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INFLUENCE OF PHENYLHYDRAZINE ON THE ANTIOXIDANT SYSTEM OF THE ERYTHROCYTES AND THE LIVER IN MICE

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Abstract—Phenylhydrazine when injected into the mouse acts in two phases. At an early stage it provokes directly in the erythrocytes as well as in the liver a decrease in the concentration of acidosoluble nonprotein thiol groups. Indirectly it causes a later and more lasting increase in glutathione *S*-transferase and glucose-6-phosphate dehydrogenase activities in the erythrocytes, due mostly to a renewal of the population of these cells, and in glucose-6-phosphate dehydrogenase activity in the liver due to a decrease in hepatic glutathione. Thus, modifications in the erythrocytes are mainly due first to a strong oxidation of hemoglobin and afterwards to the renewal of the population. In the liver, modifications are mostly induced by consumption of reduced glutathione and secondary activation of the pentose cycle. It is suggested that there is a similarity between this chemical aggression and an inflammatory process.

Phenylhydrazine ($\phi NHNH_2$)* is a powerful hemolytic agent. Oxidation of hemoglobin to methemoglobin followed by precipitation of the latter in the red blood cells is one of the most spectacular manifestations of its action. Several authors have shown that free radicals and peroxides appear in the tissue after administration of $\phi NHNH_2$ [1–7]. It is the purpose of this paper to follow the tissular modifications as a function of time of the antiradical and antioxidant defense system after administration of a single dose of $\phi NHNH_2$, in order to try to explain the modifications of resistance observed in the animals after injection. The substance provokes a strong hemolysis with formation of Heinz bodies in most of the erythrocytes, followed by an intense reticulocytes crisis. Treatment with $\phi NHNH_2$ of mice allows the study of the reactions of the organism after damage caused by increased production of free radicals. Indeed, a number of substances are known, such as alloxane, bleomycin, CCl_4 and paraquat [8], which during transformation in the organism, react partly or wholly by producing free radicals. Furthermore $\phi NHNH_2$ causes in the mouse changes in the resistance to certain damage such as ionizing radiation which could, at least partly, find an explanation in the modifications of the antiradical and antioxidant defense system in the target organ [9]. Thus we find two different phases in the reactions following injection of $\phi NHNH_2$: an early phase with an important hemolysis and the weakening of the animal occurring immediately after injection of the drug and a later repairing phase with modifications in the animal's resistance.

We investigated erythrocytes, one of the principal targets of the action of $\phi NHNH_2$, and the liver, being the central organ of metabolism and of detoxification of many foreign substances.

MATERIALS AND METHODS

Animals and preparation of samples. Male C_{57} Bl mice weighing about 25 g each were injected i.p. with phenylhydrazine hydrochloride (Merck, 120 mg/kg). The $\phi NHNH_2$ was dissolved immediately before use in 0.9% NaCl to give a concentration of 12 mg/ml. Control animals received an equal volume of 0.9% NaCl. Heparinized blood was taken by heart puncture and the livers were removed 1, 2, 5, 8, 11 and 14 days after injection of $\phi NHNH_2$ under ether anaesthesia. The erythrocytes were separated from the plasma by centrifugation, washed with 1.19% KCl and then recentrifuged. To 0.2 ml of the pellet 4 ml of water were added. The livers were frozen immediately on dry ice and homogenized in 9 vol. of 0.02 M EDTA at pH 6.6. The homogenates as well as the erythrocytes were centrifuged at 4°, 26,000 *g* for 20 min. Measurements were carried out on the supernatant.

Methods and measurements. Proteins in the liver were measured by a method of Oyama and Eagle [10], and hemoglobin after transformation to cyanohemoglobin [11]. The following enzymes were assayed owing to the important role they play in antiradical and antioxidant defense. Glutathione peroxidase (G-POD) was measured by the method of Paglia and Valentine [12]; glutathione *S*-transferase (GST) by a method of Habig *et al.* (13); glutathione reductase (G-Red) by a method described by Bergmeyer *et al.* [14]; catalase (Cat) by the method

* Abbreviations used: $\phi NHNH_2$, phenylhydrazine hydrochloride; Hb, hemoglobin; −SH, nonprotein thiol groups; G-POD, glutathione peroxidase; GST, glutathione *S*-transferase; G-Red, glutathione reductase; Cat, catalase; SOD, superoxide dismutase; G6PDH, glucose-6-phosphate dehydrogenase.

