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Glutathione diminishes Dibutyltin- and tributyltin-induced loss of lytic function in human natural killer cells

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Abstract

This study investigated whether reduced glutathione (GSH) was able to alter the negative effects of tributyltin (TBT) or dibutyltin (DBT) on the lytic function of human natural killer (NK) cells. NK cells are an intital immune defense against the development of tumors or viral infections. TBT and DBT are widespread environmental contaminants, due to their various industrial applications. Both TBT and DBT have been shown to decrease the ability of NK cells to lyse tumor cells (lytic function). The results indicated that the presence of GSH during exposure of NK cells to TBT or DBT diminished the negative effect of the BT on the lytic function of NK cells. This suggests that interaction TBT and DBT with functionally relevant sulfhydryl groups in NK cells may be part of the mechanism by which they decrease NK lytic function.

Keywords

NK cells; glutathione; TBT; DBT; lytic function

INTRODUCTION

Tributyltin (TBT) was produced in large quantities for wood preservation, marine antifouling paints, disinfection of circulating industrial cooling water, and slime control in paper mills (Roper, 1992). Detectable levels of TBT have been found in fish (Kannan and Falandyz, 1997; Kannan et al, 1995a,b,c; Alzieu et al., 1989). It has also been found in plastic products (baking parchments made from siliconized paper) that come in contact with food and was found to transfer into cookies placed on TBT-containing baking parchments (Takahashi et al., 1999). There is significant exposure of humans to TBT as evidenced by its detection in human blood (Whalen et al., 1999; Kannan et al., 1999).

DBT is used as a stabilizer in the production of polyvinyl chloride (PVC) plastic products (Roper, 1992) and has been found in some plastic food containers (Nakashima et al., 1990; Yamada et al., 1993). It has also been used as a deworming agent in poultry and some poultry products have been shown to contain measurable levels of DBT (Epstein et al., 1991). Drinking water has also been reported to contain DBT due to leaching from PVC pipes (Sidiki et al., 1996). Human exposure to DBT could come from poultry products (Epstein et al., 1991), consuming beverages stored in PVC pipes during manufacture (Forsyth et al., 1992a,b), and drinking water (Sidiki et al., 1996). DBT has also been found in human blood from donors with no specific route of exposure other than what is found in the environment (Kannan et al., 1999; Whalen et al., 1999). Exposure to DBT appears to disrupt normal embryonic

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development and to cause a variety of birth defects in rats including cleft mandible and fused ribs (Ema et al., 1996; Noda et al., 1993). It has also been shown to cause atrophy of the thymus and pancreatitis in exposed rats (Merkord et al., 1997; Pieters et al., 1994). Exposure of animals to DBT may debilitate their immune function, making them more vulnerable to infectious diseases (Kannan et al. 1997, 1998).

Natural Killer (NK) cells are a subset of lymphocytes that are capable of killing tumor cells, virally infected cells and antibody-coated cells. They are defined by the absence of the T cell receptor/CD3 complex and by the presence of CD56 and/or CD16 on the cell surface (Walzer et al. 2005). NK cells are capable of killing tumor cells without prior sensitization, putting them in the forefront of lymphocyte defense against tumor cells and virally infected cells (Vivier et al., 2004; Wu and Lanier, 2003; Biron et al, 1989; Fleisher et al., 1982; Hanna, 1980; Kiessling and Haller, 1978). Therefore, any chemical that can affect the ability of NK cells to recognize and lyse a target cell, could potentially increase the risk of tumors and viral infections.

Our laboratory has shown that TBT and DBT are able to significantly reduce the lytic function of human NK cells (Dudimah et al., 2007a,b; Whalen et al., 1999). Reduced glutathione (GSH) is a tri-peptide that serves as an electron donor in cells (Meister and Anderson, 1983). The detrimental effects of TBT in tunicates and of DBT on ox heart mitochondria have been shown to be alleviated by GSH (Cain et al., 1977; Cima and Ballarin, 2004). Thus, it is important to examine whether GSH is able to reduce the demonstrated negative effects of BTs on the function of human NK cells. Such an examination will aid in elucidation of the mechanism by which BTs may be interfering with the important anti-tumor function of NK cells. Here we determine if the negative effects of TBT and DBT on the lytic function of human NK cells can be diminished by GSH.

