TOXICOLOGICAL STUDIES OF ANEMIA BY P-METHYL-PHENYLHYDRAZINE : DRUG INDUCED PHAGOCYTOSIS OF P-METHYLPHENYLHYDRAZINE INJURED ERYTHROCYTES BY MACROPHAGES

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Summary......Hemolytic anemia was induced in dogs with repeated intravenous injections of p-methylphenylhydrazine (MPH), phenylhydrazine (PH) or nitrite for five days (10 mg/kg day each). Both erythrocyte number and hemoglobin concentration were decreased more by MPH than by PH or nitrite. Reticulocytes were also increased more by MPH than by PH or nitrite. Phagocytosis of MPH-injured erythrocytes by macrophages was observed in vitro. Erythrocyte number was decreased about 30 % by MPH upon repeated intraperitoneal administration of anti-macrophage serum compared to about 45 % effect without anti-macrophage serum. These results show that MPH-induced hemolytic anemia is caused by macrophagic phagocytosis of MPH-injured erythrocytes.

Hemolytic anemia is caused by sequestration of erythrocytes which are slightly injured by a drug. Phenylhydrazine (PH) has been used to induced hemolytic anemia or to artificially age erythrocytes in animals (Kudo 1953). Studies of erythrocyte sequestration have usually been made by microscopic observation, or by observing reticuloendothelial block which results from saturation of phagocytic cell induced by intravenous injection of colloidal carbon (Keene et al, 1965) or injured erythrocytes (Mollison 1962).

In the present study, we found that p-methylphenylhydrazine (MPH), the most specific monoamine oxidase inhibitor known (Arai 1960), was more potent than PH in causing hemolytic anemia in dogs. In order to analyze the mechanism of this MPH-induced hemolytic anemia, in interaction between macrophages and treated erythrocytes was observed in mice using a light microscope. The effect of reticuloendothelial block, caused by injection of macrophage antibodies, was also examined in MPH-induced hemolytic anemia.

MATERIAL AND METHODS

1) Erythrocytes and reticulocytes :
Blood was collected from male dogs weighing approximately 10 kg (N=5) or CBA
male mice weighing approximately 30 to 35 g (N=5). Erythrocytes in the blood were counted with a Toa Microcell-counter. Erythrocyte suspensions diluted 5,000-fold with saline was used as semiples. Oxyhemoglobin (O₂-Hb), methemoglobin (Met-Hb) and sulfhemoglobin (S-Hb) were measured spectrophotometrically with a Shimazu Spectrophotometer type MPS by Evelyn's method (Evelyn et al. 1938). Reticulocytes were observed microscopically according to Brencher's method and the count was expressed as number per 1,000 erythrocytes. For testing erythrocyte resistance to hemolysis by Parpart's procedure, salt solutions corresponding to NaCl concentrations of 0.85, 0.75, 0.65, 0.55, 0.45, 0.40, 0.35, 0.30 and 0.20 % (weight/weight) were used. Preparations were incubated for 30 min at room temp, and centrifuged at 2,000 r.p.m. for 5 min. Transmittance of the supernatant at 540 mμ was measured with a Shimazu Spectrophotometer type MPS.

2) Splicenectomized dogs:

Male dogs weighing approximately 10 kg were used. The abdominal wall was cut along the ventrimeson and all rami lienales outside the spleen were sectioned, after being tied off with gut, before removing the spleen. Sturations of tunica muscularis were conducted from the internal paries of the abdomen. All procedures were conducted under sterile condition.

3) Phagocytosis by macrophages:

Peritoneal exudates were obtained from 30-35 g male CBA mice 3-4 days after an intraperitoneal injection of 1 ml of glycoegen solution (0.2 mg/ml). These exudates were incubated for 1 hr in culture bottles with culture medium 199 containing 10 % pooled calf serum. Each culture bottle was shaken gently to put the dead cells, lymphocytes, and mast cells into a suspension which was then decanted off, leaving the macrophages. Fresh culture medium was then added to place the macrophages in suspension. The suspension of macrophages was then mixed with a suspension of erythrocytes which were either intact or pretreated by MPH, or PH, or NaNO₂ (0.125 mg/ml each). The mixture was incubated at 37° C in an air-tight culture bottle on the bottom of which was a microscope slide plate. After 30 min, those macrophages which settled onto the glass plate were stained with Giemusa's solution.

