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Induction of Anemia in Splenectomized and Nonsplenectomized Rats (40743)

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Prior studies from this laboratory have shown that the degree and severity of an induced anemia depends on the size and location of the substituent on substituted phenylhydrazine (1). Other compounds such as nitrosobenzene and phenylhydroxylamine are also inducers of anemia (2). As a result of these studies we were interested in the ability of the spleen to remove and destroy these partially damaged red blood cells and in contrasting these studies with splenectomized animals. Many investigators have been interested in the effect of the spleen on red blood cell survival studies (3–6). These studies have used various methods of inducing a mild hemolytic anemia, including phenylhydrazine (3) and acetylphenylhydrazine (5). Both of these compounds were administered in a single dose, the former by addition to an aliquot of blood obtained directly from the animal and the latter by a single subcutaneous dose. Both iron-59 and chromium-51 (radioactive markers) were employed to follow the sequestering of the red blood cells (3–6). The spleen and to a lesser extent the liver have been established as the major organs of red blood cell sequestration in the rat (4). Other parts of the reticuloendothelial system have little normal involvement in red cell sequestration (7).

In this study we examined the effect of daily subcutaneous drug injections. The time course for the induction of this anemia was followed with both splenectomized and nonsplenectomized rats. These studies were done with a variety of substituted phenylhydrazines, nitrosobenzene, and phenylhydroxylamine.

Materials and methods. The animals used in these experiments were female Sprague–Dawley rats with an average weight of 210 g. One-half of the animals were splenectomized under light ether anesthesia. The other half of the rats were given sham operations. All of the animals were allowed to recover for 10 days after surgery on *ad libitum* diet. Whole blood was obtained by tail clipping and collected in heparinized capillary tubes for microhematocrit determinations. Two microhematocrit tubes were obtained from each animal at every bleeding. The rats were put in groups of four to six. Half of the animals were splenectomized and the other half were subjected to sham operations. The drug was administered subcutaneously at a dosage of 0 to 100 μmole per rat per day for approximately 2 weeks. The rats were sacrificed at the end of this period, and their organs were removed for study.

Results. The effect of several substituted phenylhydrazines on hemolytic activity in splenectomized and nonsplenectomized rats is shown in Table I. As shown previously (1) unsubstituted phenylhydrazine was the most potent inducer of anemia. The larger the *ortho* substituent, the lesser was the degree of induced anemia. The same trend was found in rabbits (1).

The time course for the induction of this anemia is shown in Fig. 1. The hematocrits of splenectomized rats decreased more than those of nonsplenectomized animals. With a continual maintenance dose of the drug the hematocrits of the splenectomized rats remained at a lower level than those of the nonsplenectomized rats. However, the hematocrit eventually returned to normal levels in the nonsplenectomized rats even when the same drug dosage was maintained. Concomitant with this increase in red blood cell production was a dramatic increase in the size of the spleen. Normal spleens from 200-g rats weighed 563 ± 116

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TABLE I. EFFECT OF SELECTED DRUGS ON THE INDUCTION OF ANEMIA IN RATS

Drug [a]	Spleen	Hematocrit				
		Day 0	Day 5	Day 8	Day 12	Day 15
1. Control	−	47.25	42.5	42.5	43	40
	+	47.5	41.5	40	44	46
2. Phenylhydrazine	−	46.7	29.5	20.5	22	23.5
	+	43.2	29.2	31.7	33.5	38
3. 2-Fluorophenylhydrazine	−	47	31.7	27	29.5	32.7
	+	46	30.2	31.5	39.7	40.5
4. 2-Chlorophenylhydrazine	−	45	31	28.7	34.5	36.7
	+	41.2	30.7	32.2	38.5	38.2
5. 2-Bromophenylhydrazine	−	39.5	33	32.2	34	38
	+	44.5	34.2	33.2	40.7	42
6. 2-Iodophenylhydrazine	−	44.5	37	33.5	37	37.5
	+	43.5	35.5	34	40.7	42.7
7. 2,6-Dichlorophenylhydrazine	−	45.5	41.5	36.7	38.5	38
	+	44.5	39.2	36	37.5	40
8. Nitrosobenzene	−	45.2	38.5	36.2	44	41.7
	+	44.2	36	40.5	43	40.7
9. Phenylhydroxylamine	−	44	35.5	36	37.5	39
	+	45.7	34	37	47	49
10. Hydrazinobenzoic acid	−	40.2	42.2	46	43	34
	+	47.2	43.5	39.5	40.2	38

[a] All of the substituted phenylhydrazines (compounds 2–7, and 10) were administered subcutaneously at a dose of 20 μmole/rat/day. Nitrosobenzene and phenylhydroxylamine were injected at the dosage of 30 and 15 μmole/rat/day respectively.

mg. The spleen removed from animals which had recovered from a severe anemia weighed 3 g or more.

