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N-Acetylcysteine and Clotrimazole Inhibit Sickle Erythrocyte Dehydration Induced by 1-Chloro-2,4-Dinitrobenzene

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Clotrimazole, a specific inhibitor of the Ca^{2+} activated potassium (Gardos) channel, and the antioxidant *N*-acetylcysteine were found to inhibit the in vitro formation of high-density sickle cells induced by treatment with 1-chloro-2,4-dinitrobenzene (CDNB). The CDNB induced leakage of K^+ can be inhibited by treatment of SS erythrocytes with 20 mM *N*-acetylcysteine. We conclude that the effect of *N*-acetylcysteine in preventing K^+ efflux and formation of high-density sickle cells is related to its ability to protect the Gardos channel from oxidative damage caused by diminished levels of reduced glutathione. This effect is due to the ability of *N*-acetylcysteine to maintain an appropriate level of reduced glutathione and its direct antioxidant activity. Am. J. Hematol. 62:19–24, 1999.

Key words: sickle cell disease; gardos channel; dense cells

INTRODUCTION

Homozygous sickle cell patients' (SS) red blood cells (RBCs) have abnormally high levels of oxygen radicals [1,2] and the most dense portion of sickle cells have diminished levels of glutathione (GSH) for effective protection against oxidative stress [3]. The combination of these pathological conditions is thought to be responsible for oxidation of thiol groups in certain target proteins. Potential targets for oxidative damage includes the systems which control water and ion content in the sickle cells. The water content of the high-density SS erythrocytes is low causing the mean corpuscular hemoglobin concentration to be as high as 50 g/dl [4,5].

High-density SS erythrocytes are primarily (60–85%) irreversibly sickled cells (ISCs) that retain a sickled shape in well-oxygenated blood. ISCs can represent up to 45% of the circulating RBCs in SS blood and they are poorly deformable, of short life span, correlated to hemolysis, and have lower levels of fetal hemoglobin than do reversibly sickled cells (RSCs) [6]. This population of dense cells appears to block the narrow lumen of vessels [7] which contributes to vaso-oclusion. High concentration of hemoglobin S (HbS) results in acceleration of its polymerization and increased sickling of the erythrocytes [8].

It is established that two membrane transport pathways are critical to potassium and water loss in sickle cells. One of the pathways is volume and pH sensitive, and described as the K^+-Cl^- cotransport system, which uses the energy of the K^+ electrochemical gradient for efflux of K^+ and Cl^- and subsequent extrusion of intracellular water from the cells [9–11]. This system can be effectively inhibited by [(dihydroindenyl)oxy] alkanoic acid (DIOA) and its derivatives [12]. Another transport system responsible for K^+ and water loss from erythrocytes is the Gardos channel [13], which was shown to be involved in SS RBC dehydration [14]. This channel is activated by intracellular increase of Ca^{2+} and can be

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blocked by several agents: charybdotoxin [15], nifedipine [16], and clotrimazole [17].

Our recent work has demonstrated that 1-chloro-2,4-dinitrobenzene (CDNB), which rapidly lowers GSH levels within erythrocytes by forming an irreversible adduct 2,4-dinitrophenol-S-glutathione, cause a large conversion of low-density SS erythrocytes to dense cells [18]. We have also demonstrated that *N*-acetylcysteine (NAC) blocks the in vitro formation of dense cells upon oxy/deoxygenation cycling of light-density SS RBCs, and inhibits the formation of irreversibly sickled cells [18]. This effect of NAC is due to its combined antioxidant activity and the ability to increase intracellular GSH levels [3]. NAC is itself an antioxidant and is converted intracellularly to L-cysteine, which is a precursor of GSH.

In the current study, we demonstrate that CDNB induced formation of dense cells is accompanied by K^+ leakage, and that both dense cell formation and K^+ leakage are reduced by addition of NAC to CDNB-treated cells. We also demonstrated that formation of dense cells by CDNB can be blocked by inhibition of the Gardos channel with clotrimazole. In contrast, DIOA was found to be ineffective in inhibition of CDNB stimulated dense cell formation.

