In Vitro Sub-Hemolytic Effects of Butoxyacetic Acid on Human and Rat Erythrocytes

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When 2-butoxyethanol (2-BE) is administered to rats, hemolysis occurs as the active metabolite butoxyacetic acid (BAA) is formed. Human red blood cells appear to be relatively resistant to the hemolytic effects of BAA in vitro, whereas rat red blood cells undergo changes in deformability, cell swelling, and hemolysis. In this study, exposure of human red blood cells to high concentrations of BAA resulted in loss of deformability and a small increase in mean cellular volume, but no significant hemolysis. These changes resembled the changes that occur in rat erythrocytes exposed to much lower concentrations of BAA. Therefore, a comparison was made between the sub-hemolytic effects of BAA at high concentrations (up to 10 mM) on human red cells with the sub-hemolytic effects of lower concentrations of BAA (up to 0.1 mM) on rat erythrocytes. Under these conditions, human and rat erythrocyte deformability decreased, while mean cellular volume (MCV) and osmotic fragility increased. Although there was a substantial shift in rat erythrocytes to lower densities, human erythrocyte density was only slightly decreased. Human and rat erythrocyte sodium also increased. Rat erythrocytes demonstrated increased spherocytosis. In a survey of blood samples from adults and children, none demonstrated an increase in hemolysis (n = 97) or MCV (n = 65) after exposure to 10 mM BAA for 4 h. In these experiments, in which hemolysis was not evident, human erythrocytes required exposure to a 100-fold greater concentration of BAA to develop changes in red cell deformability, osmotic fragility, and sodium content similar to those observed in rat erythrocytes. These concentrations are not likely to occur under normal human use of 2-BE-containing products.

Key Words: 2-butoxyethanol; ethylene glycol monobutyl ether; butoxyacetic acid; glycol ethers; hemolysis; rat erythrocytes; human erythrocytes.

Glycol ethers are an important group of solvents with many industrial and commercial applications. Ethylene glycol butyl ether or 2-butoxyethanol (2-BE) has a remarkable species-dependent toxicity for red blood cells (RBCs) (Carpenter, et al., 1956; Ghanayem, 1989). The administration of 2-BE causes lethal hemolysis in rats, rabbits, and mice, but not in guinea pigs (Carpenter et al., 1956; Gingell et al., 1998). 2-BE alone is only a weak hemolysin in vitro, but its administration to rats is followed by metabolism in the liver and other sites to 2-butoxyacetic acid (BAA) via an aldehyde intermediate, butoxyacetaldehyde (Ghanayem, et al., 1987). Most evidence points to BAA as the principal hemolysin. Rat erythrocytes incubated in vitro with BAA demonstrate hemolysis, but little or no hemolysis occurs when red cells are exposed to butoxyacetaldehyde or 2-BE (Ghanayem, 1989). Human erythrocytes demonstrate no hemolysis when incubated in BAA at concentrations up to 2.0 mM, a level which results in greater than 30% hemolysis of rat red cells after a 2 to 4 h incubation (Bartnik, et al., 1987; Ghanayem, 1989; Udden et al., 1994). The mechanism of rat red-cell damage is not known. Similarly, there is no understanding of human resistance to BAA in vitro. Bartnik et al. (1987) found no evidence for hemolysis of human RBCs incubated with concentrations of 1–15 mM BAA for up to 3 h. Ghanayem (1989) noted a slight degree of hemolysis of human RBCs incubated in 8.0 mM BAA for 4 h. 2-BE can cause hemolysis of human RBCs, but only when cells are exposed to 125–200 mM for 2 or 3 h (Bartnik et al., 1987). At such high levels of 2-BE, rat erythrocytes are only marginally more sensitive to the solvent than humans. Non-specific detergent effects of 2-BE probably might account for the red-cell damage observed at such high concentrations of 2-BE.

