Sequence of Toxic Events in Arsine-Induced Hemolysis in Vitro: Implications for the Mechanism of Toxicity in Human Erythrocytes

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Arsine gas (AsH₃), the hydride of arsenic, is a severe hemolytic toxicant that can be acutely fatal. Human exposure can occur after accidental formation from arsenicals mixed with acid in the presence of certain metals and from its use in the manufacture of semiconductors in the electronics industry. Exposure to arsine was fatal in up to 25% of the reported human cases (Fowler and Weissberg, 1974); concentrations of 26 ppm for 1 hr were uniformly fatal to mice (Peterson and Bhattacharyya, 1985). Clinical experience with human arsine poisoning is consistent with intravascular hemolysis. Signs include abdominal pain, hematuria, and jaundice, and laboratory results demonstrate hemoglobin concentrations of <10 g/100 ml, intravascular erythrocyte fragments, ghost cells, anisocytosis and poikilocytosis, reticulocytosis, and a plasma hemoglobin of >2 g/100 ml. The only treatment is exchange transfusion to remove the erythrocytes exposed to arsine.

Arsine hemolysis has been compared to the red cell destruction caused by other oxidant drugs. Hemolysis by these agents is a multistep process that results from the denaturation of hemoglobin and oxidation of the red cell membrane (Chiu and Lubin, 1989). In the most commonly presented pathway, oxyhemoglobin is oxidized to methemoglobin (metHb) and that is followed by hemichrome formation which dissociates to the heme moiety (hematin or heme) and precipitated globin protein (Heinz body). This hemolytic process is enhanced by compounds that produce active oxygen species (Watkins et al., 1985; Goldberg and Stern, 1974; Wallace and Caughley, 1974) and is inhibited by anti-oxidant processes (Hebbel and Eaton, 1989; Shviro and Shaklai, 1987). However, comparison of many of these oxidant compounds indicates that not all of these steps are necessary for hemolysis (Maede et al., 1989). Clearly not all hemolytic compounds act by the same mechanism.

The mechanism of arsine-induced hemolysis is unknown but does share certain characteristics in common with oxidant drugs. Arsine reacts with erythrocytes directly to form toxic hemoglobin oxidation products, and this was also investigated as a potential cause of hemolysis. Essentially on contact with arsine, methemoglobin was formed but only reached 2-3% of the total cellular hemoglobin and remained unchanged for up to 90 min. There was no evidence that further oxidation products (hemin and Heinz bodies) were formed in this system. Based on these observations, hemolysis appears to be dependent on membrane disruption by a mechanism other than hemoglobin oxidation.


Arsine, the hydride of arsenic (AsH₃), is the most acutely toxic form of arsenic, causing rapid and severe hemolysis upon exposure. The mechanism of action is not known, and there are few detailed investigations of the toxicity in a controlled system. To examine arsine hemolysis and understand the importance of various toxic responses, human erythrocytes were incubated with arsine in vitro, and markers of toxicity were determined as a function of time. The earliest indicators of damage were changes in sodium and potassium levels. Within 5 min incubation with 1 mM arsine, the cells lost volume control, manifested by leakage of potassium, influx of sodium, and increases in hematocrit. Arsine did not, however, significantly alter ATP levels nor inhibit ATPases. These changes were followed by profound disturbances in membrane ultrastructure (examined by light and electron microscopy). By 10 min, significant numbers of damaged cells formed, and their numbers increased over time. These events preceded hemolysis, which was not significant until 30 min. It has been proposed that arsine interacts with hemoglobin to form toxic hemoglobin oxidation products, and this was also investigated as a potential cause of hemolysis. Essentially on contact with arsine, methemoglobin was formed but only reached 2-3% of the total cellular hemoglobin and remained unchanged for up to 90 min. There was no evidence that further oxidation products (hemin and Heinz bodies) were formed in this system. Based on these observations, hemolysis appears to be dependent on membrane disruption by a mechanism other than hemoglobin oxidation.
MATERIALS AND METHODS

Chemicals. Zinc arsine, 99%, was obtained from Pfaltz and Bauer, Inc. (Waterbury, CT). Silver diethyldithiocarbamate, ACS Grade, was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Blood collection supplies were obtained from Fisher Scientific. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Erythrocyte isolation and incubation. Blood was obtained by venipuncture from healthy male and female volunteers (ages 21–40). Volunteers were assigned randomly to sample groups, and blood was not pooled from more than one volunteer. Sample size (n) refers to the number of individual volunteers in that group. The blood was sedimented by centrifugation (900g, 10 min) and rinsed twice with isotonic saline. Plasma and buffy coat were removed during the washing procedure. Packed erythrocytes were mixed 1:1 with 2 mM arsine or buffered saline for control. To determine hematoctit, erythrocyte suspensions were centrifuged in graduated Wintrobe sedimentation tubes for 10 min at 900g. The red cell pellet was measured, and hematoctit is expressed as percentage of cell volume/total volume. The samples were gently stirred in loosely capped vials and maintained at room temperature (22°C) unless otherwise stated (Orbital environ-shaker; Labline Inc., Melrose Park, IL).