MATERIALS AND METHODS

Isolation of NK cells

Peripheral blood from healthy adult (male and female) volunteer donors was used for this study. Buffy coats (source leukocytes) obtained from the American Red Cross (Portland, OR) were used to prepare NK cells. Highly purified NK cells were obtained using a rosetting procedure. Buffy coats were mixed with 0.8 mL of RosetteSep human NK cell enrichment antibody cocktail (StemCell Technologies, Vancouver, British Columbia, Canada) per 45 mL of buffy coat. The mixture was incubated for 25 min at room temperature (~ 25° C) with periodic mixing. Following the incubation, 7−8 mL of the mixture was layered onto 4 mL of Ficoll-Hypaque (1.077 g/mL) (MP Biomedicals, Irvine, CA) and centrifuged at 1200 g for 30 min. The cell layer was collected and washed twice with phosphate buffered saline (PBS) pH 7.2 and stored in complete media (RPMI-1640 supplemented with 10% heat-activated BCS, 2 m*M L*-glutamine and 50 U penicillin G with 50 μg streptomycin/ml) at 1 million cells/mL. The resulting cell preparation was >95% CD16+, and CD56+, 0% CD3+ by fluorescence microscopy and flow cytometry (Whalen et al., 2002).

Chemical preparation

TBT and DBT were purchased from Aldrich Chemical Co. (Milwaukee, WI). DBT was dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to give a stock solution. TBT (Sigma-Aldrich) is a liquid at room temperature. TBT was dispersed initially in double de-ionized water (ddH_2O) to give a 1 mM solution. Desired concentrations of each of the compounds were prepared by dilution of the stock into complete media containing 0.5% gelatin (gelatin media) in place of the 10% bovine serum. Gelatin replaced BSA to avoid binding of the hydrophobic compounds to serum albumin, which could interfere with their delivery the cells. The final concentration of DMSO for DBT exposures did not exceed 0.01%.

A stock solution of GSH (Fisher Scientific, Pittsburgh, PA) dissolved in ddH_2O was prepared just prior to addition to the cell culture. The initial stock was appropriately diluted into gelatin media prior to addition to the cell cultures.

Cell Treatments

NK cells (at a concentration of 1.5 million cells/ mL) were exposed to 1 mM GSH either 1 h prior to or at immediately prior to the addition of various concentrations of TBT or DBT or control for 1 h, 24 h or 48 h. Following the exposures the cells were assayed tumor-destroying function.

Cell Viability

Cell viability was determined by trypan blue exclusion. Cell numbers and viability were assessed at the beginning and end of each exposure period. Viability was determined at each concentration for each of the exposure periods. The viability of treated cells was compared to that of control cells at each length of exposure (Whalen et al., 2002). Only those concentrations where viability was unaffected were used at a given length of exposure.

Cytotoxicity assay

The ability of NK cells to lyse tumor cells was measured using a ^{51}Cr release assay (Whalen et al., 1999). The target cell in all cytotoxicity assays was the NK-susceptible K562 (human chronic myelogenous leukemia) cell line. K562 cells were incubated with ^{51}Cr (Perkin-Elmer Life Sciences, Boston, MA) in 0.2−0.5 ml of BCS for 1−1.5 h at 37 °C in air/CO_2 (19:1). Following this incubation the target cells were washed twice with gelatin media. NK (effector) cells (1.2×10^5/100 μL for 12:1 ratio with target cells) were added to the wells of round-bottom microwell plates. The effectors were diluted to 6:1 ratio (0.6×10^5/100 μL) and 3:1 ratio (0.3×10^5/100 μL); each ratio was tested in triplicate. Target cells were added (1×10^4/100μL) to each well of the microwell plate and the plate was centrifuged at 300g for 3.5 min and incubated for 2 hr at 37 °C (air/CO_2 ,19:1). After incubation a 0.1 ml aliquot of the supernatant was collected and counted for radioactivity for 60 sec in a Packard COBRA gamma radiation counter (Packard Instrument Co., Meriden, CT). Target lysis was calculated as follows: 100× [(test c.p.m - spontaneous c.p.m.)/maximum c.p.m.- spontaneous c.p.m.)]. Maximum release was produced by adding 100 μL of 10% Triton X-100.

GSH assay

The stability of exogenously added GSH under the culture conditions used was determined using a glutathione colorimetric assay kit (BioVision, Mountain View, CA). Briefly, GSH was added to gelatin media at a final concentration of 1 mM and incubated for 1 h, 24h, 48h or 6 days at 37°C, 19:1 Air:CO_2. A volume of 20 μL of the culture solution was added to 160 μL of reaction buffer and incubated for 10 min at room temperature (RT), 20 μL of substrate was then added and incubated for 10 min at RT. Absorbance was read at 405 nM. Concentrations of reduced GSH in the samples were determined using a standard curve.