4) Preparation of anti-macrophage serum.

The antibodies were obtained from rabbits immunized with peritoneal macrophages. Peritoneal exudate, rich with macrophages, was obtained from 30-35 g male CBA mice 3-4 days after an intraperitoneal injection of 1.5 ml of 10 per cent proteose peptone. Peritoneal exudate cells were washed three times with ice-cold tissue culture medium 199 and incorporated with complete Freund's adjuvant. Two rabbits were each immunized with 6 x 10⁶ macrophages in 10 ml of Freund's emulsion containing Mycobacterium Tuberculosis (5 mg/ml) twice, at two week intervals. They were bled 4 weeks after the last injection. The serum obtained from these rabbits was pooled, heated at 56°C for 30 min, and stored at -20°C. CBA mice weighing 30 to 35 g were treated in three groups: one group had 2.5 ml anti-macrophage serum per day administered peritoneally.
HEMOLYTIC ANEMIA BY P-METHYLPHENYLHYDRAZINE

for five days; another group was peritoneally administered normal rabbit serum on the same schedule; the third group was not treated. After 3 days 15 mg per kg of MPH was intravenously administered each day for 3 days to all mice. After 4 days of MPH injection, blood samples were collected and erythrocytes were counted.

RESULTS

1) Effect of MPH, PH and NaNO₂ on hemolysis:

Figure 1 shows the effects of MPH, PH and NaNO₂ on hemolysis in dogs. The number of erythrocytes decreased as much as 60% after administration of MPH. Phenylhydrazine was not as potent as MPH in decreasing erythrocytes. The decrease in erythrocyte number by NaNO₂ was slight. Dogs treated with MPH restored their erythrocytes to 70% of normal in 18 days, while the restoration of erythrocytes in dogs treated with PH was very slow. The dose response curve of MPH effect on hemolysis is shown in figure 2.

2) Effects of MPH, PH and NaNO₂ on hemoglobin concentration in vivo, and on formation of reticulocytes.

Hemoglobin concentration was measured in blood of dogs treated with MPH, PH and NaNO₂ according to the dose schedule described in the METHODS section. While oxyhemoglobin decreased by 75% and 50% in dogs injected with MPH and PH respectively, NaNO₂ had a negligible effect. Neither methemoglobin nor sulfhemoglobin was

![Graph](image-url)

Fig. 1. Effects of MPH, PH and NaNO₂ on erythrocyte count. MPH, PH and NaNO₂ were injected intravenously in dogs at 10mg per kg per day on 1, 2, 3, 4 and 5, days. A : NaNO₂  B : PH  C : MPH

— 253 —
Fig. 2. Dose–response relationship of hemolysis to MPH in vivo. MPH was intravenously injected in dogs with each dose for 4 days. Each point represents mean ± S.E. from 5 experiments.

Fig. 3. Effects of MPH, PH and NaNO₂ on hemoglobin concentration in blood. Drugs were injected as described in Fig. 1. A: NaNO₂  B: PH  C: MPH
HEMOLYTIC ANEMIA BY P-METHYLPHENYLHYDRAZINE

detected in the blood of dogs treated with MPH, PH or NaNO₂. A significant increase
of reticulocytes was observed in each case after administration of these drugs. As shown
in Table 1, the reticulocytes formed in the blood of dogs treated with MPH, PH, NaNO₂
or saline were 108±11, 61±8, 23±6 and 2±2 per 1,000 erythrocytes respectively. After
administration of MPH was stopped, hemoglobin concentration remained almost the same,
whereas erythrocytes were restored. In contrast, both erythrocyte number and hemoglobin
concentration recovered slowly after cessation of PH or NaNO₂ administration. These resul-
ts suggest that abnormal reticulocytes may be produced by MPH in vivo (Fig. 1 and 3).