Generation of arsine in solution. AsH$_3$ was produced from zinc arsine in the presence of 50% sulfuric acid as described (Hatlelid et al., 1995). The generated gas was trapped in 10 mM phosphate-buffered saline (PBS), pH 7.4. AsH$_3$ concentration was determined spectrophotometrically (510 nm) by reaction with 0.55% silver diethyldithiocarbamate in pyridine. (Caution: Arsine is a toxic gas and appropriate precautions should be taken. Experiments should be conducted in an approved fume hood. A saturated potassium permanganate solution trap, in-line after the aqueous trap, should be used to oxidize excess AsH$_3$ before it escapes into the air. For safety reasons, a rubber hose attached to a gas trap, should be used to oxidize excess AsH$_3$ before it escapes into the air. Potassium permanganate solution should also be on hand in the event of loss of containment of the AsH$_3$-generating mixtures or the AsH$_3$-saturated aqueous solutions.)

Measurement of hemolysis. Hemoglobin leakage into the extracellular fluid was considered a direct measure of total cell death or hemolysis. An aliquot of the cell suspension was centrifuged (8700g, 30 sec), and the resulting supernatant was assessed for hemoglobin by reaction with Drabkin's solution (Sigma kit 525A). Data are expressed as percentages of released hemoglobin relative to total hemoglobin in a noncentrifuged sample.

Determination of sodium (Na$^+$) and potassium (K$^+$). Intracellular Na$^+$ and K$^+$ were determined by flame emission photometry. A 25–29 µl sample of cells was washed twice with 1 ml of 310 mOsm Tris–HCl (pH 7.4). The cells were lysed in 1 ml distilled water and proteins precipitated by the addition of 20 µl concentrated perchloric acid. The resulting supernatant was assessed for hemoglobin by reaction with Drabkin's solution (Sigma kit 525A). Data are expressed as percentages of released hemoglobin relative to total hemoglobin in a noncentrifuged sample.

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Morphologic characterization. To view by scanning electron microscopy (SEM), cells were fixed overnight in 4% formaldehyde/1% glutaraldehyde (4%FG) and sealed between 0.5 µm PTFE filters for ease of handling. The samples were postfix for 1 hr in 1% osmium tetroxide. (Caution: Osmium tetroxide is a highly toxic gas, and skin and eye protection should be worn at all times. All procedures should be conducted in an approved fume hood.) After three rinses with distilled water, samples were dehydrated in a graded series of ethanol solutions and critical point dried in ethanol/carbon dioxide. The filters were removed, mounted, and gold sputter coated (Technics Hummer Sputter Coat, Alexandria, VA). Samples were viewed with a dis 130 scanning electron microscope (International Scientific Instruments, Paramus, NJ) at an accelerating voltage of 20 kV. The morphologic changes were classified according to Bessis (1973). To quantify damage, cells were lightly fixed before viewing by light microscopy (Olympus BH-2 RFCA, Tokyo, Japan). At least 200 cells from three separate, random fields were photographed and later scored for the presence of spherical cells.

RESULTS

Hemolysis of erythrocytes. Figure 1A depicts the progression of hemolysis in arsine-treated human erythrocytes and the effect of temperature on cell death. Cells incubated at 22 and 37°C exhibited toxicity, but only after a 30-min lag period. Following this, hemolysis progressed rapidly and relatively linearly for both groups. Decreasing temperature appeared to inhibit hemolysis, and cells maintained at 4°C had virtually no lysis. To determine if the decrease in temperature was protective, erythrocytes were incubated for 2 hr at 4°C then heated to 37°C for an additional hour (Fig. 1B). Following heating, the rate of hemolysis was equivalent to that of cells heated continuously from the time of exposure. Clearly, the decrease in temperature did not prevent toxicity but rather delayed the manifestation of the damage. Because of the speed of the reaction and high levels of hemolysis, subsequent markers of cell injury were determined at 22°C.

