mice

by Marta Roque*, Cecilia D’Anna, Christian Gatti & Tania Veuthey
Laboratorio de Fisiología Humana, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, Argentina

Summary
In this study we developed a mouse model of Phenylhydrazine (PHZ)-induced hemolytic anemia to study erythropoietic regenerative response through clinical, pathological, and morphological studies. Hemolytic anemia was induced in female mice (CF1) using PHZ at a wide range of doses (up to 100 mg/Kg) on days 0 and 2. Hemolytic anemia was observed at 60 mg/kg PHZ on day 4 and was evidenced by decreased HCT (34.3±0.28%), reticulocytosis (51.6±2.10%), anisocytosis, poikilocytosis, leukocytosis, and increased Heinz body count. A time-course and dose-dependence analysis of the regenerative response was performed. HCT decreased on days 2 and 4 in a dose-dependent manner, returning to basal levels on day 8. PHZ only induced reticulocytosis (day 4) at the highest doses tested (60-100 mg/kg). Heinz body formation was dose-dependent. These changes were accompanied by splenomegaly and splenic erythroid hyperplasia. Results revealed that the presence of erythroblastic islands was most clear in the spleen, followed by the liver and kidney. SEM showed Heinz body-containing erythrocytes and spherocyte-like erythrocytes. Anemia recovery results from coordinated action of extramedullary tissues depending on the time post injection and the dose applied. In conclusion, this mouse model allowed us a better understanding of murine erythropoietic regenerative response.

Introduction
In vertebrates, anemia is a common hematological disorder associated with several conditions such as drug toxicity, parasites, genetic or acquired defects, and blood loss (Criswell et al., 2000; Jollow & McMillan, 2001). The hemolytic activity of aryldrazines, such as phenylhydrazine (PHZ), dapsone hydroxylamine, divicine, may lead to acute hemolytic anemia in vertebrates (Jollow & McMillan, 2001; Kozlov et al., 1980; Magnani et al., 1986). The main action of the classical hemotoxicant PHZ has long been associated with drug-induced oxidative stress occurring within erythrocytes (Kinuta et al., 1995; Jain & Hochstein, 1980). This process produces an increase in the oxidation of oxyhemoglobin, thus leading to the formation of metahemoglobin which is subsequently converted into irreversible hemichromes which, in turn, lead to the denaturation and precipitation of hemoglobin in the form of Heinz bodies (Rifkind & Danon, 1965; Rifkind, 1965). Skeletal protein damage and lipid peroxidation as well as glutathione and ATP depletion, cation imbalances, and reduced membrane deformability have been proposed to be involved in the hemolytic response that is induced by oxidant drugs (McMillan et al., 1998).

On the other hand, it has also been reported that PHZ-induced anemia is associated with immune
activation (Naughton et al., 1990). In this respect, PHZ can cross-link red cell band 3 protein (senescent antigen), resulting in the binding of circulating autologous antibodies (Magnani et al., 1986). Recognition of this antigen-antibody complex by macrophage receptors triggers phagocytosis in the spleen and possibly the liver.

The spleen plays important roles in processes other than blood storage and immune competence, and in PHZ-induced hemolytic anemia in rodents and rabbits it acts as the main erythropagocytic organ (Latunde-Dada et al., 2006). This implies that damaged cells are removed intact by the spleen, on the one hand, and that intravascular lysis is minor with respect to extravascular lysis, on the other.

In adult mice, the erythroid regenerative response to PHZ-induced acute hemolysis is mainly performed by the spleen (Kozlov et al., 1980). Pluripotential stem cells proliferate and migrate from the bone marrow to the spleen under erythropoietic stimuli (Rencricca et al., 1970), a finding that finds support in later studies, which indicate an increase in precursor erythropoietic cells in the peripheral blood of animals with PHZ-induced hemolytic anemia (Hara & Ogawa, 1976; Hara & Ogawa, 1977).