MATERIALS AND METHODS

Density Separation of SS RBCs

Light-density SS RBCs (LDSS) were prepared as described previously [18,19]. After washing the cells were transferred into the incubation buffer containing 20 mM HEPES, 130 mM $NaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 1 mM glucose, 2 mM Na_2 HPO_4, 2 mM $CaCl_2$, 1 mM adenosine, 0.5% BSA and 100 U/ml penicillin G, 100 μg/ml steptomycin, 0.25 μg/ml ampotericin B (pH 7.4). For all experiments LDSS were resuspended to 50% suspension. After incubation under varying conditions of CDNB, NAC, DIOA, or clotrimazole, the cells were vortexed and 1.5 ml of suspension was placed over discontinuous Percoll gradients in 12 ml tubes, consisting of 45%, 50%, 55%, 60% and 65% Percoll (Pharmacia, Sweden) in 18% Reno M-60 (Squibb Diagnostics, Princeton, NJ), 20 mM HEPES, 10 mM $MgCl_2$, 10 mM glucose (pH 7.4). The cells were centrifuged at 907 g for 45 min at 4°C. After centrifugation the tubes were photographed and the cells from the upper three layers (45%, 50%, and 55%) carefully separated from the bottom two layers (60% and 65%). Cells were washed twice in buffer containing 10 mM Na_2 HPO_4 150 mM NaCl (pH 7.6), fixed in 1% glutaraldehyde and counted by using the Cell Dyn (Sequoia-Turner, Mountain View, CA). Percent of the RBCs in 60% and 65% Percoll was determined, versus the total number of RBCs, and presented as percent of dense cells.

The Effect of Various Reagents on CDNB Induced Formation of Dense Cells

NAC: A 50% suspension of LDSS (5 ml) was treated with 20 mM NAC (Sigma Chemical Co., St. Louis, MO) for 1 h at 4°C and then incubated in the presence of 1 mM CDNB for various times as described in the Results section of the text. *Clotrimazole*: Clotrimazole (Sigma) was dissolved in DMSO and was added to 50% LDSS (5 ml) alone or in combination with 1 mM CDNB. The final concentration of clotrimazole was 10 μM. *DIOA*: DIOA (RBI, Natic, MO) was dissolved in 95% ethanol and was added to 50% LDSS (5 ml) alone, in combination with 1 mM CDNB or 10 μM clotrimazole or both. Final concentration of DIOA was 1 mM. *CDNB*: CDNB (Sigma) was dissolved in 95% ethanol and was added to 50% LDSS (5 ml) in the presence of various drugs and incubated for times described in the Results section of the text. All incubations were carried out at 4°C.

Measurement of Intracellular GSH

The intracellular GSH was determined as described by Gibson et al [18].

Measurement of Extracellular K^+ Concentration

Potassium leak was determined by measurement of extracellular K^+ using a potassium specific electrode (Orion Research, Inc., Beverly, MA). LDSS were isolated and quickly washed in the incubation buffer with no KCl and 140 mM NaCl instead of 130 mM NaCl. The 50% suspension of LDSS (20 ml) was treated with 20 mM NAC, 20 mM NAC plus 1mM CDNB, 1 mM CDNB or were not treated with any drugs. Voltage was measured by immersing the K^+ electrode directly into the suspension until the signal was stabilized. Measurements were conducted at 15, 30, 45, 60, and 300 min of incubation. After each measurement the electrode was rinsed with distilled water and shaken dry. For each experiment a separate calibration curve was prepared by plotting millivolt values on the linear axis and the standard K^+ concentrations on the logarithmic axis. By using these calibration curves the K^+ concentration was determined.

Statistical Analysis

Using one-way and two-way analysis of variance (ANOVA), we first tested for significant differences among the means observed. Once the difference was found to be significant, multiple comparisons by using Fisher's least significant difference procedure and Dunnett's test (for comparing different treatments with each other and control, respectively) were performed. The *P* values were derived through these test procedures.

