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EFFECTS OF SULFHYDRYL REAGENTS ON THE ANTI-SICKLING ACTIVITY OF SOME MEMBRANE-INTERACTING COMPOUNDS

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Under deoxygenated conditions (P_{O_2} = 0 mmHg), sulfhydryl reagents such as *N*-ethylmaleimide and iodoacetamide had no effect on sickling, but they did inhibit the anti-sickling activity of chlorpromazine. At the same concentration, these sulfhydryl reagents inhibited the cup formation of chlorpromazine in an oxygenated state (P_{O_2} = 143 mmHg). This supports our previous finding that the anti-sickling effect of membrane-interacting compounds is related to their ability to form a cup-shaped red cell.

Introduction

It is known that certain types of membrane-interacting drugs such as chlorpromazine, produce a 'cup' shaped erythrocyte [1]. These investigators provided an explanation based upon the interaction of these drugs with the lipid bilayer of the erythrocyte membrane. Haest et al. have reported that a sulfhydryl-interacting reagent such as *N*-ethylmaleimide inhibits the cup formation caused by these drugs due to a loss of erythrocyte deformability [2]. Sato has found that other sulfhydryl reagents such as iodoacetamide also inhibit the cup formation induced by chlorpromazine (Sato, unpublished data). Allen and Cadman [3] reported that iodoacetamide did not change the deformability of erythrocytes. Since sulfhydryl groups are an earmark of protein, these results suggest that protein may also be involved in the drug-erythrocyte membrane interaction.

We have reported that chlorpromazine and other membrane-interacting drugs [4–7] have in vitro anti-sickling activity. However, the mechanism of the anti-sickling effect of these drugs is still unknown. In order to study the relationship between cup formation and anti-sickling, as well as to investigate the role of protein, we have undertaken this study. Using the ability of *N*-ethylmaleimide and iodoacetamide to inhibit cup formation, we have tested whether or not these compounds also inhibit the anti-sickling effect of membrane-interacting drugs. The data suggest a correlation between cup formation and the anti-sickling effect.

Materials and Methods

Blood. Heparinized venous blood was obtained from an adult sickle-cell anemia subject (27-years old). Normal blood was drawn from a healthy donor with normal hemoglobin.

Drugs. Chlorpromazine was a gift from Smith, Kline and French (Philadelphia, PA). All other chemicals were purchased from Sigma Chemical Company.

Incubation of red blood cells with drugs. A medium used in this experiment contains 110 mM NaCl, 5 mM KCl, 20 mM sodium phosphate buffer, 27 mM sodium bicarbonate, 1 mM $MgCl_2$, 5 mM glucose, and 0.5% human serum albumin.

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The medium was equilibrated before use with a gas mixture of 95% air/5% CO_2 at 37°C in a rotating flask [7] and the pH was adjusted to 7.4. Red blood cells were suspended in the medium to make the hematocrit value 1%. The suspension was pretreated for 30 min in the presence or absence of sulfhydryl agents. The cells were washed-centrifuged twice with the medium and resuspended (same hematocrit value).

The suspension was then incubated for 30 min in the presence or absence of chlorpromazine. Finally, the suspension was equilibrated at several oxygen partial pressures (with the gas mixture of 95% air/5% CO_2 and 95% N_2/5% CO_2) for another 30 min. The oxygen partial pressure was determined by a Clark electrode.

Morphological studies. An aliquote of the suspension was taken out and fixed in 0.9% glutaraldehyde solution (in 0.1 M phosphate buffer; pH 7.4) which had been equilibrated with the same gas mixture as that used for cell suspension.

Measurement of sickling. The degree of sickling was measured under a light microscope by the method reported previously [7].

Oxygen affinity of sickle cells. $P_{O_2}^{50}$ values were determined by the TCS Hemox Analyzer (Southampton, PA) using the method described previously [7]. The standard deviation was calculated from four measurements.

Scanning electron microscopy. The shape of the erythrocyte was observed under a scanning electron microscope, type JSM-35, the Japan Electron Optic Co. (Tokyo, Japan), after fixation with 0.9% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and a coating of carbon and gold.

Statistical method. The Student's *t*-test was used for statistical analysis of differences between control and experimental groups.