In addition, it is worthy of note that, although murine hematopoiesis has served as a model for the study of human hematopoiesis, there are differences between those species. In humans, the spleen plays no significant role in erythropoiesis, and adaptation to anemia is not as effectively accomplished as in rodents (Mattson et al., 1984). On the other hand, the liver, which has a role in the development of the murine hematopoietic system, has been reported to be a site of extramedullary hematopoiesis in adult mice and rats (Ploemacher & Van Soest, 1977; Redondo et al., 1995).

In view of the above, time-course and dose-dependence studies were conducted in the present research in order to characterize hematologically and morphologically the erythroid regenerative response following anemia induction in mice. To this end, the experimental approach followed included clinical, pathological, and ultrastructural evaluations. The role of extramedullary organs in the regenerative response was also evaluated.

Materials and Methods

Animals

Adult virgin female mice (CF1, at 10 weeks of age) were provided, bred and housed at the Bioterio of the Universidad Nacional del Sur. The animals were kept in cages -three animals per cage- in a room at controlled room temperature (22±2 °C) and humidity (60 ±10%) during 10 days prior to the beginning of the study. During the experiment the animals were fed on a certified diet (batch 10006/06/01, Ganave ratas y ratones, Pilar, Argentina) consisting of 25.0% protein, 10.0 % fibre, 4.0 % fat, 9.0 % minerals, 12.0 % moisture and access to water ad libitum, under standard conditions with a 12 h light-dark period. Body weights were controlled throughout the study. The body weight of each mouse in the group was similar at the beginning of the experiment (35 ± 2.3 g). The procedures followed were in agreement with the protocols approved by the Guide for the Care and Use of Laboratory Animals.

Experimental Design

Adult mice were divided into a) a control group receiving saline solution intraperitoneally (0.9% NaCl) on days 0 and 2 (n=3, for each time point), and b) experimental groups receiving PHZ dissolved in 0.5 ml of saline solution intraperitoneally on days 0 and 2. The doses applied ranged between 10-100 mg/kg (n=3, for each time point). Mice were subject to blood controls on day 0, prior to PHZ or saline solution administration, and samples from three individual mice of each group were subsequently collected to determine hematological and morphological parameters on days 2, 4, 6, and 8, respectively. Erythropoietic recovery was assessed using conventional hematological tests on days 6 and 8. Animals were anesthetized with halothane for blood collection. Samples were collected throughout the study from the retro-orbital venous plexus (25 µl each).
Phenylhydrazine preparation
A phenylhydrazine hydrochloride solution (PHZ) (Sigma Chemical Co.) in 0.1 M potassium phosphate buffer pH 7.4 was prepared on day 0. The solution was sterilized by filtration prior to use and administered by intraperitoneal injection on day 0 and 2.

Hematology
The following tests were performed on EDTA samples: RBC, Hb, HCT, reticulocytes, MCV, MCH, WBC, and differential WBC. Red cell data were obtained using a CELL DYN 3500 Abbott and a SEAC Genius counter. Reticulocytes were counted in blood smears stained with Brilliant Cresyl Blue. Each reticulocyte count was based on a count of 1000 RCB. Blood smears were prepared immediately after blood sample collection and were stained with May Grünwald-Giemsa. Differential Heinz body RCB count was obtained by counting 1000 cells in a Brilliant Cresyl Blue-stained preparation. Cell count was performed three times for each time point and for each group.

Tissue preparation for necropsy
On days 0, 2, 4, 6, and 8, a subset of each group (n=3) was anesthetized for euthanasia by diethyl ether inhalation. The spleen, liver, and kidney of both control and PHZ-treated mice (60 mg/kg) were removed under sterile conditions by abdominal incision and they were separated from their attachments to the surrounding organs. All the tissues were kept in sterile saline solution and the appropriate arterial branch was cannulated. Histological techniques were followed using Ringer solution plus heparin (0.25% w/v) and Bouin solution as perfusion liquids. Approximately 100 ml of solution was perfused in each organ to wash out the red cells.