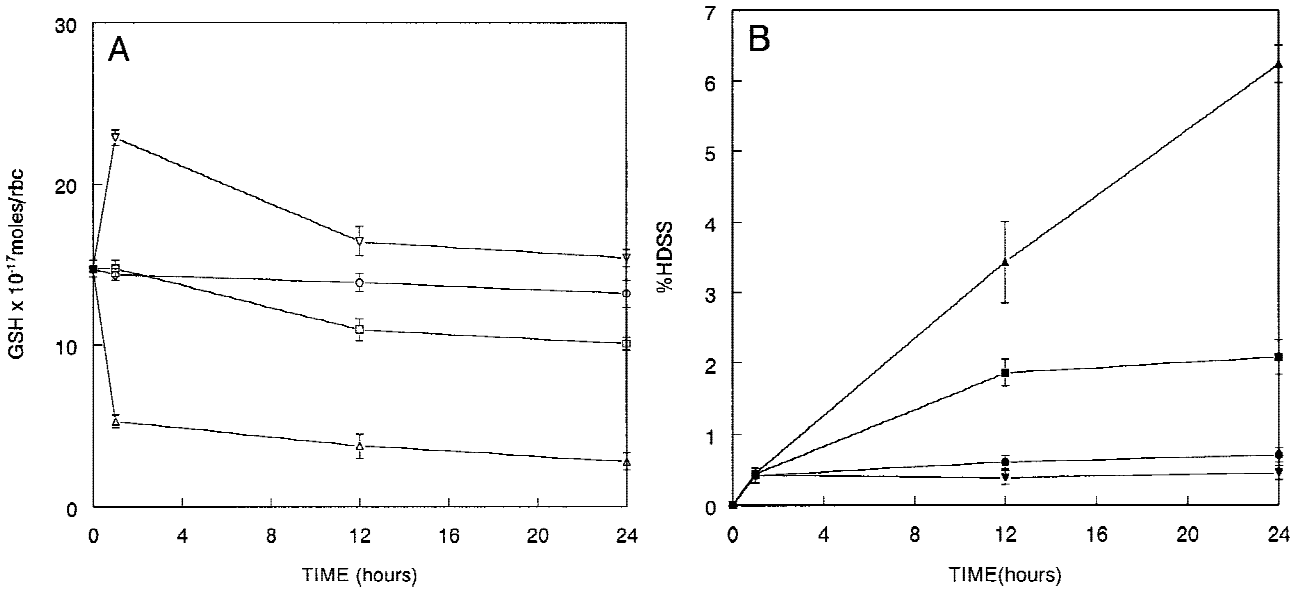

Fig. 1. Determination of GSH and % HDSS after incubation with CDNB and NAC. LDSS erythrocytes were suspended in incubation buffer and maintained at 4°C for varying times. A: GSH was determined on samples in incubation buffer alone (-○-), incubation buffer plus 20 mM NAC. (-▽-), incubation buffer plus 1 mM CDNB (-△-) and incubation buffer plus 20 mM NAC and 1 mM CDNB (-□-). B: % HDSS was determined at the same time points for the same samples in incubation buffer (-●-), plus CDNB (-▲-), plus NAC (-▼-), and plus NAC and CDNB (-■-). Data is presented as mean ± standard error of three independent experiments. Values of GSH and % HDSS were significantly different ($P < 0.05$) at 1 h, 12 h, and 24 h (except values for % HDSS at 1 h) when comparing samples receiving 1 mM CDNB with the rest of the samples. Note that diminishment of GSH correlates with formation of dense cells.

RESULTS

Effect of NAC and CDNB on the Formation of Dense Cells and Level of Intracellular GSH

Addition of NAC (20 mM) to LDSS erythrocytes, incubated at 4°C, causes a 50% increase in GSH levels after 1 h, which returns close to control levels by 24 h of incubation (Fig. 1A). CDNB (1 mM) results in a 65% decrease in GSH after 1 h of incubation and 80% after 24 h. As Figure 1A indicates this dramatic decrease in GSH was significantly inhibited if the LDSS were pretreated with NAC for 1 h before addition of CDNB. In the same experiments the percentage of high-density (SS) erythrocytes (those found in the 60% and 65% Percoll layers) increased constantly at 1 h, 12 h, and 24 h of CDNB treatment (Fig. 1B). CDNB induced cellular dehydration was effectively reduced by NAC. NAC reduced the CDNB induced formation of dense cells from control LDSS by 65% at 24 h. Therefore, by forming an irreversible adduct, 2,4-dinitropheno-*S*-glutathione, CDNB diminishes GSH levels in LDSS, which is sufficient to cause dramatic cellular dehydration. By raising intracellular GSH, NAC is inhibiting this effect of CDNB.

K^+ Leakage

It is well known that sickle cells exhibit an abnormal potassium loss and as a result become increasingly dehydrated [5]. Our results indicate that CDNB induced increases in cell density are preceded by, and correlated with, increased K^+ leakage (Fig. 2). LDSS erythrocytes treated with CDNB, over a 5-h incubation period, revealed significantly higher release of K^+ into the incubation media than those cells which were not influenced by CDNB (Fig. 2). CDNB induced increase in K^+ efflux was completely reversed by treatment with 20 mM NAC. As shown in Figure 2, potassium loss from the untreated LDSS erythrocytes was also significantly decreased by NAC. Therefore, the oxidative damage to sickle cells, due to the effect of CDNB on decreasing the level of intracellular GSH, results in increased K^+ efflux. The elevated K^+ efflux is followed by the dehydration of cells and increase in their density. NAC has the ability to reverse this multistep process by stabilizing the level of intracellular GSH (Figs. 1 and 2).