Na$^+$, K$^+$, and hematocrit. Changes in erythrocyte Na$^+$ and K$^+$ were examined as a more sensitive indicator of toxicity. Human erythrocytes were exposed to 1 mM arsine and Na$^+$ influx and K$^+$ efflux were monitored for 60 min; results are presented in Fig. 2. Within 5 min of arsine exposure, the cells had altered Na$^+$ and K$^+$. Intracellular levels of K$^+$ rapidly decreased matching the rapid increase in intracellular Na$^+$. These trends in ion levels continued for approximately 30 min then remained unchanged without any apparent recovery. Not surprisingly, these changes were accompanied by cellular swelling. This was indicated by increase in red cell volume without an increase in cell number, measured by a significant increase in hematocrit (Fig. 3). In the erythrocyte, Na$^+$ and K$^+$ are primarily maintained by the membrane bound ion pump, Na$^+$, K$^+$-ATPase. Because of the apparent loss of volume regulation, the effect of arsine on
TOXIC EVENTS IN ARSINE-INDUCED HEMOLYSIS

ATP levels and ATPase activity was determined. ATP determined enzymatically in arsine exposed erythrocytes (Sigma Diagnostic Kit 366) did not significantly decrease compared to control cells (data not shown). ATPase activity was determined as described (Jenkins and Marshall, 1984; modifications by Kakimoto et al., 1995). Activity was not inhibited by arsine regardless of whether arsine was added before or after ATPase was isolated from the cells (data not shown).

Morphologic changes. Human erythrocytes exposed to arsine were fixed and analyzed by a combination of scanning electron microscopy and transmission electron microscopy to characterize changes. Figure 4A depicts an array of cell types that were observed after arsine exposure. Profound disturbances in the cellular membrane occurred with frequent formation of echinocytes (e), burr cells (b), and spheroechinocytes (s). The classification of erythrocyte injury based on morphological changes was described by Bessis (1973). Discocytes are normal biconcave erythrocytes. Echinocytes are erythrocytes that have a crenated appearance, but are still flat. This change in shape represents reversible injury. Progression to spheroechinocytes, erythrocytes that have become spherical and have spicules, represents an irreversible change and is prelytic. The occurrence of spheroechinocytes was quantitated by light microscopy (Fig. 5); these cells appeared after 10 min of incubation and continued to increase up to 45 min. After 60 min, the numbers of cells decreased, presumably due to the significant hemolysis that occurred by this time (Fig. 1). These morphologic changes are due to alterations in the membrane and do not originate from an intracellular structural source, such as Heinz bodies or vacuoles. This is demonstrated by transmission electron microscopy of the damaged cells that are homogeneous inside the cell, near the membrane, and within the actual protrusions (Fig. 4B).

Interactions with hemoglobin. It is clear that arsine can interact with hemoglobin, and many have suggested that...

FIG. 1. Temperature dependence of arsine-induced hemolysis. (A) Hemolysis of cells incubated with 1 mM arsine at 4°C (●), 22°C (▲), and 37°C (●) and control incubations (■). The cell suspensions incubated at 4°C were subsequently heated to 37°C for an additional hour (B), demonstrating that 4°C did not negate the effects of arsine. Values represent the percentage of hemolysis, mean ± SD (n = 3, *p < 0.05).

FIG. 2. Changes in sodium and potassium in human erythrocytes exposed to arsine (●) compared to control (■). Values represent μmol (Na⁺ or K⁺)/g Hb, mean ± SD (n = 3, *p < 0.05).
hemoglobin oxidation products contribute to hemolysis. To assist in understanding the importance of hemoglobin oxidation products, human erythrocytes were exposed to arsine and analyzed for methemoglobin formation and Heinz bodies. In arsine-exposed cells, methemoglobin formed essentially on contact but constituted less than 3% of the total pigment and remained unchanged after this (Fig. 6). One hemoglobin oxidation product, hemin, is highly toxic to the cell by targeting the Na\(^+\),K\(^+\)-ATPase (Yasuhara et al., 1991). Attempts to measure hemin directly in this system were largely unsuccessful. It is known, however, that desferrioxamine is protective of hemin-induced hemolysis (Baysal et al., 1990). Addition of 1 mM desferrioxamine before arsine treatment had no effect on the progression of hemolysis (data not shown). The formation of HBs would also indicate instability of hemoglobin; therefore, cells were examined by TEM and light microscopy for the presence of HBs. No detectable HBs were formed in either preparation for up to 120 min of incubation (data not shown).