^{14}C-DBT accumulation

Purified NK cells were exposed to 10 μM and 5 μM ^{14}C-DBT for 1 h or 1 μM and 0.5 μM ^{14}C-DBT for 24 h plus or minus 1 mM GSH. Following the exposures the cells were centrifuged and the supernatant was removed and placed in an appropriately labeled scintillation vial. The cell pellet was then resuspended in 500 μL of medium and centrifuged. The supernatant was removed and added to the scintillation vial containing the initial supernatant. This process was repeated. Following the wash step, 100 μL of 0.1 M NaOH was added to the pellet and the dissolved pellet was transferred to a scintillation vial. The total radioactivity added to the cells at a given DBT concentration was determined by adding that

same amount of ^{14}C-DBT to a separate scintillation vial and counting it with the samples. Results are given as μM DBT accumulated in the cells. The volume of the cells was estimated using the number of NK cells that were present and multiplying that number by the volume of an individual NK cell (approximately 7.35×10^{-10} mL) to get the total volume of the cells (Whalen et al., 2008).

Statistical Analysis

Statistical analysis of the data was carried out utilizing ANOVA and Student's t test. Data were initially compared within a given experimental setup by ANOVA. A significant ANOVA was followed by pair wise analysis of control versus exposed data using Student's t test.

RESULTS

Effect of GSH added immediately prior or 1 h prior to TBT exposures on the TBT-induced inhibition of NK cell lytic function

GSH (1 mM) was added to NK cells either immediately or 1 h prior to a 1 h, 24 h, or 48 h exposure to various concentrations of TBT. Figure 1A shows that a 1 h exposure to 500 nM TBT decreased NK lytic function by greater than 90% and 300 nM TBT caused a 67.5% decrease . However, when 1 mM GSH was added to the NK cell just before the addition of the TBT, 500 nM TBT was able to decrease lytic function by only 29% and 300 nM decreased function by only 13.7%. There was very significant protection of lytic function at both concentrations of TBT ($P < 0.00001$). A 1h pre-incubation with GSH prior to adding the TBT diminished the effectiveness of the GSH at protecting against 500 nM TBT but did not significantly decrease its effectiveness at protecting against 300 nM TBT.

When NK cells were exposed to 200 nM, 100 nM , or 50 nM TBT for 24 h the losses of lytic function were 89.4%, 59.1%, and 25%, respectively. The presence of 1 mM GSH during the exposure to TBT for 24h diminished the negative effects of the TBT to a significant ($P<0.02$) extent at 100, and 50 nM TBT (Figure 1B). However, the extent of protection was greatest at the 100 nM exposure where the cells that received 1 mM GSH prior to TBT exposure showed a 22.8% loss of lytic function as compared to the nearly 60% loss of function in the absence of GSH (Figure 1B). Effects were similar whether the GSH was added immediately prior to TBT exposure or 1h prior.

A 48 h exposure to 100 nM or 50 nM TBT decreased the lytic function of NK cells by 72.9% and 42.8%, respectively (Figure 1C). There was some significant protective effect of having added GSH. The decrease at 100 nM went from about 73% to only 53% ($P<0.04$). The decrease in effectiveness of GSH is likely due to the fact that the GSH has been oxidized under the incubation conditions such that essentially no reduced GSH remains after 48h. This was determined by incubating GSH in cell culture medium for 48 h and assaying for reduced GSH (data not shown).

Effect of GSH added immediately prior or 1 h prior to DBT exposures on the DBT-induced inhibition of NK cell lytic function

GSH (1 mM) was added to NK cells either immediately or 1 h prior to a 1 h, 24 h, or 48 h exposure to various concentrations of DBT. Exposure to 10 μM DBT decreased NK lytic function by 83% and 5 μM DBT caused a 41.8% decrease . When 1 mM GSH was present at the time of the addition of the DBT, 10 μM DBT was able to decrease lytic function by only 9% and 5 μM did not decrease function at all (Figure 2A). There was very significant protection of lytic function from the DBT-induced decreases in function by GSH ($P < 0.00001$). A 1h pre-incubation with GSH prior to adding the DBT showed essentially the same results as were seen with no pre-incubation.

NK cells exposed to 1 μM or 0.50 μM DBT for 24 h showed decreases in lytic function of 93.1% and 67.1%, respectively (Figure 2B). The presence of 1 mM GSH during the exposure to DBT for 24h diminished the negative effects at both DBT concentrations to a significant extent ($P<0.00001$). When GSH was present during the exposure to 1 μM DBT the decrease in lytic function was only 26.6% and there was no decrease seen with 0.5 μM DBT (Figure 2B). Effects were similar whether the GSH was added immediately prior to DBT exposure or 1h prior.