Results

Anti-sickling effect of sulfhydryl reagents

On studying the effect of sulfhydryl reagents on sickling, we have observed that the drugs themselves have an anti-sickling effect which begins to become significant above a P_{O_2} of about 10 mmHg (Fig. 1). Fig. 2 shows the dose-response relationship of these reagents on sickling. At a P_{O_2} of 0, they have no effect, but at a P_{O_2} of 30 mmHg the sickling can be suppressed from a control level of 90% +, down to 17% (see also Fig. 3). Some sulfhydryl reagents are known to have an anti-sickling effect by virtue of a direct interaction with the hemoglobin molecule [8–10]. Therefore, we have

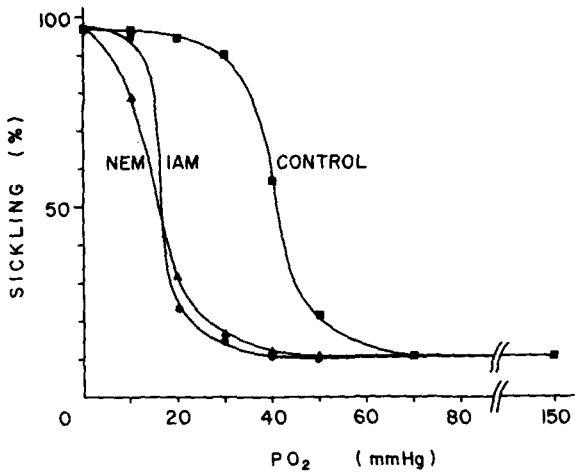

Fig. 1. Effect of P_{O_2} on anti-sickling activity of 9.5 mM *N*-eythylmaleimide (NEM) (▲) and 9.5 mM iodoacetamide (IAM) (●). Experimental conditions: 110 mM NaCl, 5 mM KCl, 20 mM sodium phosphate buffer (pH 7.4), 27 mM sodium bicarbonate, 5 mM glucose, 0.5% serum albumin, 1 mM $MgCl_2$. Deoxygenation was done with a gas mixture of 95% air/5% CO_2 and 95% N_2/5% CO_2. Temperature 37 ± 0.1°C.

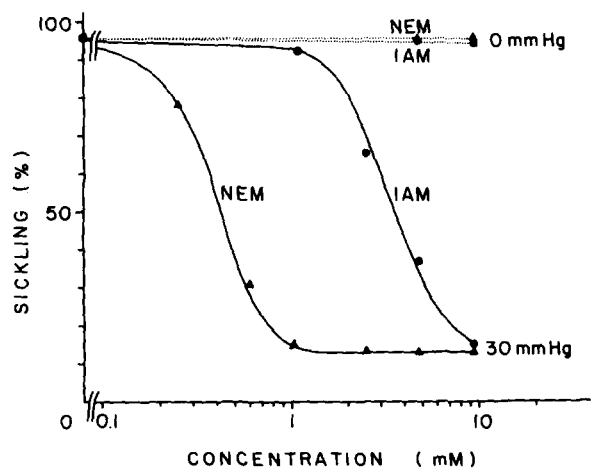

Fig. 2. Anti-sickling effect of 9.5 mM *N*-ethylmaleimide (NEM) (▲) and 9.5 mM iodoacetamide (IAM) (●) at P_{O_2} of 30 mmHg (solid lines) and 0 mmHg (dotted lines). Experimental conditions are the same as those in Fig. 1.

examined whether or not these reagents interact with hemoglobin to change the oxygen affinity.

The $P_{O_2}^{50}$ value from this sickle cell patient was 36.5 ± 1.0 mmHg. We have observed that the $P_{O_2}^{50}$ value decreased (i.e., the oxygen affinity of hemoglobin increased) to 10.5 ± 1.5 ($P < 0.001$) and 22.0 ± 1.3 ($P < 0.001$) in the presence of *N*-ethylmaleimide (5 mM) and iodoacetamide (5 mM), respectively.