Effect of Clotrimazole and DIOA Upon CDNB-Induced Dense Cell Formation

LDSS erythrocytes were incubated for 12 h at 4°C with various combinations of CDNB, clotrimazole, and DIOA to determine whether CDNB induced K^+ leakage was via the Gardos channel and /or K^+-Cl^- cotransport channel (Fig. 3). After 12 h incubation the sickle cells were density separated (Fig. 3A) and % HDSS (Fig. 3B) determined. As can be seen in Figure 3A and B, 10 μM

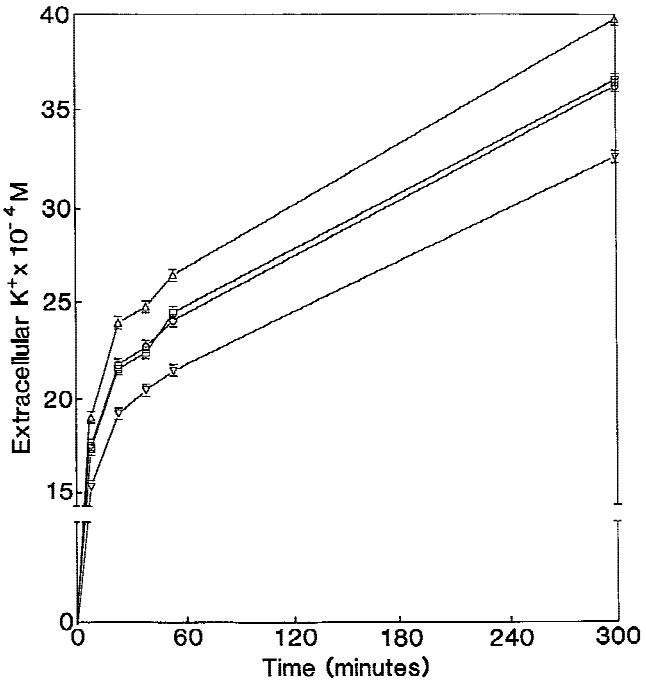

Fig. 2. Potassium efflux is induced by CDNB and inhibited by NAC. Cell treated with 20 mM NAC (-▽-), 20 mM NAC and 1 mM CDNB (-□-), and no addition (-○-) have significantly lower release of K^+ ($P < 0.05$) into the extracellular medium over time, than those which were treated with 1 mM CDNB (-△-). Data is presented as mean ± standard error of three independent experiments.

clotrimazole reduces the CDNB induced dense cell formation to control levels, whereas 1 mM DIOA had no effect. Therefore, CDNB induced K^+ leakage is through the Gardos channel. Measurement of GSH in clotrimazole and DIOA treated samples (Fig. 3C) indicates no effect of these reagents upon GSH concentrations. Therefore, clotrimazole and DI0A are inhibiting the Gardos pathway and the K^+-Cl^- cotransport channel respectively, and not influencing glutathione metabolism in the sickle cell erythrocytes.

DISCUSSION

The goal of this study was to understand the molecular details of the mechanism responsible for the CDNB induced formation of high-density sickle cell [18]. We wanted to determine whether CDNB induced sickle cell dehydration is preceded by increased K^+ leakage. If so, we wanted to determine which K^+ channel is responsible for the CDNB induced K^+ leakage. Finally we wanted to determine whether NAC could block the CDNB induced K^+ leakage and cell dehydration.

Hebbel et al. [1] have shown that sickle cell generate about twice the amount of activated oxygen species found in control RBCs. The reason for this increase in oxygen radicals is the result of accelerated autoxidation of HbS to methemoglobin, a conversion that causes release of heme [20]. Heme is increased in content on the cytoplasmic surface of sickle cell membranes and this correlates with membrane thiol modification [21]. The amount of heme and free iron associated with the cytoplasmic surface of the SS erythrocyte membrane is particularly increased in the most dense fractions of SS RBCs [20]. GSH levels are diminished by about 20% in SS RBCs as compared with high reticulocyte controls, and is lower in high-density sickle cells erythrocytes compared with low-density erythrocytes [22,23]. While earlier studies have indicated only a 20% decrement in intracellular GSH [22,23], our recent work has shown that dense cells (found in 60% Percoll layer) contain only 1.5% control levels of GSH and the highest density sickle cells (65% Percoll) have undetectable intracellular GSH (Shartava et al., submitted for publication). The diminished levels of GSH are related to decreased glutathione reductase activity, increased glutathione peroxidase activity, and inhibition of the pentose phosphate shunt in SS erythrocytes [22]. Therefore, SS have increased activated oxygen species, and dense cells also have diminished levels of GSH to protect against oxidative damage. This combination is probably responsible for oxidation of thiol groups in many target proteins.