Exposures of NK cells to 1 μM or 0.50 μM DBT for 48 h decreased the lytic function by 94.6% and 50.8%, respectively (Figure 2C). There was some significant protective effect of having added GSH. The decrease with1 μM went from about 95% to 60% ($P<0.01$) and the decrease seen with 0.5 μM was only 11% as compared to 50.8% in the absence of GSH. However, there did seem to be somewhat of decrease in effectiveness after 48h (Figure 2D).

Effect of GSH on the uptake of DBT by NK cells

The ability of GSH to prevent DBT from entering NK cells, as a mechanism for its effect on decreasing the toxicity of DBT, was examined using a ^{14}C-DBT. Figure 3A shows the uptake of 10 μM and 5 μM at 1h in NK cells with and without added GSH (1 mM). The uptake was not significantly affected by the presence of 1 mM GSH at either of the DBT concentrations. Figure 3B shows the effect of GSH on the uptake of 1 μM and 0.5 μM DBT by NK cells after a 24 h incubation. Again, there was no statistically significant alteration of DBT uptake when 1mM GSH was present in the incubation.

DISCUSSION

Past studies have shown that sulfydryl containing compounds such as GSH were able to block the negative effects of TBT on the function of phagocytes in tunicates (Cima and Ballarin, 2004) as well as the negative effects of DBT on oxidative phosphorylation (Cain et al., 1977). Additionally, the ability of TBT to directly interact with sulfydryl groups has been demonstrated using column chromatography (Wronski, 1976). Previously, we had shown that both TBT and DBT were able to decrease the lytic function of human NK cells (Dudimah et al., 2007a,b; Whalen et al., 1999). The current study examined the capacity of exogenous GSH to alleviate the negative effects of both TBT and DBT on the lytic function of human NK cells.

The decreases in lytic function that occurred after 1 h, 24 h, and 48 h exposures of NK cells to TBT were diminished when GSH was present in the incubation medium. The protective effect of the GSH was not enhanced by allowing the cells to pre-incubate with the GSH before adding the TBT. The protective effect of the GSH on NK lytic function was consistent with the results of past studies showing that GSH was protective of hemocytes from *B. Schlosseri* (Cima and Ballarin, 2004). Additionally, we found that as the reduced GSH levels decreased presumably due to oxidation that the protective effect also decreased (the protective effect was greatest for a 1 h exposure to TBT and least for a 48 h exposure to TBT). This suggested that the direct interaction of the TBT with the sulfhydryl group of GSH may be responsible for the protective effect, possibly by preventing the TBT from interacting with sulfhydryl groups in biomolecules important to the function of the NK cell. As mentioned above, direct interaction of TBTwith sulfhydryl groups has been demonstrated (Wronski, 1976).

NK cells exposed to DBT showed dramatic decreases in lytic function at each time point that was examined (1 h, 24 h, 48 h). The presence of GSH in the incubation medium had a very significant protective effect on lytic function. As with TBT, there was no advantage seen when the GSH was added 1 h prior to the addition of the DBT. However, the capacity of the GSH to diminish the negative effects of DBT on NK lytic function was greater with DBT than with TBT. For example, a 1 h exposure to the highest concentration of TBT caused an 89% loss of

lytic function which was reduced to 29% when GSH was present, while the highest concentration of DBT caused an 83% decrease in function which was decreased to only 9% in the presence of GSH. This same trend was true at other concentrations and lengths of incubation. This suggested that the interaction of DBT with GSH may be stronger than that of TBT. This may be in part due to the fact that the tin atom of DBT is less sterically hindered in terms of being able to coordinate with an additional atom (such as the sulfur of GSH) than that of TBT (Smith and White, 1972).

We found that the antioxidant vitamin E had no remedial effect on the butyltin-induced loss of NK lytic function, suggesting that the potential interaction with the sulfur group and not the antioxidant effect of GSH was important in its ability to diminish the negative effects of the organotins. Finally we found that GSH in the incubation medium did not decrease the uptake of DBT by NK cells, which would have been a possible explanation for its ability to spare NK lytic function.

CONCLUSIONS

TBT- and DBT-induced decreases in the lytic function of human NK cells can be decreased by the presence of reduced glutathione in the incubation medium during exposure to the compounds. The ability of GSH to protect NK cells from the negative effects of DBT was greater than for TBT. The protective effect of GSH did not appear to be simply due to its capacity to act as an antioxidant. Finally, the capacity of GSH to form a complex with the organotins and prevent their entry into the NK cell did not appear to be the explanation for the protection that it exerted.