DISCUSSION

We report here the time-dependent changes in human erythrocytes exposed to arsine in an in vitro system. The most significant change observed was an immediate disturbance in cell volume control; exhibited by potassium leakage, sodium influx, and increases in hematocrit. The obvious target that would elicit these changes would be the membrane ion pump, Na\(^+\), K\(^+\)-ATPase, which is primarily responsible for maintaining volume control. Indeed, Levinsky and co-workers (1970) suggested that the membrane pump was a target for arsine through alteration of erythrocyte glu-

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**FIG. 3.** Changes in hematocrit of human erythrocytes exposed to arsine (●) compared to control (■). Values represent the percentage of hematocrit, mean ± SD (n = 3, *p < 0.05).

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**FIG. 4.** Morphological changes associated with arsine exposure (30 min). (A) Arsine-induced transformation of human erythrocytes. The above cells typify the morphologic response with formation of echinocytes (e, rounded spicules or protrusions), burr cells (b, spiky protrusions), and spheroechinocytes (s, spheres with many small protrusions). Scale bar, 5 μm. (B) Transmission electron micrograph of spheroid cells (*) demonstrating the absence of vacuoles or inclusion bodies in damaged cells. Scale bar, 3 μm.
and glutathione showed no alteration in arsine stability, indicating that no direct interaction was occurring (Hatlelid et al., 1995). In the system reported here, glutathione (GSH) and glutathione disulfide (GSSG) were determined by UV-HPLC as described by Fariss and Reed (1987) with modifications by Winski and Carter (1995). No significant changes in GSH and GSSG were observed after 1 hr of incubation with 1 mM arsine (data not shown), clearly at a time after significant hemolysis had occurred.

There is significant evidence that arsine interacts with hemoglobin both in vivo and in vitro. After arsine exposure in mice, circulating erythrocytes contained Heinz bodies and there were large increases in metHb (Blair et al., 1990). Chiu and Lubin (1989) described the process of hemoglobin oxidation leading to erythrocyte destruction in general terms, proposing that the first step is formation of metHb, followed by formation of hemichrome and breakdown to precipitated globin (Heinz bodies) and the toxic heme moiety. In the data presented here, arsine-exposed human erythrocytes do form metHb, but levels never increase above 3%.

Based on the data presented here, in vitro hemolysis of human erythrocytes by arsine does not occur through an oxidative mechanism involving hemoglobin. The changes observed in sodium and potassium are sufficient to explain the appearance of irreversibly damaged cells and resulting
tathione. In the results described here, neither ATP nor ATPase activity were decreased by arsine treatment. In addition, dog erythrocytes, which do not have Na⁺,K⁺-ATPase, still hemolyze in the presence of arsine (Hatlelid et al., 1995). One difference in the response of dog and human erythrocytes to arsine was that human erythrocytes exhibited temperature dependence and dog erythrocytes did not (Hatlelid et al., 1995). Based on these observations, Na⁺, K⁺-ATPase may not be a direct target of arsine but may contribute to the manifestation of damage, particularly at higher temperatures. Although there is little information available concerning the effects of arsine on ion channels, there are other compounds which alter Na⁺ and K⁺ by routes other than inhibition of Na⁺/K⁺-ATPase. One of these compounds is tert-butyl hydroperoxide (tBH). Although tBH does moderately inhibit Na⁺/K⁺-ATPase, this does not explain the extent of damage to ion levels (Dwight and Hendry, 1996).

It has been postulated that membrane damage occurs through additional pathways, including formation of a "leak" pathway, crosslinking of membrane thiols, and formation of lipid peroxides (Deuticke et al., 1992; Dwight and Hendry, 1996).

It is possible that arsine also increases membrane permeability through one of these mechanisms, but further work is required to make that distinction.

The effect of arsine on cellular thiols and its importance is controversial in the literature. Pernis and Magistretti (1960) reported that thiol depletion preceded hemolysis, but later reports demonstrated that thiol depletion followed hemolysis (Hatlelid et al., 1995). Blair and co-workers (1990) also reported that thiol depletion occurred following in vivo exposure, but only 50% depletion of reduced thiols occurred and this was after 4 hr. In addition, aqueous incubations of arsine and glutathione showed no alteration in arsine stability, indicating that no direct interaction was occurring (Hatlelid et al., 1995). In the system reported here, glutathione (GSH) and glutathione disulfide (GSSG) were determined by UV-HPLC as described by Fariss and Reed (1987) with modifications by Winski and Carter (1995). No significant changes in GSH and GSSG were observed after 1 hr of incubation with 1 mM arsine (data not shown), clearly at a time after significant hemolysis had occurred.

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hemolysis. The cause of membrane damage remains unclear due to the fact that ATP and ATPase remain at control levels. Arsine does, however, immediately react with hemoglobin and methHb is formed. We propose that arsine and hemoglobin interact to form arsenic metabolites which are the ultimate toxic species, perhaps through alteration of membrane constituents.

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REFERENCES