In the rat, an increase in mean cellular volume (MCV) accompanies hemolysis during in vivo exposure to 2-BE (Ghanayem et al., 1990). A similar degree of cell swelling occurs in vitro with exposure of red cells to BAA. Measurements of MCV were used by Ghanayem to identify susceptible species for 2-BE toxicity by demonstrating an increase in MCV after in vitro incubation of RBCs with 2.0 mM BAA (Ghanayem et al., 1993). Rat erythrocyte deformability, as assessed by a polycarbonate filtration method, also decreases after exposure to BAA in vitro. Human erythrocyte deformability was unaffected by incubation with 2.0 mM BAA for 4 h, while rat erythrocytes were considerably more rigid after exposure to 0.2 mM BAA, which caused no in vitro hemolysis (Udden and Patton, 1994). Rat erythrocyte morphology undergoes a transformation from a normal discocytic shape to spherocytic and stomatocytic, or cup-shaped, red blood cell when exposed to...
BAA in vitro or after 2-BE administered by gavage (Udden, 2000). Human erythrocyte morphology was not affected by exposure to 2.0 mM BAA (Udden and Patton, 1994).

In the present study, changes in human RBC, deformability along with changes in other functional aspects of the red cell, are described after exposure to high concentrations (up to 10 mM) of BAA in vitro. Blood samples from healthy donors and hospitalized patients including children were screened for evidence of increased erythrocyte susceptibility to hemolysis or cell swelling. A comparison is also presented of similar pre-hemolytic RBC changes observed at high concentrations of BAA (up to 0.1 mM). The absence of an in vitro threshold for hemolysis of human RBCs has made it difficult to infer from animal studies an estimate of relative toxicity of BAA, and therefore of 2-BE for man. The experiments presented here offer a new approach to making this comparison by focusing instead on pre-hemolytic RBC changes observed at high concentrations of BAA for human RBCs and similar changes in rat RBCs exposed to 100-fold lower concentrations of BAA.

MATERIALS AND METHODS

Red blood cells. Heparinized blood samples were obtained by venipuncture from healthy adult volunteers, after informed consent was obtained. Blood samples from hospitalized patients anticoagulated with EDTA and stored at 4°C for 1 to 3 days were also examined. Blood samples anticoagulated with EDTA were obtained from children by venipuncture or by finger or heel stick. Rat erythrocytes were obtained from Fischer 344 males aged 9–11 weeks. Rats were maintained in the Baylor College of Medicine Vivarium and fed on a standard NIH diet for one week before heparinized blood samples were obtained, during euthanasia by cardiac puncture under CO₂ anesthesia. These studies were approved by the Baylor College of Medicine Animal Care and Use and Institutional Review Board for Human Research Committees. Blood samples were then centrifuged at 1000 × g for 10 min before removal of plasma and buffy coat. The cells were then washed free of plasma with a buffer containing 10 mM TRIS, pH 7.4; 140 mM NaCl; 2.0 mM CaCl₂; 4.0 mM KCl; 10 mM glucose; and 0.1% bovine albumin. The RBCs were adjusted to a packed cell volume of 10% and incubated in the presence of BAA for 1–4 h at 37°C in a water bath.

Butyroyacetic acid. A 99% pure reagent was obtained from the Shell Development Company, Westhollow Technical Center, Houston, Texas.

Red blood cell indices: Morphology and hemolysis. Erythrocyte suspensions were sampled at intervals for determination of microhematocrit, hemoglobin, RBC count, and percent hemolysis, utilizing standard methods. The mean cellular volume (MCV) for blood samples obtained in heparin from healthy donors and for rat RBCs was determined from the microhematocrit and the red-cell count. The distribution of RBC size was determined using a Coulter model Z2 cell-size analyzer. For blood samples obtained from the clinical laboratory and stored in EDTA, the MCV and size distribution were determined using the Z2 analyzer.