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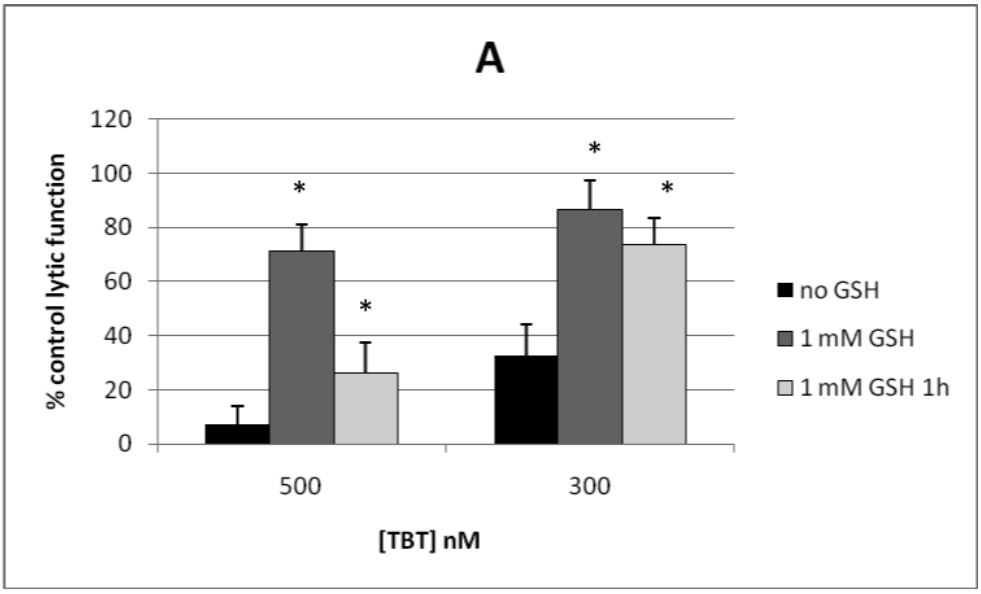
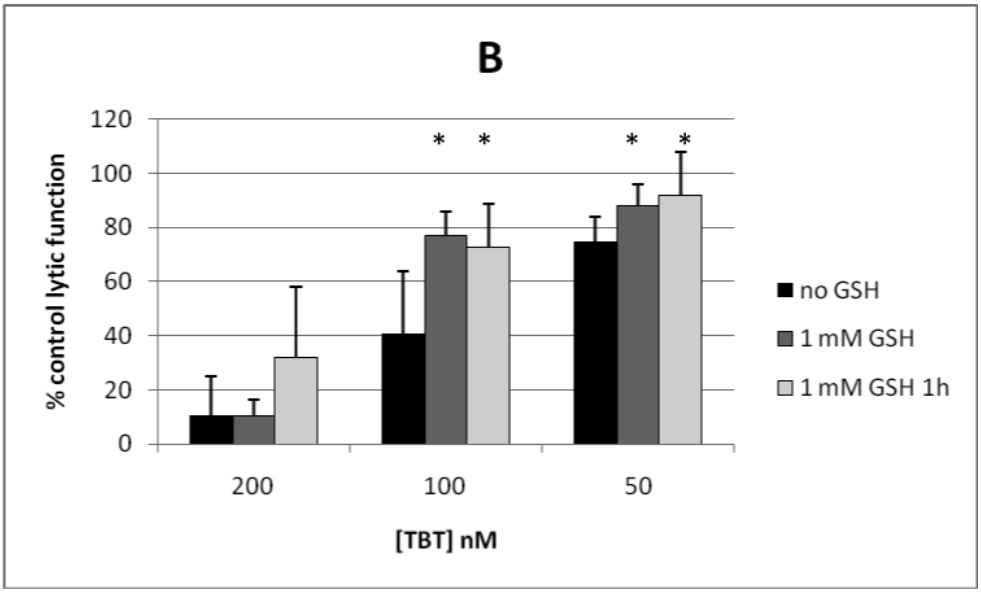