In Table II we have summarized the effect of dihydroxymaleic acid on induction of anemia. Autooxidation of dihydroxymaleic acid generates the superoxide anion (8). The superoxide anion has been implicated as the mediator of hemolytic agents on blood (8). If the superoxide anion were responsible for inducing an anemia, then at the doses tested we should have seen a dramatic effect of this drug. However, even at 100 μmole/rat/day the change in hematocrit was not significant.

Discussion. The spleen has been established as the major site of red blood cell sequestration in rats (3–6). In the case of splenectomized animals the liver quite adequately takes over some of this splenic function. There is abundant evidence that the spleen is highly selective toward erythrocytes with various qualitative alterations, such as spherocytic shape change (9) or red cell inclusion bodies (5).

The mechanism of oxidative denaturation

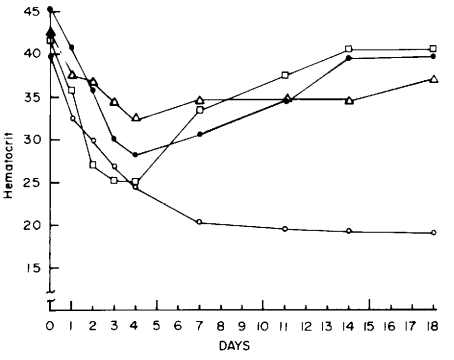

FIG. 1. Time course for the induction of anemia by phenylhydrazine and acetylphenylhydrazine. □, 20 μmole phenylhydrazine/nonsplenectomized rat/day; ○, 20 μmole phenylhydrazine/splenectomized rat/day; ●, 10 μmole acetylphenylhydrazine/nonsplenectomized rat/day; △, 10 μmole acetylphenylhydrazine/splenectomized rat/day.

TABLE II. INDUCTION OF ANEMIA BY DIHYDROXYMALEIC ACID

Dose (μmol/rat/day)	Spleen	Hematocrit					
		Day 0[a]	Day 4	Day 7	Day 11	Day 14	Day 18
10	−	44[b]	43.5	43.2	42	38.5	43.2
	+	46	43.7	42.5	37	40	43.7
20	−	42.7	42.7	43.2	40.7	36.5	41.7
	+	44.7	43.5	43.5	41.2	39.5	44.2
50	−	43	41.7	42.2	41.2	38.5	41.7
	+	43	40.5	44	39.7	39.5	44.2
100	−	45.5	41.2	42	40	40	41
	+	44.5	41	43.5	41.7	40.5	42.5

[a] Number of days after first drug injection.
[b] The hematocrit was determined on at least two different animals in each group. Each determination was made in duplicate.

of hemoglobin by the phenylhydrazines (1, 10), nitrosobenzene (2), and phenylhydroxylamine (2) has similarities. All of these compounds generate H_2O_2 which can attack either the hemoglobin molecule, the red cell membrane, or both. References (1) and (2) have a detailed description of the proposed mechanism of action of phenylhydrazine and phenylhydroxylamine, respectively.

These data presented here show that rats with their spleen become anemic more rapidly than rats without their spleen. In addition, even though a daily dose of the drug was administered to the rats with spleens, they were able to recover from the drug-induced anemia. Splenectomized animals on the other hand were not able to regenerate sufficient red blood cells to overcome the effect of the drug. These splenectomized rats whose hematocrits remained low throughout the study deposited large amounts of iron in the kidney and liver. The localization and effect of this iron deposition are part of another study.

The observed splenic enlargement is similar to that observed by Azen and Schilling (5). This enlargement is probably related to proliferation of the reticuloendothelial system and erythrogenic cells in addition to trapped cells. Giblett *et al.* (11) used methylcellulose to cause an increase in spleen size. When the spleen was enlarged it became much more efficient at sequestering red blood cells and would result in an anemia in the rat. We noticed no apparent increase in the size of the liver in splenectomized animals even though it is the primary organ of red blood cell sequestration in the absence of a spleen. However, a slight change in the weight of the liver would be more difficult to detect than changes in weights of the spleens.

Summary. Daily subcutaneous injections of phenylhydrazine and related compounds resulted in the induction of an anemia in splenectomized and nonsplenectomized rats. The severity of the anemia induced depended on the nature of the ligand or the functional group on the drug. Dihydroxymaleic acid, a potent free radical generator, was ineffective at inducing any anemia. The animals that had not been splenectomized became anemic more rapidly than the splenectomized rats. This effect was attributed to the sequestering of damaged red blood cells by the spleen. The rats with spleens gradually recovered from anemia despite continual daily injections of the test compounds. During this recovery phase the spleen became enlarged. Splenectomized animals did not recover from the anemia until the daily injections were terminated.

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