Histopathological studies
Spleen, liver, and kidney samples from control and PHZ-treated mice (60 mg/kg) were post-fixed by immersion in fresh fixative solution (Bouin and 10% neutrally buffered formalin, pH 7.2). Tissues were embedded in paraffin, sectioned, and stained with hematoxilin and eosin using standard methods.

The extent of extramedullary erythropoietic activity was expressed as the number of erythroblastic islands per 10 microscopic fields on each tissue. Erythroblastic islands (EI) were determined semi-quantitatively in stained histopathological sections of tissue, and were scored into the following categories according to the level of extramedullary erythropoiesis: a) 0-4 EI/10 fields: +; b) 5-10 EI/10 fields: ++; c) 11-15 EI /10 fields: +++; and d) more than 16 EI/10 fields: ++++. Samples from both groups were examined on the same day to exclude day-to-day variability as well as inter-group differences. Cell counts were made on 10 microscopic fields with high cellularity. The staining procedures and microscope settings carried out in the present study were performed cautiously to reach the highest standards of reproducibility and stringency.

Scanning Electron Microscopy
The morphology of erythrocytes and splenic tissue in control and PHZ-treated mice (60 mg/kg) was observed by scanning electron microscopy (SEM). Small fragments of tissue were fixed in glutaraldehyde, washed, postfixed in osmium tetroxide, dehydrated, and embedded in epoxy resin. Control and experimental mice spleens were cut into small pieces and fixed using 1% glutaraldehyde. Thin sections were stained and examined at variable angles (1000x, 3000x, 6000x).

Statistical Methods
All values are expressed as mean ± SD. Statistical differences between treated and control groups were determined by Student’s t-test and between treated groups by one-way ANOVA. Bartlett’s test was performing for homogeneity of variance. Analysis of variance was followed by Tukey multiple comparison test. The level of statistical significance was set at $P < 0.05$. 

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Results

Hematological Studies

The erythropoietic regenerative response to anemia was firstly assayed at a single PHZ dose (60 mg/kg). Hematological data from control and PHZ-treated mice were registered (Table 1). The hematological parameters of untreated mice inoculated with physiological solution remained constant during the whole experimental procedure. However, on day 2 the experimental animals showed an important decrease in Hb, RBC count, and HCT. These changes were more marked on day 4, whereas on day 6 a tendency to return to control values was observed. Reticulocyte count remained constant on day 2 but it surprisingly revealed a 200% increase on day 4. This behavior is associated with an increase in MCV on day 6, in coincidence with the appearance of reticulocytes in peripheral blood. The increase in MCH occurring on day 4 could be interpreted as indicative of intravascular hemolysis (Table 1). An important increase of Heinz bodies with respect to the control group was observed in blood smears stained at different time points, being more significant on day 2. The above-mentioned changes were accompanied by leukocytosis. This augmented leukocyte count was observed on day 2, being more significant on day 4 (Table 1).

Interestingly, the analysis of the leukocytary formula showed that the lymphocyte contribution significantly increased (data not shown). RBC, HCT and Hb returned to normal values on day 8.

