Vol. 80, No. 4, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1978 Pages 879-885

EFFECT OF DICHLOROMETHANE ON SICKLE CELLS. AN *in vitro* WATER PROTON MAGNETIC RESONANCE RELAXATION STUDY

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Received November 14,1977

SUMMARY: To examine the manner in which dichloromethane inhibits sickling, sickle blood was subjected to both prevention and reversal schemes over a range of CH_2Cl_2 vapor pressures. Following CH_2Cl_2-treatment, the rotating frame spin lattice relaxation time ($T_{1\rho}$) of water protons in deoxygenated packed sickle cells was measured, cell types in a deoxygenated fixed sample were counted, and the extent of hemolysis determined. At CH_2Cl_2 vapor pressures above 200 mm, the NMR relaxation rate decreased sharply, the extent of hemolysis increased, the fraction of sickled cells and other abnormal erythrocytes decreased, and the fraction of biconcave discs increased. Apparently CH_2Cl_2 is absorbed by the cell membrane and preferentially lyses sickled cells and other abnormal cells. Part of the decrease in NMR relaxation rate with increased CH_2Cl_2 pressure is due to a larger fraction of discs, but an additional factor probably arises from CH_2Cl_2 inhibition of hemoglobin S gelation.

INTRODUCTION: When erythrocytes from patients homozygous for sickle cell disease are deoxygenated, the hemoglobin S in the erythroctyes aggregates into long tactoids forming a gel with accompanying deformation of the cell into a characteristic sickle shape. The difference between hemoglobin S (HbS) and normal adult hemoglobin A (HbA) is simply the substitution of a valine residue for a glutamic acid residue in the sixth position in the β chain of HbS (1). This single substitution permits tactoid formation of HbS molecules apparently *via* hydrophobic interactions (2) with concommitant change in cell morphology. The debilitating symptoms of the disease arise from the mechanical properties of sickled cells, rather than a lack of HbS function. The sickled cells are more rigid than normal erythrocytes making passage through microcapillaries difficult (3).

There has been much public and research interest in the development

0006-291X/78/0804-0879$01.00/0

of agents which inhibit HbS gelation and sickle cell formation (4-9). Although a variety of compounds have been demonstrated to inhibit sickling *in vitro*, many of the treatments or their side effects make *in vivo* application impractical. Promising studies have indicated that dichloromethane vapor inhibits HbS gelation in solution (8) and sickling in intact cells (9). The value of CH_2Cl_2 as a potential drug is its demonstrated low toxicity as an anesthetic and as an industrial solvent (10). Dichloromethane has been shown to reduce the number of sickled cells in a deoxygenated smear using either a prevention or reversal scheme (9). The antisickling behavior was attributed to interaction of CH_2Cl_2 with HbS. Although four unique CH_2Cl_2-HbS binding sites have been predicted (8), the actual mechanism by which gelation is inhibited is unknown. The most effective inhibition was attributed to reversible CH_2Cl_2 interaction with hydrophobic regions of HbS, thus interfering with the aggregation sites in deoxy HbS.

In our current investigation of the antisickling behavior of CH_2Cl_2 at the molecular level, we have examined the rotating frame spin-lattice relaxation rate $1/T_{1\rho}$ for water protons in packed sickle cells. As we have previously demonstrated, the value of $1/T_{1\rho}$ is sensitive to the extent of HbS tactoid formation (11,12). Slow motions, especially those with a rotational correlation time τ_r greater than 10^{-6} sec, affect the value of $1/T_{1\rho}$. Our $T_{1\rho}$ measurements of water protons in erythrocytes show that some of the water molecules experience the lower mobility of HbS in the tactoids of deoxygenated sickle cells and exhibit a correlation time $\tau_r \approx 10^{-5}$ sec (11,12), while water molecules associated with HbS monomers in solution possess a correlation time $\tau_r \approx 10^{-7}$ sec.

The NMR experiments led to microscopic cell counts and measurements of the extent of hemolysis in sickle cell samples subjected to different amounts of CH_2Cl_2.