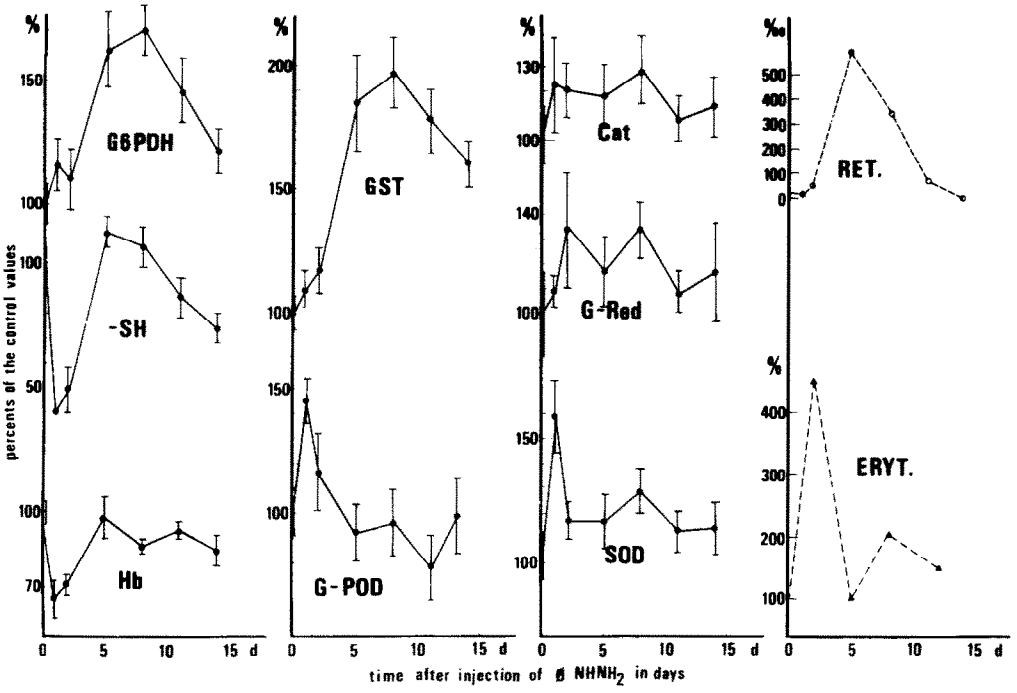

Fig. 1. Modifications of the Hb concentration (8.6 ± 0.4 mg/ml hemolysate in the control animals), of the —SH concentration (39 ± 2 μg/ml hemolysate in the control animals) and of different enzymatic activities in the erythrocytes as a function of time in days after injection of $\phi NHNH_2$ (120 mg/kg). In the control animals the activities per g soluble Hb were: G6PDH: 15 ± 1 U; G-POD: 182 ± 40 U; G-Red: 12 ± 2 U; GST: 2860 ± 200 U; Cat: 110 ± 13 k; SOD: 1900 ± 100 U. Erythropoietin was measured in total blood. Reticulocytes were counted on a thousand erythrocytes. Values are expressed in % of control animal values (=100% at time 0).

of Aebi [15]; superoxide dismutase (SOD) by the method of Rigo and Rotilio [16]; glucose-6-phosphate dehydrogenase (G6PDH) by a method of Richterich [11] and the acidosoluble nonprotein thiol groups (—SH) (practically equivalent to reduced glutathione) by the method of Sedlack and Lindsay [17].

Furthermore we measured the aldolase in the liver by a method described by Bergmeyer and Bernt [18] and erythropoietin in the plasma by the method of Moccia *et al.* [19]. Enzymatic activities were calculated in units (μM of substance transformed per min) per g soluble proteins in the supernatant for the liver or per g soluble Hb for erythrocytes. The concentration of —SH was calculated in μM per ml supernatant, and erythropoietin in cpm per ml of erythrocytes. The results are means of 10 measurements. They were submitted to an analysis of variance. In order to simplify the presentation, results in the figures are not given in absolute values but in percentage of the values obtained with the controls (=100% at time 0).

RESULTS

Figure 1 shows the sudden decrease of soluble Hb in the erythrocytes as well as the reticulocytes crisis with a maximum on the fifth day. The concentration of the —SH decreased rapidly while GST and G6PDH activities increased considerably. Early peaks appeared for SOD and G-POD. In the liver (Fig. 2) the concentration of —SH decreased as early as in the erythrocytes, while G6PDH activity increased spectacularly between the fifth and the eighth day. The other enzymatic activities measured were not significantly affected in comparison to the control values. Erythropoietin measured in the plasma was also increased early, $\phi NHNH_2$ being a classical agent to increase its concentration.

DISCUSSION

According to the postulate of Michaelis [20], $\phi NHNH_2$ passes through intermediate radical stages. These radicals can react with O_2 forming reactive O_2^- and $OH^\cdot$.

$$\phi NHNH_2 \xrightarrow[-H^+]{-e^-} \phi NH{-}NH^\cdot \xrightarrow[-H^+]{-e^-} \phi N{=}NH \xrightarrow[-H^+]{-e^-} \ldots$$

$$\downarrow$$

$$O_2 \rightarrow O_2^- \rightarrow H_2O_2 \rightarrow HO^\cdot$$

The existence of these by-products of oxygen after injection of $\phi NHNH_2$ has been demonstrated [1–7]. The decrease of reduced glutathione which seems to play a central role in antiradical defense, in the erythrocytes as well as in the liver, is an indication of important intracellular oxidation occurring immediately after injection of $\phi NHNH_2$. The modifications of the enzymatic activities we observed in the erythrocytes are mainly due to the destruction of hemoglobin and the ensuing renewal of the cell population, revealed also by the reticulocytic crisis (Fig. 1). The progressive and substantial increase in G6PDH activity in the erythrocytes is a sign of this