Deformability. Erythrocytes were diluted with buffer to 2.5 × 10⁸ cells/ml and then infused via a Harvard syringe pump at 2.0 ml/min through a 3.0-micron polycarbonate filter (lot number 8084011, Nuclepore Filter Corporation, Pleasanton, CA). During filtration at a constant rate, pressure increases were measured by a Statham P23Db pressure transducer with graphic output, on a Gould Chart recorder. The typical pressure-time curve and a mathematical expression describing the curve as a function of initial filtration pressure (P₀), parameters determined by the electronic filter in the chart recorder, and filling of the filter chamber are described in detail elsewhere (O’Rear et al., 1979). The initial filtration pressure, or P₀, is a function of RBC deformability and was determined by a software program that analyzed digitized curves using a Marquardt non-linear regression numerical method (Biological Research Systems, Houston TX).

Erythrocyte osmotic fragility, density, and cations. Osmotic fragility was determined on washed RBCs as described by Beutler (1995). Density distribution of red cells was determined on mixtures of phthalate esters (Danon and Marikovsky, 1964). Erythrocyte sodium and potassium concentrations were determined using an atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, CT) in the emission mode. Measurements were made on erythrocytes washed 4 times with isotonic MgCl₂ and then lysed in 0.1% LiCl.

Microscopy. Red blood cells were prepared for light-phase microscopy (1000 × magnification) by addition of 20 µl of cell suspension to 1.0 ml of buffer containing 1.0% glutaraldehyde.

Statistics. Mean and SE for the number of different subjects tested, n, were used to characterize the effects of BAA at varying concentrations when compared to a control (0 mM BAA) group. Significance was determined by one-way analysis of variance, with additional use of an all pair-wise multiple comparison procedure (Tukey) test to determine significance of difference in effect due to different concentrations of BAA, compared to control (no BAA) incubation. Paired t-tests were used for normally distributed data, and the Wilcoxon Rank Sum test was used to characterize the differences in effect of BAA on non-normally distributed results of studies on patients’ blood samples. Calculations were made using a Sigmastat software program.

RESULTS

The time dependence of the effect of a 1 to 4 h exposure to 10 mM BAA on human RBC deformability, as measured by filtration, is shown in Figure 1. Increased red-cell rigidity was indicated by an increase in the initial pressure developed during filtration (P₀) after 4 h. No hemolysis was observed. To compare the sub-hemolytic effects of BAA, human RBCs were challenged with 0–10 mM BAA for 4 h while rat erythrocytes were exposed to BAA at a 100-fold lower concentration (0–0.1 mM) previously shown to be sub-hemolytic (Udden and Patton, 1994). As shown in Figure 2, there was a comparable increase in red-cell rigidity without hemolysis in both the rat
and human experiments, even though there was a 100-fold difference in BAA concentration. At the highest concentrations of BAA used, there was a 64% increase in P₀ of rat RBCs and an increase in P₀ of 40% for human RBCs. A significant change in human erythrocyte deformability occurred at 7.5 and 10 mM BAA, whereas decreased deformability in the rat erythrocyte was noted first at 0.05 mM BAA.

Because the filtration of RBCs is greatly affected by changes in cell size, MCV was also measured (Fig. 3). There was a small but significant increase in human red blood cell volume after exposure to the highest concentration of BAA. The size distribution, as determined using an impedance cell counter, is shown (Fig. 4) for human and rat erythrocytes incubated with 10 mM and 0.1 mM BAA respectively. The distribution demonstrates a main peak that is very close to the mean cell volume as determined by the packed cell volume and red cell count corrected for coincidence. A “shoulder” was also present, due to the presence of two red cells caught at the same time in the aperture of the impedance counter. Both rat and human erythrocytes appeared to shift in size to a larger cell volume, as the number of smaller red blood cells diminished.

Osmotic fragility, a sensitive indicator of changes in the surface area to volume ratio of red cells, was also determined (Fig. 5). Human RBCs showed no significant changes in osmotic fragility until they had been exposed to either 7.5 or 10.0 mM BAA (Fig. 5A). Rat erythrocytes demonstrated a remarkable increase in osmotic fragility after exposure to lower concentrations of BAA (Fig. 5B).