Time-course evaluation of the dose-dependence of PHZ hemolytic action

After the hematological and morphological analysis of the response to a fixed PHZ dose, experiments were conducted in order to establish the dose-dependence nature of the regenerative response variation through-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Untreated Mice</th>
<th>PHZ-treated Mice</th>
<th>PHZ-treated Mice</th>
<th>PHZ-treated Mice</th>
<th>PHZ-treated Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 2</td>
<td>Day 4</td>
<td>Day 6</td>
<td>Day 8</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>15.1 ± 0.50</td>
<td>11.5 ± 0.80</td>
<td>7.4 ± 0.63</td>
<td>11.1 ± 0.35</td>
<td>14.4 ± 0.6</td>
</tr>
<tr>
<td>RBC (x10⁶/ L)</td>
<td>8.1 ± 1.5</td>
<td>7.4 ± 1.4</td>
<td>5.2 ± 1.9</td>
<td>6.4 ± 1.8</td>
<td>7.9 ± 1.2</td>
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<tr>
<td>HCT (%)</td>
<td>47.0 ± 1.8</td>
<td>38.3 ± 0.57</td>
<td>34.3 ± 0.28</td>
<td>39.5 ± 1.7</td>
<td>45.8 ± 1.1</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>51.5 ± 3.5</td>
<td>57.4 ± 3.9</td>
<td>60.9 ± 4.2</td>
<td>81.0 ± 3.5</td>
<td>69.0 ± 2.4</td>
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<tr>
<td>MCH (pg)</td>
<td>18.1 ± 2.6</td>
<td>27.7 ± 2.7</td>
<td>32.3 ± 2.8</td>
<td>26.8 ± 2.9</td>
<td>23.2 ± 2.1</td>
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<tr>
<td>Reticulocytes (%)</td>
<td>3.4 ± 1.2</td>
<td>2.9 ± 0.84</td>
<td>51.6 ± 2.12</td>
<td>30.3 ± 3.5</td>
<td>10.0 ± 1.2</td>
</tr>
<tr>
<td>Heinz Body (%)</td>
<td>1.2 ± 1.2</td>
<td>90.0 ± 3.2</td>
<td>12.3 ± 0.98</td>
<td>5.8 ± 1.96</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>Leucocytes (x10⁶/ L)</td>
<td>7.2 ± 0.20</td>
<td>15.2 ± 0.85</td>
<td>93.0 ± 3.4</td>
<td>27.9 ± 0.6</td>
<td>8.4 ± 0.5</td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
<td>100 ± 30</td>
<td>310 ± 40</td>
<td>580 ± 50</td>
<td>460 ± 40</td>
<td>150 ± 30</td>
</tr>
<tr>
<td>Liver weight (mg)</td>
<td>1320 ± 30</td>
<td>2000 ± 30</td>
<td>1850 ± 40</td>
<td>2200 ± 30</td>
<td>1500 ± 40</td>
</tr>
<tr>
<td>Kidney weight (mg)</td>
<td>200 ± 20</td>
<td>230 ± 10</td>
<td>360 ± 30</td>
<td>245 ± 10</td>
<td>230 ± 15</td>
</tr>
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</table>
out post-injection time. Both the magnitude and the
effect pattern of different PHZ doses for each individ-
ual postinjection day were firstly determined and sub-
sequently compared with those observed at 60 mg/kg.
Mice were randomly allotted to groups and were
inoculated intraperitoneally with PHZ at a concen-
tration range between 10 and 100 mg/kg on day 0
and 2. For each time point, samples were collected
from three individual mice of each group.
We first analyzed the effects of different PHZ doses
on the parameters evaluated on each experimental
day (Fig. 1). The decrease in HCT clearly showed a
dose-dependence behavior in all doses tested on
days 2 and 4. On day 6, values tended to return to
control ones at all doses tested. The same pattern
was observed in Hb variation (data not shown).
In the range 0-20 mg/kg, PHZ produced no increase
in reticulocytes. Nevertheless, at higher doses (60
and 100 mg/kg), a drastic increment in this param-
eter was evidenced on days 4 and 6 but it was not
correlated to the applied dose.
Heinz body formation on day 2 was strictly related
to the applied dose of up to 60 mg/kg. On days 4
and 6 it showed a dose-dependent behavior in the
range 0-20 mg/kg. In contrast, at high PHZ doses a
non-dose-dependent decrease ranging between 60
and 100 mg/kg in Heinz body count was observed
on days 4 and 6.