MATERIALS AND METHODS: Heparinized whole blood was obtained by venipuncture from patients homozygous for sickle cell disease. Cells were oxygenated by passing humidified air through a freshly drawn sample for ∿ 10 minutes. Cells were deoxygenated by passing a humidified stream of 5% CO_2/95% N_2 through a blood sample for ∿ 10 minutes.

The experimental procedures for exposure of the sickle cells to dichloromethane are similar to those previously reported (9). Septum bottles (100 ml) were flushed for 5 minutes with a mixture of nitrogen gas and dichloromethane vapor. The vapor pressure of CH_2Cl_2 in the gas mixture was varied by changing the temperature of the flask containing CH_2Cl_2 through which nitrogen was bubbled to produce the gas mixture. The amount of CH_2Cl_2 in the gas mixture was determined by gas chromatography. For reversal schemes, 0.5 ml of deoxygenated blood (20% hematocrit) was placed in a flushed septum bottle, the bottle was reflushed with the CH_2Cl_2/N_2 mixture for an additional 30 seconds and then allowed to incubate for 60 minutes. A control sample, i.e., with zero CH_2Cl_2 vapor pressure, was prepared in the same manner using pure nitrogen. For prevention schemes, the above procedure was followed with flasks containing oxygenated sickle blood. The blood was then allowed to deoxygenate for 60 minutes in the presence of CH_2Cl_2.

Most of the blood was transferred under anoxious conditions into 5 mm NMR tubes and the cells were packed by centrifugation at ∿ 1000 g for 10 minutes. A small aliquot of treated blood was transferred to a hematocrit tube, the stroma was centrifuged down, and the supernatant was tested spectrophotometrically by cyanomethemoglobin assay for hemoglobin content. The extent of hemolysis was quantitated by this method. The percent hemolysis reported is the ratio of hemoglobin in the supernatant to that found in a lysed cellular suspension.

A portion of the CH_2Cl_2-treated blood was fixed with 4% glutaraldehyde and microscope slides prepared. Cells were counted from microscopic

observation in three categories: (a) sickle cells, (b) normal biconcave discs, and (c) abnormal cells fitting neither of the other two categories; most abnormal cells were pinched cloverleaf forms.

NMR rotating frame spin-lattice relaxation rates for water protons were measured using the spin-locking technique (13) on the spectrometers in this lab described previously (11, 14). All $T_{1\rho}$ measurements were made at a single spin-locking frequency of 0.7 KHz and a Larmor frequency of 44.4 MHz.

RESULTS AND DISCUSSION: We have subjected sickle blood to both prevention and reversal schemes using dichloromethane over a range of vapor pressures. The water proton spin-lattice relaxation time in the rotating frame for packed erythrocytes was measured immediately following CH_2Cl_2-treatment; the results are recorded in Fig. 1. Cell counts determined microscopically for fixed cells and the amount of hemolysis for each sample of CH_2Cl_2-treated sickle blood are also shown in Fig. 1.

It is evident in Fig. 1 that the reversal and prevention schemes yield similar results. With the reversal experiment a gradual change is seen in each of the curves up to about 200 mm CH_2Cl_2 pressure, then each curve sharply increases in slope. At this point, the percentage of sickled forms and abnormal cells decreases, and the percentage of discs rises, but hemolysis also sharply increases. At 260 mm CH_2Cl_2, hemolysis increases to an intolerable 25%, while the fraction of sickled cells has dropped only from 60 to 45%. Dichloromethane in sufficient amounts to inhibit sickling is apparently capable of lysing erythrocytes.