Since these drugs do not show any anti-sickling

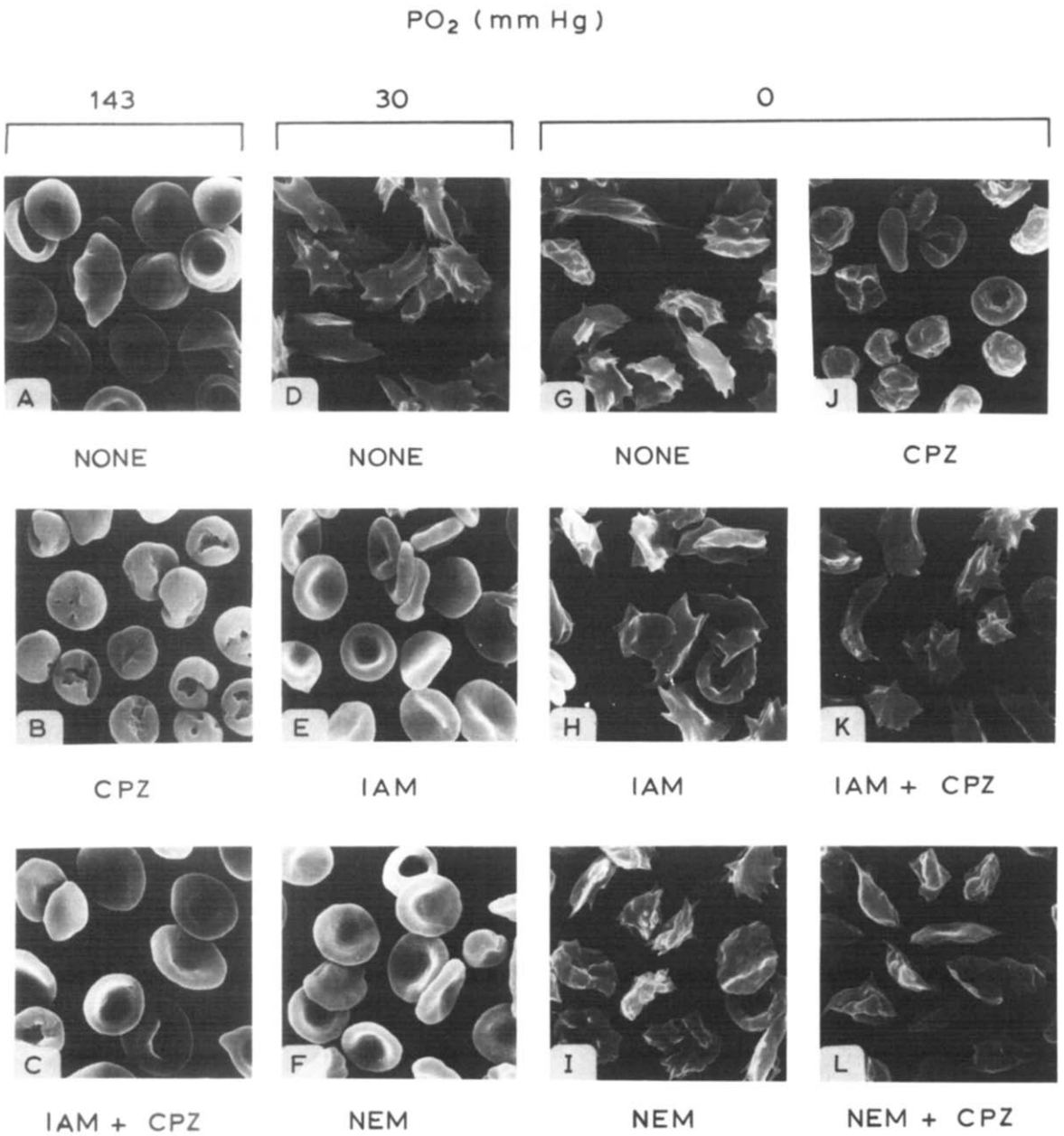

Fig. 3. Scanning electron micrographs of sickle cells. Drug concentrations are: (A), (D) and (G): no drug: (C), (E), (H) and (K): pre-treatment with 9.5 mM iodoacetamine (IAM); (F), (I) and (L): pre-treatment with 9.5 mM *N*-ethylmaleimide (NEM); and (B), (C), (J), (K) and (L): 140 μM chlorpromazine (CPZ). Oxygen partial pressures (P_{O_2}) are: (A), (B) and (C): 143 mmHg; (D), (E) and (F): 30 mmHg; and (G)–(L): 0 mmHg. For details of incubation conditions see Methods.

TABLE I

EFFECT OF PRE-TREATMENT BY SULFHYDRYL REAGENTS ON THE ANTI-SICKLING ACTIVITY OF CHLORPROMAZINE

The number in the table indicates the sickling percentages in the blood suspension incubated 30 min at zero oxygen partial pressure. Prior to the deoxygenation, cells were pre-treated for 30 min with sulfhydryl reagents, wash-centrifuged, and incubated with chlorpromazine for another 30 min.