Histopathological Study
At 60 mg/kg PHZ, spleen weight increased on all
post-injection days (2, 4, 6, and 8), being highest
(near 6 fold) on day 4 (Table 1). Kidney weight
changes were smaller but temporarily in agreement
with those observed in the spleen. In the liver, the
highest weight gain was registered on day 6 although
an increment in this parameter was evidenced all
along the experimental period.
The histological examination of the spleen, liver,
and kidney batches from the control group showed
uniform cellularity (Fig. 2 A, B, C). PHZ-treated
mice revealed severe splenomegaly and marked
splenic erythroid hyperplasia on day 6 (Fig. 3 A).
Histologically, the enlarged spleen revealed conges-
tion with red pulp expansion. The red pulp showed
excessive erythrocytic congestion, abundant macro-
phages, megakaryocytes, erythrophagocytosis, and
erythroblastic islands (EI).
Erythroblastic islands were observed in the spleen,
liver, and kidney, this beingindicative of compensa-
tory erythropoietic activity (Fig. 3 A, B, C). The
evaluation of erythropoietic activity, expressed as
the number of erythroblastic islands per ten fields
on each tissue, was performed at 60 mg/kg of PHZ
(Table 2). On day 6, the spleen evidenced a higher

Figure 1. Time course and dose-dependent study
of the hemolytic action of Phenylhydrazine.
Hematological parameters of mice injected with 10
mg/Kg (+), 20 mg/Kg (▲), 60 mg/Kg (▼), and 100
mg/Kg (♦) of Phenylhydrazine on day 0 and 2.
Untreated mice (■) were injected with physiologi-
cal solution (days 0 and 2). Data are expressed as
mean ± SD (p <0.05), (n=3 for each time point and
for each group).
number of erythroblastic islands (++++) with respect to the liver (+++) and the kidney (++). Splenic erythropoiesis showed equal distribution of nodular and non-nodular erythroblastic islands in the red pulp area.

Figure 2. Morphology of the spleen, liver and kidney of control mice. Histological examination showed uniform cellularity in splenic (A), hepatic (B), and renal tissues (C). WP: white pulp; RP: red pulp; V: blood vessel. Hematoxilin-eosin x 5 (A); x 45 (B,C).

Figure 3. Erythropoietic re-population in the spleen, liver, and kidney of Phenylhydrazine-treated mice. Arrow: erythroblastic islands in the spleen (A), liver (B), and kidney (C) on day 6. Hematoxilin-eosin x 5 (A); x 45 (B,C).

Ultrastructural study
Scanning electronic microscopy examination of the spleen showed normal cellularity in the untreated group and significant changes in the splenic tissue of PHZ-treated mice (60 mg/kg). Tissue samples
were collected from control as well as PHZ-exposed mice on day 4. The typical reticular meshwork in the red pulp and discocyte cells were observed in untreated mice (Fig. 4 A). Sequential transformation of PHZ-induced hemolytic anemia as well as transformation of typical erythrocytes (I) into erythrocytes full of protuberances and transformation of Heinz bodies (II) into damaged erythrocytes by splenic lytic activity (III) could be all clearly observed (Fig. 4 B). Splenic erythrocytes containing both prominent Heinz bodies and some cells injured by “pitting” (Fig. 4 C) as well as severely damaged erythrocytes exhibiting a spherocyte–like morphology (end-stage) and non-deformable membrane (Fig. 4 D) could also be observed.

**Discussion**

A hematological and morphological analysis of the erythropoietic regenerative response following PHZ-induced acute hemolytic anemia in mice was conducted in the present research by means of time-course and dose-dependence studies. The hematological response observed, which was evaluated at a single dose of PHZ 60 mg/kg, was evidenced by erythropoiesis restoration manifested by a reticulocyte increment consistent both with hematocrit recovery and spleen hypertrophy. As observed in other species, the number of circulating reticulocytes coincided with the increase in MCV, thus suggesting that erythrocyte precursors become anucleated at a more differentiated stage of erythropoiesis (Criswell et al., 2000). On the other hand, the increase in MCH observed during the experimental period could be indicative of a certain degree of intravascular hemolysis (Criswell et al., 2000).

The decrease in red cell mass was accompanied by a marked peripherai leucocytosis, which could be attributed mainly to an increase in the number of lymphocytes. This mechanism, which is not as relevant as the oxidative damage in PHZ-induced anemia, could be indicative of an immunological process involved in PHZ-induced hemolysis.