Centrifugation of RBCs over layers of mixtures of 2 phthalate esters in microhematocrit tubes was used to demonstrate changes in erythrocyte density. Human red blood cells showed only a very small shift towards lower density (Fig. 6A). However, the rat erythrocytes showed a marked shift to lower cellular density (Fig. 6B).
Erythrocyte cation content was determined by measuring the concentration of sodium and potassium, the principal intracellular cations in human and rat erythrocytes. There was a significant increase in both human and rat erythrocyte sodium concentration with exposure to high and low levels of BAA respectively (Figs. 7A and 7B). However, human red blood cell potassium was slightly decreased and this occurred after incubation in the highest concentrations of BAA (Fig. 7C). Rat red blood cell potassium levels decreased after exposure to 0.025 mM BAA, the lowest concentration used (Fig. 7D). Although there was a trend towards increased total cation content in the human (Fig. 7E), only the rat erythrocytes showed a significant net change in total cation content—a small net increase after their incubation with 0.1 mM BAA for 4 h (Fig. 7F).

The morphology of human erythrocytes was not changed after exposure to 10 mM BAA for 4 h (Fig. 8). However, rat erythrocytes exposed to 0.1 mM BAA showed increased numbers of spherocytes compared to the controls.

The dose-response curve (Fig. 2) for the effect of BAA on human RBCs demonstrates a sub-hemolytic effect for a small sample (n = 7). To examine the possibility that certain individuals might possess red blood cells that are unusually susceptible to the hemolytic or cell-swelling effects of BAA, erythrocytes obtained from heparinized blood samples from 11 additional well individuals were exposed to 10 mM BAA in vitro for 4 h. There was no substantial difference in hemolysis in a pair-wise comparison test (Table 1). MCV, as determined from a cell count and a measurement of the packed cell volume, did increase as expected (Table 2). In order to extend these studies to erythrocytes from children, a screening study of blood samples from hospitalized children and adults was undertaken. EDTA-anticoagulated blood samples from a clinical laboratory service were retrieved prior to discard, washed, and suspended in buffer. These samples had been stored for 1 to 3 days in plasma at 4°C. Hemolysis was slightly increased.
after exposure of hospitalized adult erythrocytes to 10 mM BAA for 4 h (Table 1). The degree of hemolysis, although statistically significant, was not physiologically significant given the storage conditions and the degree of illness of some of the patients from whom the blood samples derived. The hemolysis associated with BAA exposure in this group was no more than the hemolysis seen in the controls for well adults, obtained in heparin and examined fresh. The greatest degree of hemolysis above that of the control was never more than 1.5%. There was no difference in hemolysis of red blood cells obtained from children due to exposure to BAA (Table 1). The degree of hemolysis was not affected by gender of the donor or storage time of the sample in EDTA (data not shown).

Because the children’s blood sample volumes were small, MCV was determined from the size distribution of red blood cells by the impedance-cell analyzer at very low red cell counts. Under these conditions, coincidence was minimized. For the blood samples obtained from the adult patients, the

### TABLE 1
Survey of Hemolytic Effect of BAA on Human Erythrocytes

<table>
<thead>
<tr>
<th>Subjects</th>
<th>% Hemolysis</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Well adults (n = 11)</td>
<td>0.74 ± 0.10</td>
</tr>
<tr>
<td>Hospitalized adults (n = 40)</td>
<td>0.54 ± 0.07</td>
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<tr>
<td>Hospitalized children (n = 46)</td>
<td>0.68 ± 0.06</td>
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Note. Well-adult blood samples were obtained in heparin and studied on the same day. Blood samples from hospitalized adults and children were obtained in EDTA and stored for 1 to 3 days.