Pre-treatment	Chlorpromazine		
	0	140 μM	200 μM
None	95.0	51.0	39.4
9.5 mM *N*-ethylmaleimide	96.0	88.5	76.0
9.5 mM iodoacetamide	93.5	87.2	75.3

activity by themselves at $P_{O_2} = 0$, we have used $P_{O_2} = 0$ in the following experiments to simplify the interpretation of data.

Inhibition of anti-sickling activity of chloropromazine by sulfhydryl reagents

As we reported earlier, chlorpromazine can inhibit sickling in vitro even at $P_{O_2} = 0$. Table I demonstrates how sulfhydryl reagents inhibited the anti-sickling effect of chlorpromazine.

Scanning electromicroscopy

Fig. 3 shows the effect of these drugs on the red cell shape as observed by a scanning electron microscope. Photos (A), (B) and (C) show the effects of the drugs at the oxygenated state. Photo (C) shows that the pretreatment by 9.5 mM iodoacetamide inhibits the cup formation by chlorpromazine. The pre-treatment itself did not change the cell shape (photo not shown). The pre-treatment by 9.5 mM *N*-ethylmaleimide had the same effect (photo not shown). Photos (E) and (F) show the anti-sickling effects of iodoacetamide and *N*-ethylmaleimide at $P_{O_2} = 30$ mmHg. Photos (H) and (I) show that these sulfhydryl reagents do not affect sickling at $P_{O_2} = 0$. Photos (K) and (L) demonstrate that the anti-sickling effect of chlorpromazine (as shown by photo (J)) is inhibited by the pre-treatment with these reagents.

Similar phenomena of cup-formation by chlorpromazine and the inhibition by the pre-treatment with these sulfhydryl reagents were also observed in normal red blood cells (data not shown).

Discussion

We have previously demonstrated that many membrane-interacting compounds inhibit sickling without altering the oxygen affinity of sickle hemoglobin [4–6]. A common property of these compounds is their ability to cause the erythrocyte shape to change to a spherocyte ('cup' form). As we reported in this paper, the anti-sickling effect of chlorpromazine is inhibited when cup formation is blocked with sulfhydryl reagents. This confirms the close relationship between cup formation and the anti-sickling effect.

Why do cup-formed cells not sickle? Asakura et al. [4] has postulated that an increase in volume may occur accompanying the cup formation, which lowers the mean corpuscular hemoglobin concentration, thereby delaying the polymerization of deoxy-hemoglobin S. We still could not rule out other possible mechanisms. Shibata et al. [11] proposed that the erythrocyte membrane might have some enhancing effect on the gelation of deoxy-hemoglobin S in the cell. Goldberg et al. [12] argued against this result by reporting that the enhancing effect is only three times, and thus is not significant. The inhibition of cup formation by sulfhydryl reagents suggests that proteins are also playing an important role in the drug-membrane interactions. Further studies are necessary to elucidate the mechanism of cup formation by membrane-interacting drugs as well as to investigate the mechanism of anti-sickling effect of these drugs.

As far as the pharmacological efficacy of membrane-interacting compounds is concerned, we must solve certain problems. For example, a negative aspect in the use of these compounds is that the erythrocyte membrane is more rigid in the cup shaped erythrocyte than in a normal erythrocyte [13]. These cells may be sequestered by the spleen as abnormal cells. However, there is some evidence that these compounds improve the general health condition of the sickle-cell patients. Lewis et al. [14] have tried a phenothiazine compound for sickle-cell patients with some improvement. Cabannes [15] has reported that cetiedil also has a beneficial effect on sickle-cell patients. Although

an exact blood level of cetiedil has not been established, it is obvious that the blood level of this compound never reaches the point where we observe a significant in vitro anti-sickling effect (150–200 μM). Then, what kind of mechanism could account for the observed efficacy? A possibility is that we study the in vitro anti-sickling effect at $P_{O_2} = 0$, while in the circulation, such an extreme condition seldom occurs. Therefore, a smaller concentration of the drug may be sufficient to cause an appreciable anti-sickling effect. Another mechanism of action may be the prevention of potassium loss from sickle cells by the drug [16,17]. The loss of potassium causes the dehydration of cells and may lead to an increased possibility of sickling as well as the formation of irreversibly sickled cells.

Further studies are needed to elucidate the mechanism of in vivo anti-sickling activity of membrane-interacting drugs as well as a determination of their minimum beneficial blood level.

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