3) Effects of MPH, PH NaNO₂ and hydroxylamine on erythrocytes in vitro :

Effects of MPH (0.08 mM), PH (0.08 mM), NaNO₂ (0.18 mM) and hydroxylamine
(0.18 mM) on erythrocytes were examined in vitro using almost the same relative blood
volume doses of these drugs as those used in vivo. Oxyhemoglobin concentrations were
decreased to 64 % of control by MPH and to 38 % by PH. Both Met–Hb and S–Hb were
produced by these treatments. In contrast, most of the O₂–Hb was converted to only
Met–Hb by treatment with NaNO₂ or hydroxylamine. The number of erythrocytes was
not changed significantly by these drugs.

4) Changes in corpuscular fragility by MPH, PH and NaNO₂ :

Figure 4 shows changes in corpuscular fragility caused by MPH, PH and NaNO₂.
The measure of mean corpuscular fragility (MCF) is that concentration, referred to 0.9 %
NaCl, in which 50 % hemolysis occur. MCF after injection of MPH, PH, NaNO₂ or

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<th>Drugs</th>
<th>number of reticulocytes counted for 1,000 erythrocytes</th>
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<tr>
<td>control</td>
<td>2± 2 (3) ***</td>
</tr>
<tr>
<td>MPH</td>
<td>10 mg/kg</td>
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<tr>
<td>PH</td>
<td>10 mg/kg</td>
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<tr>
<td>NaN0₂</td>
<td>10 mg/kg</td>
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MPH, PH and NaN0₂ were intravenously injected in dogs (10 mg/kg for 3 days). The reticulocytes were stained with new methylene blue**
and observed microscopically.

* Mean value± standard error (number of experiments)
** 2,8-dimethyl-3,7-bis(ethylamino)-phenathionium chloride

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<tr>
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<th>O₂–Hb mg/dl</th>
<th>Met–Hb mg/dl</th>
<th>S–Hb mg/dl</th>
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<tbody>
<tr>
<td>control</td>
<td>7.30</td>
<td>0.12</td>
<td>0.00</td>
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<tr>
<td>0.08 mM MPH</td>
<td>4.66</td>
<td>1.23</td>
<td>1.29</td>
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<tr>
<td>0.08 mM PH</td>
<td>2.77</td>
<td>0.96</td>
<td>2.92</td>
</tr>
<tr>
<td>0.18 mM NaN0₂</td>
<td>0.00</td>
<td>7.43</td>
<td>0.00</td>
</tr>
<tr>
<td>0.18 mM Hydroxylamine</td>
<td>0.00</td>
<td>6.35</td>
<td>0.74</td>
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Each value represents the mean of 3 experiments.
saline was 0.60, 0.55, 0.50 and 0.45, respectively. Thus, MPH was the most potent in increasing MCF, followed by PH and NaNO₂, in that order.

5) Effects of MPH and PH on hemolysis in spleenectomized dogs:

Erythrocytes in the spleenectomized dogs were decreased by 47.6±4.8% and 21.1±3.5% of normal by MPH and PH, respectively. Thus, the decrease in erythrocyte number by these drugs in spleenectomized dogs was almost the same as in control animals injected with the same respective drugs. From these results, it seems reasonable to conclude that removal of the spleen has practically no influence on decrease of erythrocytes caused by MPH or PH.
Fig. 5. Photomicrographs of macrophages and erythrocytes treated with saline. a; MPH (0.08 mM), b; PH (0.08 mM), c; NaNO₂ (0.18 mM), d; Incubated at 37°C under airtight conditions for 30 min after mixing macrophages and erythrocyte suspensions. (x 400)
HEMOLYTIC ANEMIA BY P-METHYLPHENYLHYDRAZINE

6) Phagocytosis:

When macrophage suspension was mixed with an erythrocyte suspension pretreated with MPH, and the mixture was incubated for 30 min, active phagocytosis by the macrophages was observed (Fig. 5b). These photomicrographs clearly show capture of drug injured erythrocytes by pseudopods and the subsequent process of digestion in the macrophage cytoplasm. While erthrophagocytosis was observed after treatment with all three drugs, the greatest effect was definitely caused by MPH (Fig. 5b). Figure 5C shows similar action due to PH and Fig. 5d shows results of NaNO₂.