We should note that the important observation of hemolysis might have gone unnoticed in this study, as it did in previous studies, were it not necessary to pack the cells in NMR tubes. The occurrence of lysis was obvious from visual inspection of the hemoglobin in the supernatant. The simple experimental procedure of centrifuging to test

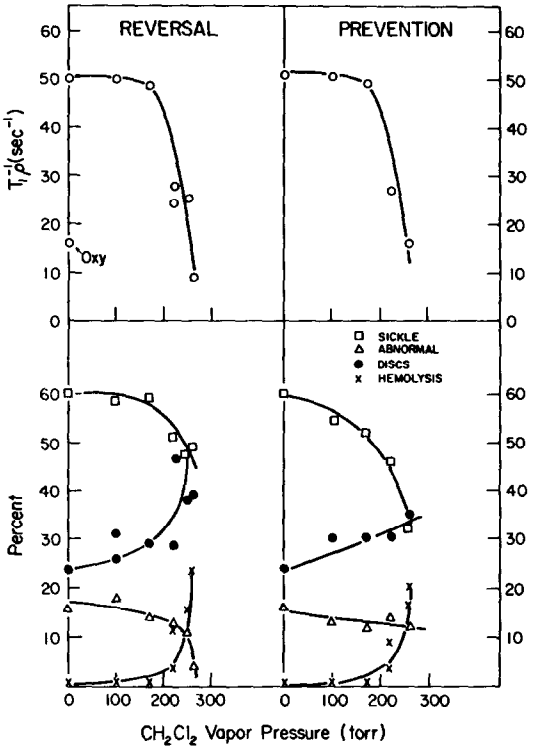

FIGURE 1: Reversal and prevention of sickling by dichloromethane vapor pressure. The upper curves show the rotating frame spin-lattice relaxation time of water protons in deoxygenated packed sickle cells subjected to a range of CH_2Cl_2 pressures. The lower graphs show the observed percentages of cell types in fixed deoxygenated smears along with the hemolysis resulting from treatment with CH_2Cl_2. Deformed cells that could not be clearly identified as either discs or sickle cells were counted as abnormal. As the drug pressure is raised to inhibit sickling, hemolysis becomes an increasing problem.

for hemolysis may be useful in other hematological studies as well.

The results for the sickling prevention scheme presented in Fig. 1 are similar, but not identical, to those for the reversal scheme. The trends are in the same direction, but the sharp changes above 200 mm CH_2Cl_2 pressures for the abnormal cell counts and extent of hemolysis are not observed in the prevention experiment as they are in the reversal studies. The fraction of sickle cells decreases more impressively with the prevention scheme from about 60% to 30%. However, the amount of hemolysis is nearly equivalent for both schemes, and the NMR relaxation data are similar.

The NMR $T_{1\rho}$ relaxation data reveal little inhibition of HbS tactoid formation up to CH_2Cl_2 vapor pressures of 200 mm since the $T_{1\rho}$ relaxation rates remain the same as for untreated deoxy packed cells. For higher pressures of CH_2Cl_2, the relaxation rates are decreased.

The conclusion derived from the results of Fig. 1 is that CH_2Cl_2 is absorbed by the cell membrane and, if a sufficiently large amount is absorbed, the cell lyses. It also appears that the membranes of sickle cells and abnormal cells are preferentially lysed since the cell counts for the remaining unlysed cells yield an increasing fraction of normal erythrocytes and a decreasing fraction of sickle and other abnormal cells with increasing CH_2Cl_2 pressure. Part of the decrease in the observed $T_{1\rho}$ relaxation rate with CH_2Cl_2 pressure is probably due to the smaller fraction of sickle cells in the packed erythrocyte samples. The relaxation rate decrease is sufficiently large, however, the possibility remains that some of the CH_2Cl_2 enters the cell and inhibits tactoid formation. Nevertheless, the large amount of hemolysis that occurs at CH_2Cl_2 pressures which may inhibit sickling leads to the conclusion that CH_2Cl_2 treatment cannot be recommended for an *in vivo* study for antisickling therapy.

Acknowledgements: We gratefully acknowledge Dr. Wayne Settle for the use of his private laboratory in determining the CH_2Cl_2 vapor pressures, and Mr. Edward Martin for his aid in preparing the samples. This work was supported by research grants AM 19047 from the National Institutes of Health and PCM-18156 from the National Science Foundation, and by grant RR-00892-01A1 from the Division of Research Resources of the National Institutes of Health.

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