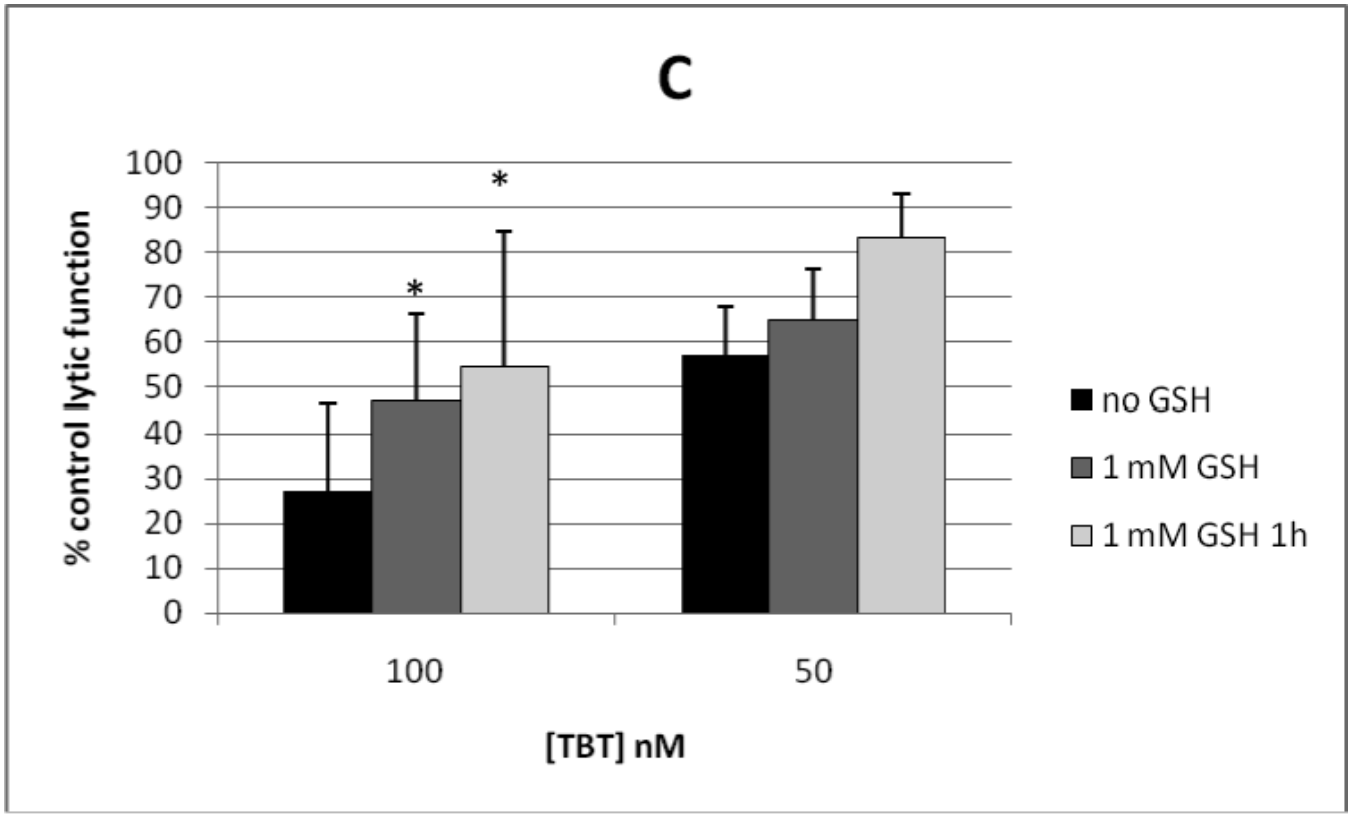
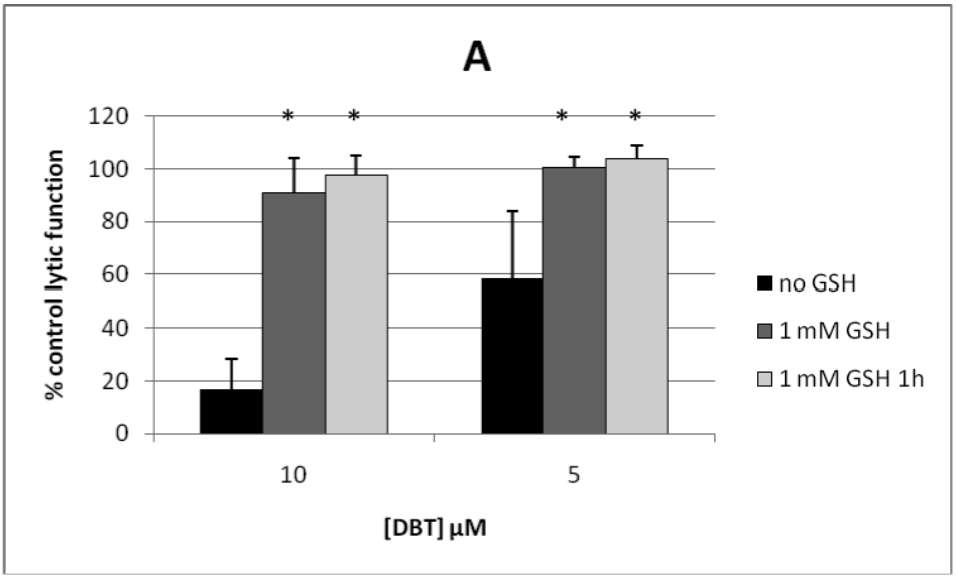