According to the integrated red cell model [24] the two channels that play a key role in the formation of dense cells are the Ca^{2+}-activated K^+ channel (Gardos pathway) and the K^+-Cl^- cotransport channel. The model suggests that deoxygenation induces a transient increase in intracellular free Ca^{2+}. This leads to activation of the Gardos Pathway with resultant loss of K^+, Cl^-, H_2O, and cytoplasmic acidification. Because the K^+-Cl^- cotransport system has an optimal pH of 6.8, this acidification would lead to further K^+ loss and dehydration. Recently we demonstrated that CDNB, which rapidly lowers GSH within LDSS erythrocytes, causes a large conversion of low-density cells to high-density cells [18]. Indeed, CDNB lowers GSH by 80% (this study) while high-density sickle cells in vivo have $\leq$1.5% control levels of GSH; therefore, oxidative damage to the Gardos Channel may be more severe in vivo than in our in vitro model system. In the current study we show that this CDNB induced formation of dense cells is preceded by K^+ leakage, and that both dense cell formation and K^+ leakage can be blocked in two different ways: (1) by addition of NAC to CDNB-treated cells thereby protecting K^+ channels from oxidative damage, or (2) by inhibition of the Gardos channel with clotrimazole. DIOA, a specific inhibitor of K^+-Cl^- cotransport system, had no effect on CDNB stimulated K^+ leakage and formation of dense cells. The lack of effect of DIOA could be due to our studies being performed at pH 7.4, rather than the pH optima of the K^+-Cl^- cotransport system (pH 6.8) [5].