*p < 0.017 by Wilcoxon signed rank test.

after exposure of hospitalized adult erythrocytes to 10 mM BAA for 4 h (Table 1). The degree of hemolysis, although statistically significant, was not physiologically significant given the storage conditions and the degree of illness of some of the patients from whom the blood samples derived. The hemolysis associated with BAA exposure in this group was no more than the hemolysis seen in the controls for well adults, obtained in heparin and examined fresh. The greatest degree of hemolysis above that of the control was never more than 1.5%. There was no difference in hemolysis of red blood cells obtained from children due to exposure to BAA (Table 1). The degree of hemolysis was not affected by gender of the donor or storage time of the sample in EDTA (data not shown).

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Survey of Effect of BAA on Human Erythrocytes

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Control</th>
<th>10 mM BAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well adults (n = 14)</td>
<td>95.1 ± 1.56</td>
<td>99.8 ± 1.97*</td>
</tr>
<tr>
<td>Hospitalized adults (n = 29)</td>
<td>87.1 ± 2.14</td>
<td>89.2 ± 2.23*</td>
</tr>
<tr>
<td>Hospitalized children (n = 25)</td>
<td>92.8 ± 2.0</td>
<td>95.2 ± 2.0*</td>
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Note. Blood samples described here are the same as those presented in Table I. The MCV was determined by microhematocrit and cell count for the samples from well adults, and by Z2 Coulter counter analysis for the samples obtained from hospitalized adults and children.

*p < 0.001 by paired t-test.

*p < 0.001 by Wilcoxon signed-rank test.

MCV obtained by impedance sizing was a little smaller but correlated linearly with the MCV determined from packed cell volume (data not shown). The frequency distribution of cell size determined for each blood sample subjected to 10 mM BAA and its control was inspected for changes in pattern due to BAA. The control MCV determinations for the adult patients (Table 2) were somewhat smaller than the control for well adults. This is due in part to the smaller MCV determined by impedance sizing and also due to the fact that some of the adult patients had microcytic red blood cells caused by iron deficiency. There may also be an effect of storage for one day in EDTA of the red blood samples obtained from the hospitalized patients, although there were no differences in MCV for samples stored for 2 or 3 days in EDTA compared to those samples stored for only one day. For these reasons, a statistical comparison was not made between the well adults and the hospitalized adult controls. The MCV determinations of control red cells from hospitalized children were larger than those observed in the adults. Again, microcytosis in some of the adults could account for this. However, some of the blood samples from children were obtained from neonates. Neonatal red blood cells characteristically have a larger MCV than red cells obtained from older children or adults. No statistical comparisons were made for MCV control values for the hospitalized children and well or sick adults because of these differences in the populations examined. As expected, the MCV did increase as a result of BAA exposure (Table 2), but no individual had a significant difference in the pattern of the frequency distribution of cell size. No effects of gender of the blood donor were found on hemolysis or cell swelling due to BAA.

DISCUSSION

Human red blood cells, including red cells from patients with hemolytic anemia, possess a remarkable resistance to the hemolytic effects of BAA (Udden, 1994). Nevertheless, sub-hemolytic change was observed in the experiments reported here. Thus, sub-hemolytic changes in red blood cell deformability were consistently observed in red cells from humans and rats, but the red cells from these two species differed strikingly in the BAA concentration required to cause these changes. Pre-hemolytic changes consistently occurred when rat erythrocytes were exposed to a 100-fold lower concentration of BAA.

Normal RBC deformability depends upon an increased surface area to volume ratio of the cell, intrinsic membrane deformability, and viscosity of the internal hemoglobin milieu (Chien, 1977). The polycarbonate sieve method used in these experiments is very sensitive to the ratio of surface area to volume (Chien, 1977; O’Rear et al., 1979). This is because a relatively under-filled, or collapsed balloon-like object is easier to push through a small pore than a filled, spherical object. Thus, the RBC deformability changes observed here could be related to cell swelling and greater sphericity. An effect on membrane structure causing a change in deformability is also possible since the increase in cell size was relatively modest. In these measurements, filters with the same pore size (3.0 micron) were used for rat and human RBCs. The human red blood cell is larger than the rat red cell, accounting for the higher P0 shown in the human control erythrocytes.