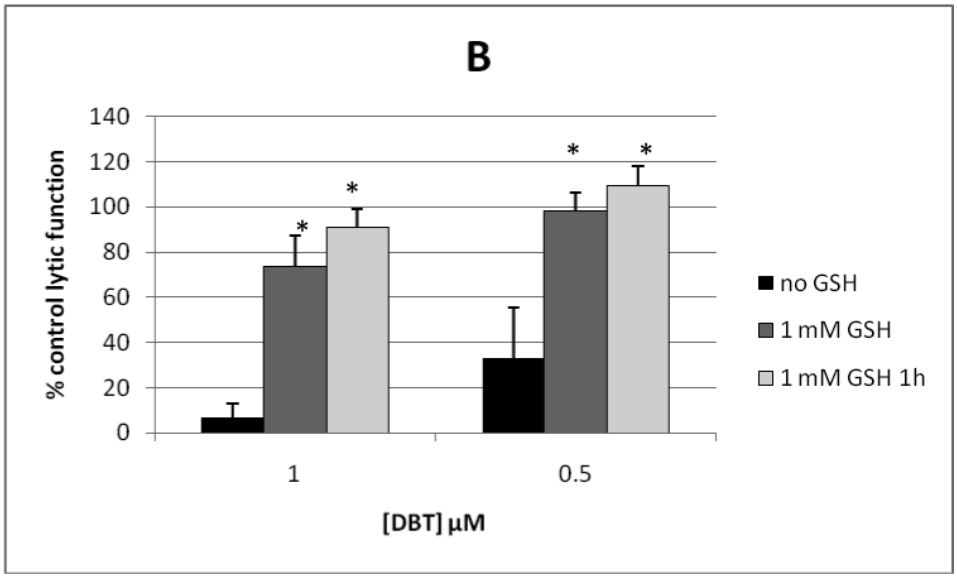
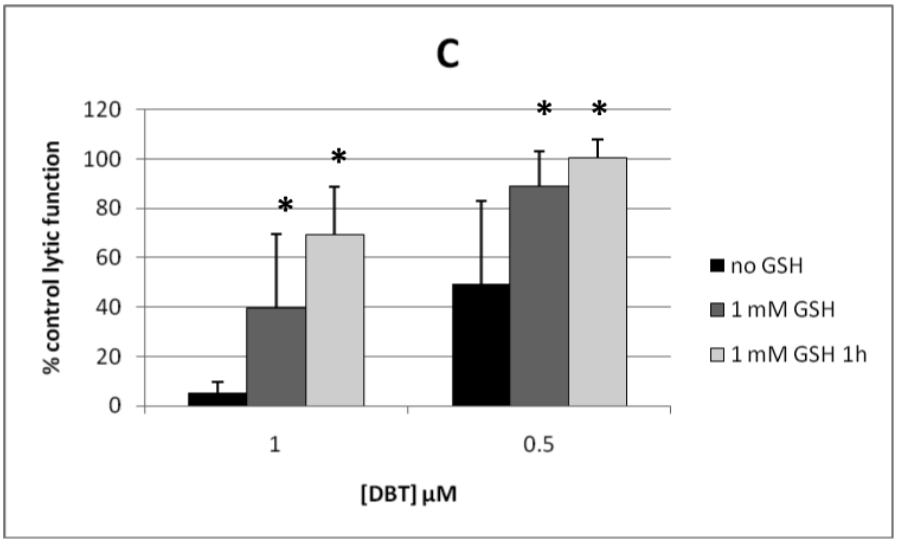