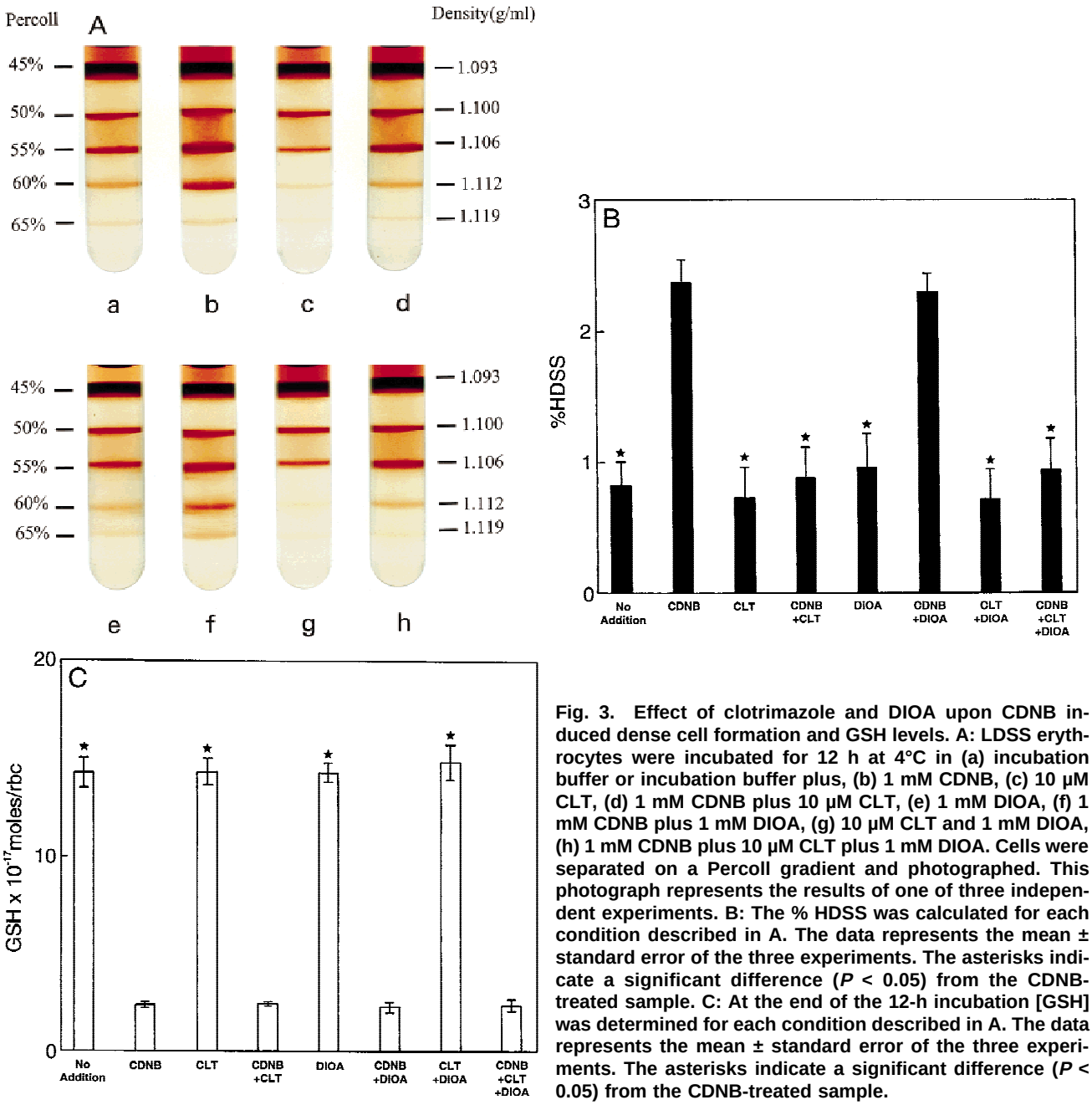

Fig. 3. Effect of clotrimazole and DIOA upon CDNB induced dense cell formation and GSH levels. A: LDSS erythrocytes were incubated for 12 h at 4°C in (a) incubation buffer or incubation buffer plus, (b) 1 mM CDNB, (c) 10 μM CLT, (d) 1 mM CDNB plus 10 μM CLT, (e) 1 mM DIOA, (f) 1 mM CDNB plus 1 mM DIOA, (g) 10 μM CLT and 1 mM DIOA, (h) 1 mM CDNB plus 10 μM CLT plus 1 mM DIOA. Cells were separated on a Percoll gradient and photographed. This photograph represents the results of one of three independent experiments. B: The % HDSS was calculated for each condition described in A. The data represents the mean ± standard error of the three experiments. The asterisks indicate a significant difference ($P < 0.05$) from the CDNB-treated sample. C: At the end of the 12-h incubation [GSH] was determined for each condition described in A. The data represents the mean ± standard error of the three experiments. The asterisks indicate a significant difference ($P < 0.05$) from the CDNB-treated sample.

However recent studies have indicated that DIOA is also ineffective at blocking CDNB induced dense cell formation at pH 6.8 (Shartava et al., submitted for publication). NAC, as the N-acetyl derivative of L-cysteine, is a powerful antioxidant with low toxicity [25]. It is highly permeable to cell membranes, and its ability to protect cells and tissues against oxidative damage has been attributed to radical scavenging and to the enhancement of the availability of L-cysteine a substrate for GSH synthesis [26]. We demonstrated in this study that by protecting the Gardos pathway against malfunctioning, due to oxidative stress in the sickle cells, NAC can eliminate the stress induced activation of the K^+-Cl^- cotransport system, because the later's activation is directly linked to the ability of the Gardos channel to maintain its proper function.

Our in vitro results would suggest the following sequence of events in dense cell formation: (1) Lowering of GSH leads to oxidative damage to various target proteins including the Gardos Channel (this study) and β-actin [18,19]. (2) Oxidative damage to the Gardos Channel leads to increased K^+ leak and H_2O loss from low-density SS converting them to high-density sickle cells. (3) NAC which raises intracellular GSH levels, as well as serving as an antioxidant, can reverse the oxidative dam-

age to the Gardos Channel. It thereby returns K^+ and H_2O homeostasis to normal. Interestingly NAC decreases K^+ efflux even in the absence of CDNB indicating that it can also reverse basal levels of oxidative damage to the Gardos Pathway. Whether these in vitro results indicate the role of GSH in dense cell formation in vivo is currently being tested in a phase II human trial. Homozygous sickle cell patients receiving placebo or various doses of NAC are being tested for percent dense cells, percent ISCs, and intracellular erythrocyte GSH and NAC, as well as other parameters. When complete, this study will indicate whether raising GSH in sickle cells leads to fewer dense cells and ISCs in vivo.

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