The changes in rat erythrocyte deformability are very consistent with cell swelling and increased water content. During exposure to sub-hemolytic concentrations of BAA, the MCV increased, as did the osmotic fragility. Lysis occurs in the osmotic fragility test because red cells behave as osmometers—taking on water as the external environmental osmolality decreases. When the cell reaches a size in which the surface area is fully expanded, the critical hemolytic volume is attained, after which further uptake of water results in lysis of the cell (Beutler, 1995). The rat erythrocyte osmotic fragility was consistent with cells being closer to their critical hemolytic volume after exposure to BAA. A remarkable shift towards lower erythrocyte density shown in the phthalate ester density distribution of the rat RBCs is consistent with cell swelling and dilution of hemoglobin, hemoglobin concentration being the most important determinant of red cell density (Mohandas et al., 1980).

Although the change in MCV in the human and rat red cells was of about the same magnitude (3.7 μm³ for human, 5.7 μm³ for the rat), the relative increase in the human red cell, which starts out as a larger cell (98 μm³ versus 56 μm³ in rat), was smaller. The size distribution as determined by impedance cell sizing is demonstrably changed after exposure to BAA in vitro. In these studies the coincidence of cells makes it difficult to detect a sub-population of enlarged cells, although a loss of the smallest cells and a shift of the main peak to a higher cell volume is apparent. Certain size analyzers are unable to detect such changes in rat MCV after BAA exposure (Ghanayem et al., 1990). There was a significant increase in osmotic fragility for human RBCs, indicating a greater surface area to volume ratio. However, the absence of a significant shift towards lower...
density, demonstrated by the phthalate ester density distribution, suggests that water content did not greatly increase. A possible explanation is that the changes occurred in a subpopulation of human erythrocytes, or that exposure to BAA renders the human red blood cell more vulnerable to osmotic stress. However, most of the rat erythrocytes subjected to a lower concentration of BAA had an increased osmotic fragility and decreased cell density. Spherocytosis was evident in the rat red blood cells exposed to BAA, but human red blood cell morphology was not affected by BAA at much higher concentrations.

Both human and rat RBCs showed a clear increase in cell sodium as a result of exposure to BAA. Rat erythrocytes appeared to immediately respond with a small potassium loss that failed to compensate for the net increase in sodium. Because water follows cations into the red cell, the net increase in total cation content of the rat erythrocytes exposed to 0.1 mM BAA for 4 h could have contributed to increased cell size and decreased density. The redistribution of cell density to lower values and the increased osmotic fragility are consistent with increased cell volume due to increased water.

Although similar (quantitatively and qualitatively) changes occurred for human and rat RBC deformability, osmotic fragility, cell sodium levels, and perhaps MCV, it is not certain that these changes happened for the same reasons. The absence of a major decrease in cell density and the absence of morphologic changes in the human red blood cell suggests that the mechanism for pre-hemolytic changes are different in the two species. However, the clear increase in sodium content of human and rat erythrocytes suggests that the effect of BAA is to disturb cation transport. Even so, it is now possible to assess the concentration-dependent effects of BAA on human RBCs.

These studies are consistent with a 100-fold difference between concentrations at which pre-hemolytic effects of BAA are observed on rat and human red blood cells. Although there was a very small (but statistically significant) increase in hemolysis of red blood cells exposed to BAA from hospitalized adults, none of the blood samples from 97 adults and children demonstrated an increase in hemolysis of a degree that would predict an increased susceptibility to BAA accumulating in the blood from an exposure to 2-BE. There was an increase in the MCV, as expected. There was no evidence of differences due to age or sex. A human pharmacokinetic model (Corely et al., 1994) indicated that the predicted level of BAA in humans exposed continuously by inhalation to a saturated vapor of 2-BE or by dermal exposure to a 40% 2-BE solution was less than 2 mM. Thus, the pre-hemolytic changes described here are unlikely to occur in humans under normal exposure conditions. These results are particularly important when uncertainty factors used in risk assessment are based on inter- or intra-species differences in hemolytic effects.

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REFERENCES