Our results demonstrate that the erythropoietic regenerative response following PHZ-induced acute hemolytic anemia in mice is time- and dose-dependent. This results from the fact that i) HCT decreased on days 2 and 4 in a dose-dependent manner, returning to basal levels on day 8; and ii) PHZ only induced a marked reticulocytosis (day 4) at the highest doses tested (60-100 mg/kg).

In addition, red cell removal from peripheral blood proved to be concentration-dependent. Mice receiv-
ing a 60 mg/kg dose showed a marked increase in Heinz body-containing cells in peripheral blood on day 2, and a drastic decrease on day 4. In mice receiving higher PHZ doses (60 and 100 mg/kg), the Heinz body count on days 4 and 6 was indicative of an increased removal by sequestration of injured RBC. In contrast, Heinz body-containing cell clearance was lower in mice that received lower doses of PHZ. These cells thus remained longer in blood circulation. Our results also indicate two distinct hemolytic responses elicited by PHZ. At low concentrations, PHZ produced moderate sequestration whereas high concentrations induced premature splenic sequestration, the main mechanism of cell removal.

Ultrastructural studies were also carried out in order elucidate the process involved in cell removal in mice under our experimental conditions. The erythrocyte membrane alterations observed in the present study provide strong evidence of spleen erythrophagocytosis. On the other hand, the examination of SEM images allowed us to identify three different stages in the process. Protruded Heinz bodies were pitted by erythrophagocytic activity. Then, in a post-pitting phase, erythrocytic cells became severely damaged and finally, spherocyte-like erythrocytes could be observed. The presence of Heinz bodies impairs RBC deformability by altering intracellular fluidity leading, in turn, to increased entrapment in splenic sinusoids when blood is being filtered through the spleen (Hasegawa et al., 1993).

In the light of our results, splenic sequestration is the main mechanism by which anemia is produced.
under PHZ induction. In this respect, and in agreement with our data, previous studies demonstrated that extravascular hemolysis is the principal mechanism in PHZ-induced anemia (Jollow & McMillan, 2001; Latunde-Dada et al., 2006).

On the other hand, and although the role of the spleen in hematopoietic repopulation of anemic adult mice has been previously reported (Kozlov et al., 1980; Latunde-Dada et al., 2006), we carried out an histopathological study to evaluate the role of the spleen, liver, and kidney in the regenerative response. In agreement with previous studies (Kozlov et al., 1980; Rencricca et al., 1970; Hara & Ogawa, 1976), our analysis demonstrated that anemia is prevented mainly by compensatory erythrocytic spleen hyperplasy. Thus, the increased number of erythroblastic islands in splenic tissue and the HCT recovery observed in our research are coincident with erythropoietic repopulation. Such islands have been reported to be anatomic units consisting in a macrophage surrounded by a ring of maturing erythroblasts (Yokoyama et al., 2003). In addition, our histological examination revealed erythroblastic islands in liver tissue. Furthermore, it is well known that either in the absence of the spleen or when the erythropoietic demand is highly increased, mice liver can partially perform the erythropoietic function of the spleen (Ploemacher & Van Soest, 1977; Wolber et al., 2002).

Morphological changes in kidney, indicative of erythropoietic recovery, were also observed in the present study. The presence of EI in renal tissue under our experimental conditions led us to speculate about the possibility that the kidney is another site for extramedullary hematopoiesis in adult mice. However, renal erythropoiesis has not been reported to date and further studies are therefore necessary to clarify its possible involvement in acute anemia. Finally, and in view of the data collected from the present study, it can be concluded that the erythropoietic regenerative response in PHZ-induced hemolytic anemia in mice results from the coordinated action of extramedullary tissues, depending both on the time post-injection and the dose applied.

This study also shows that several mouse tissues may contribute to hematopoietic homeostasis in response to PHZ treatment.

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