ANTI-SICKLING ACTIVITY OF NITROSOUREAS

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SUMMARY - Incubation at physiological conditions of erythrocytes from a person bearing the sickle-cell trait with 1-methyl-1-nitrosourea, 1,3-bis-(2-chloroethyl)-1-nitrosourea, or 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea prevented sickling of the cells upon subsequent treatment of the cells with sodium metabisulfite. In these experiments these compounds were more effective than potassium cyanate as inhibitors of sickling. It is assumed that the inhibition results from the carbamoylation of the hemoglobin S by the isocyanic acid or the isocyanates derived from the nitrosoureas.

The effectiveness of cyanate in preventing the sickling of red blood cells has aroused much interest, and Cerami et al. (1) have recently reviewed experimental and clinical studies with this agent. The evidence indicates that the anti-sickling activity derives from the carbamoylation of the terminal amino groups of the β-chain of hemoglobin S.

Studies in this laboratory and elsewhere have shown that 1,3-disubstituted-1-nitrosoureas decompose at physiological conditions to generate isocyanates (2, 3), which can react with the ϵ-amino groups of lysine moieties of proteins to yield substituted carbamoyl derivatives (4, 5). We also have unpublished evidence that the isocyanates generated from such nitrosoureas can react with the terminal amino groups of peptides. Therefore, we speculated that these nitrosoureas might serve indirectly as inhibitors of the sickling phenomenon. We also expected 1-substituted-1-nitrosoureas to serve indirectly as inhibitors, since they decompose at physiological conditions to yield isocyanic acid, which is the

active molecule derived from cyanate. Since these nitrosoureas are uncharged, they might diffuse easily into red cells, where they would give rise to isocyanates or isocyanic acid in situ in proximity to the hemoglobin. We have experimental evidence (6) that the substituents on N-1 and N-3 influence the solubility and the stability of the nitrosoureas and that the substituent on N-3 influences the reactivity of the isocyanate that is generated from the nitrosourea. There is also evidence (7) that the steric properties of the alkyl group of alkyl isocyanates can convey specificity for reaction with specific sites on proteins. Therefore, it seemed that nitrosoureas potentially might have some advantages over cyanate.

These facts and thoughts led us to undertake experiments to determine if nitrosoureas would in fact prevent sickling. The results of the initial experiments are reported here.

METHODS - Heparinized blood from a person with the sickle-cell trait was refrigerated until used in the experiments. Experiments 1 and 2 were performed 48-72 hours after the blood was drawn, and experiment 3 was performed approximately 1 week after it was drawn. The procedures for the three experiments were the same except that in experiments 1 and 2 the slides were scored 30 minutes after their preparation, and in experiment 3 they were scored 1 hour after their preparation.

The blood was centrifuged, and the sedimented cells were washed 3 times with 5 volumes of Dulbecco's phosphate-buffered saline (8) and then suspended in approximately 4.5 volumes of this saline. Air was bubbled through this suspension for 3 minutes to oxygenate the cells. To portions of the suspension were added appropriate volumes of solutions of the test compounds[1] to yield the desired concentrations, and the mixtures were incubated in a water bath at 37°C for the indicated periods of time. KCNO was dissolved in phosphate-buffered saline; MNU, in dimethyl sulfoxide; BCNU,

[1] Potassium cyanate, KCNO; 1-methyl-1-nitrosourea, MNU; 1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU; 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, CCNU.

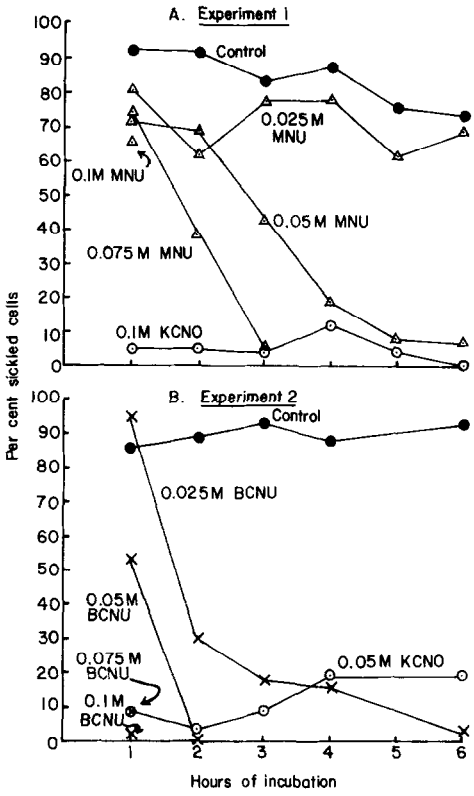

Figure 1 - Effectiveness of various concentrations of MNU(A) and BCNU(B) for various periods of incubation upon the in vitro sickling of cells.

in absolute ethyl alcohol or in dimethyl sulfoxide; and CCNU, in dimethyl sulfoxide. Appropriate volumes of absolute alcohol or of dimethyl sulfoxide were added to the control samples or to the samples containing KCNO, so that the compositions of the solvents in all samples within an experiment would be the same. At selected times during the incubation, portions of the samples were removed and mixed with an equal volume of a 1% solution of sodium metabisulfite in phosphate-buffered saline, and wet slides were prepared. After 30 minutes (experiments 1 and 2) or 1 hour (experiment 3) the slides were scored for normal cells and sickled cells. The results are presented in the charts.