7) Effects of anti-macrophage serum on erythrophagocytosis:

To verify that the in vivo phagocytosis of erythrocytes injured by MPH was by macrophages, a reticuloendothelial block was caused. When mice (N=5) pretreated with anti-macrophage serum were treated with MPH, erythrocytes count was decreased by only 30% compared to the 45 to 50% decrease caused by MPH in mice treated with saline or normal rabbit serum. Thus macrophage activity was approximately halved by anti-macrophage serum.

DISCUSSION

It is a well known fact that aged erythrocytes are sequestered primarily in the spleen and liver. When the erythrocytes are coated with incomplete antibody (Jandl et al. 1957; Hughes–Jones et al. 1957) or treated with SH-reagents (Jacob et al. 1962) or complexed with metalloprotein (Jandl et al. 1957) or changed in shape (Harris et al. 1957), these erythrocytes are usually sequestered in the spleen. On the other hand, erythrocytes incubated under sterile conditions (Jandl et al. 1958), or treated with complement fixing antibodies (Mollison 1959), metallic cation (Jandl et al. 1957) or sodium arsenate (Harris et al. 1957) are sequestered in the liver. Richard proposed the mechanism of sequestration of erythrocytes (Richard 1965) as follows: 1) Sequestration in the spleen involves selective accumulation of damaged cells within the vascular space of Brilloth cords; 2) More severely injured cells may undergo intravascular hemolysis within the splenic red pulps; however, no evidence for the intravascular sequestration of injured cells is observed in the liver. The present experiment presented evidence that erythrocytes were decreased by the administration of MPH even in spleenectomized dogs, thus leading

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<th>Table 4. Effect of anti-macrophage serum on MPH hemolysis in mice.</th>
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<td>relative number of erythrocytes after MPH administration (%)</td>
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<tr>
<td>control: 55.4±6.5 (3) ※</td>
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<tr>
<td>NRS: 52.9±3.6 (3)</td>
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<td>AMS: 71.2±4.5 (3)</td>
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Anti-macrophage serum (AMS) and normal rabbit serum (NRS) were peritoneally injected for 6 days. MPH was intravenously injected for 3 days from 4th day after anti-macrophage serum administration.

* Mean value±standard error (number of experiments)
to the conclusion that MPH-damaged erythrocytes are probably destroyed in the liver. Phenylhydrazine also causes hemolytic anemia by the same mechanism as that caused by MPH, although it is less effective than MPH. \( \text{NaNO}_2 \) has slight hemolysis producing effect but does cause methemoglobinemia to a greater extent than PH or MPH. Although there is no report describing the source of macrophages which gather in the peritoneal cavity after peritoneal injection of stimulators, or whether they have same characteristics as other other macrophages, these experiments were conducted in vitro using macrophages collected from the peritoneal cavity.

In order to verify that macrophages phagocytize the MPH-damaged erythrocyte in vivo, macrophage antibodies were prepared and injected into mice treated with MPH. As shown in Table 4, the antibody protection afforded MPH-damaged erythrocytes from macrophages was only 20%. It has been reported that macrophages not killed by macrophage antibodies show marked cytological aberration from 6-48 hr after injection of antimacrophage serum (Utanue 1968). From these facts, we concluded that all macrophages did not come into contact with macrophage antibodies as a result of being bound to tissue in the spleen or liver.

Maruta reported that the specific discrimination of an erythrocytes state by a macrophage is accomplished by interaction between the erythrocyte surface and the macrophage surface, and that no other component such as a specific antibody is required (Maruta et al. 1971; 1973). It seems that macrophages can discriminate the surface structure of erythrocytes and will phagocytize only those whose surfaces have been changed by MPH.

It may be asked whether it is the MPH or its metabolites which affect the surface structure of erythrocytes. As demonstrated in the present paper, our results clearly indicate the MPH, itself, causes changes in the erythrocyte’s membrane in vitro. This was observed in phagocytosis by macrophages. However, the possibility still remains that the erythrocyte membrane may be changed by metabolites of MPH in vivo, since MPH is easily metabolized as are other monoamine oxidase inhibitors (Axelrod 1954).

REFERENCES


— 258 —
HEMOLYTIC ANEMIA BY P-METHYLPHENYLHYDRAZINE