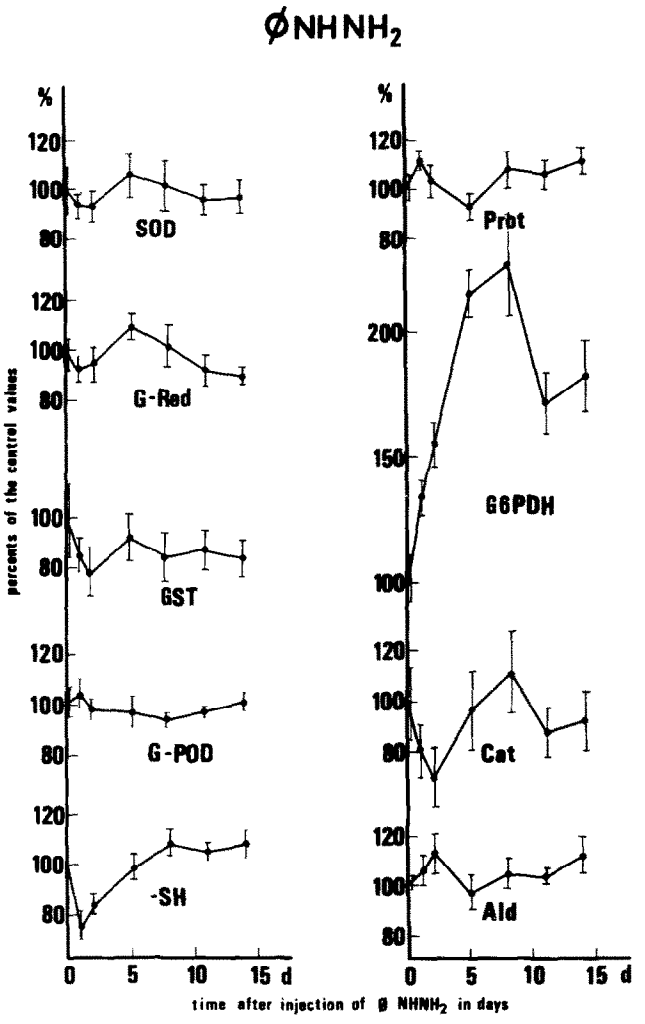

Fig. 2. Modification of soluble protein concentration and of different enzymatic activities in the liver as a function of time in days after injection of $\phi NHNH_2$ (120 mg/kg). Values are expressed in % of control animal values (=100% at time 0). Measurements were carried out on liver supernatants. In the supernatants of the control animals, the protein concentration was 10.6 ± 0.6 mg/ml; —SH: 251 ± 15 μg/ml; G6PDH: 7.2 ± 0.6 U; aldolase: 118 ± 4 U; G-POD: 979 ± 25 U; G-Red: 59 ± 2 U; GST: 2000 ± 230 U; Cat: 366 ± 80 k; SOD: 4310 ± 390 U per g protein respectively.

rejuvenation of the cell population rather than a change in the enzymatic activity [21]. Since the curves of the increase in erythrocytic GST activity and that of G6PDH are superposable, they are probably due to the same cause. The transitory increase in SOD and G-POD activities in the erythrocytes is only a relative one due to an important decrease of the Hb concentration to which these activities are correlated. In the liver we find an early decrease of the reduced glutathione concentration followed by an induced increase of the G6PDH activity. The large increase in G6PDH activity in the liver is a sign of increased activity of the pentose phosphate cycle and therefore of the reducing capacity of liver tissues, while the glycolysis does not seem to be modified (normal aldolase activity). According to Eggleton and Krebs [22] oxidation of glutathione increases the flux through the phosphate pentose pathway allowing the organism to regenerate the oxidized glutathione.

The analogy between the above results and those described by Sinet *et al.* [23] of the action of diethyldithiocarbamate *in vitro* on the erythrocytes shows that for these two substances of totally different chemical structure and pharmacological activity there is a resemblance between some of their actions due to the production of identical, non-specific free radicals [7].

Furthermore, injection of $\phi NHNH_2$ provokes in the organism non-specific modifications comparable to those of acute inflammation: hyperleukocytosis [24], decrease of zincemia, increase of zinc in the liver and of plasma haptoglobins [25]. Thus, these reactions provoked in the organism by injection of $\phi NHNH_2$ correspond to those induced by a biostimulant [26]; hence the proliferation of hemopoietic stem cells [24] and the increase in radioresistance by $\phi NHNH_2$ [9, 24]. They could be induced by an endogenous leukocytic mediator [25].

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