RESULTS - The curves of experiment 1 (Figure 1A) show that the effective-

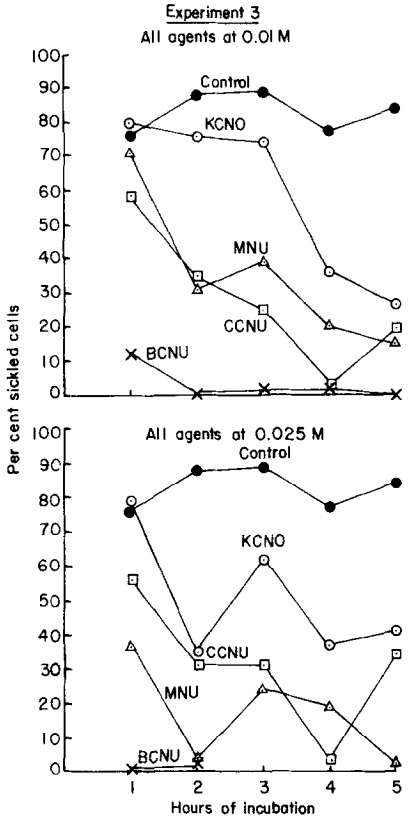

Figure 2 - Effectiveness of KCNO, MNU, CCNU, and BCNU at concentrations of 0.01 M and 0.025 M upon the in vitro sickling of cells.

ness of MNU as an anti-sickling agent is concentration and time dependent. This is not surprising, since time is required for the MNU to decompose to yield isocyanic acid, which would then react similarly to cyanate. At a concentration of 0.1 M, MNU inhibited sickling to some extent after incubation for 1 hour, but by 2 hours the agent at this concentration caused lysis of the cells. After an incubation period of 3 hours, MNU at a concentration of 0.075 M was as effective as 0.1 M KCNO, but by 4 hours MNU caused lysis. After incubation for 5 or 6 hours 0.05 M MNU was essentially as effective as 0.1 M KCNO, and cell lysis did not occur.

Experiment 2 (Figure 1B) showed that BCNU was considerably more effective than MNU and that sickling was completely prevented without lysis by 0.05 M BCNU upon incubation for 2 hours. At concentrations of 0.075 M

and 0.1 M, BCNU inhibited sickling after incubation for 1 hour but caused lysis upon incubation for 2 hours or longer.

In experiment 3 (Figure 2) KCNO, MNU, BCNU, and CCNU were compared at equal concentrations (0.01 M and 0.025 M). All of the nitrosoureas were more effective than KCNO, and BCNU was considerably more effective than MNU. It appears that BCNU was also more effective than CCNU, but a direct comparison in this experiment is complicated by the fact that some of the CCNU came out of solution even at the lower concentration. At a concentration of 0.01 M for incubation periods of 2-5 hours BCNU almost completely prevented sickling, but at 0.025 M it caused cytolysis upon incubation for 3 hours or longer.

A possible explanation for the greater effectiveness of MNU and of BCNU in experiment 3 in comparison to experiments 1 and 2 is that the cells had been stored for a considerably longer time when experiment 3 was performed.

In these three experiments slides were also made of the incubation mixtures without the prior addition of sodium metabisulfite. After 30-60 minutes the cells on the control and KCNO slides were crenated to a large extent, but the cells treated with MNU, BCNU, and CCNU under non-lysing conditions retained essentially normal appearances. The cells treated with KCNO plus sodium metabisulfite that did not sickle had irregular, abnormal appearances, while the cells treated with the nitrosoureas plus sodium metabisulfite that did not sickle had essentially normal appearances.

CONCLUSIONS - Although these preliminary experiments leave many questions unanswered, including the feasibility of using these agents clinically, they do show certain things.

1. Isocyanic acid generated from a 1-substituted-1-nitrosourea can prevent sickling similarly to exogenous cyanate.

2. The effectiveness of 1,3-disubstituted-1-nitrosoureas indicates that the generated organic isocyanates can prevent sickling similarly to

cyanate. This suggests that structural similarity to carbon dioxide, such as that of cyanate (1), may not be essential for the prevention of sickling. It even appears that the organic isocyanates are more active than isocyanic acid.

3. The different degrees of effectiveness of BCNU and CCNU indicates that the group on N-3 of the nitrosourea, and hence on the generated isocyanate, is important in determining the anti-sickling effectiveness of the agent. This points to the possibility that more effective compounds might be found among compounds having other substituents on N-3.

We plan to test other 1-substituted- and 1,3-disubstituted-1-nitrosoureas as inhibitors of sickling in vitro and to study the reactions of compounds of these types with hemoglobins.

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