Figure 1.

Effect of 1 mM GSH on the TBT-induced decreases in NK lytic function. A.) NK cells were exposed to either 500 nM or 300 nM TBT for 1 h in the absence (black bars) or presence of 1 mM GSH, which was added immediately prior (dark gray bars) to or 1 h in advance of (light gray bars) the addition of the TBT. * indicates a statistically significant difference from cells exposed to TBT in the absence of GSH ($P<0.00001$). B.) NK cells were exposed to 200 nM, 100 nM, or 50 nM TBT for 24 h in the absence or presence of GSH, as described above for the 1 h exposure. * indicates a statistically significant difference from cells exposed to TBT in the absence of GSH($P<0.02$). C.) NK cells were exposed to 100 nM or 50 nM TBT for 48 h in the absence or presence of GSH, as described for the 1 h exposure. * indicates a statistically significant difference from cells exposed to TBT in the absence of GSH ($P<0.04$). The concentrations of TBT tested at each length of exposure were based on prior studies (Dudimah et al. 2007b) and the fact that these concentrations do not decrease the viability of NK cells at those lengths of exposure. Data are the mean±S.D. for a minimum of 9 determinations from a minimum of three individual donors.







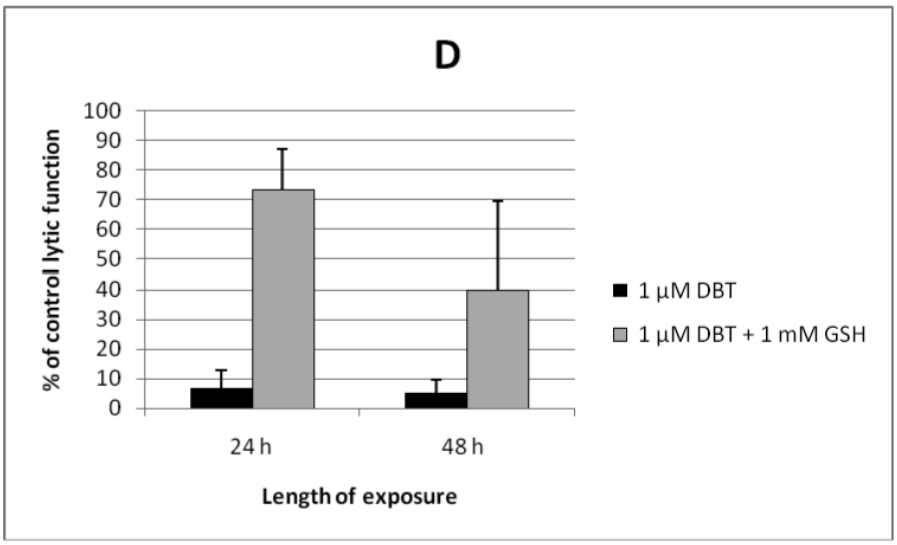
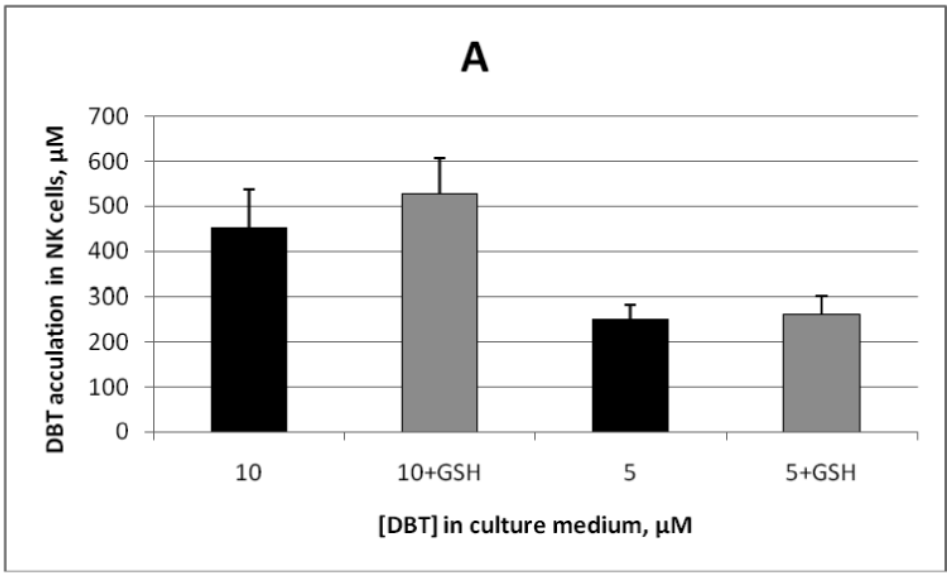


Figure 2.

Effect of 1 mM GSH on the DBT-induced decreases in NK lytic function. A.) NK cells were exposed to either 10 μM or 5 μM DBT for 1 h in the absence (black bars) or presence of 1 mM GSH, which was added immediately prior (dark gray bars) to or 1 h in advance of (light gray bars) the addition of the DBT. * indicates a statistically significant difference from cells exposed to TBT in the absence of GSH ($P<0.00001$). B.) NK cells were exposed to 1 μM or 0.50 μM DBT for 24 h in the absence or presence of GSH, as described above for the 1 h exposure. * indicates a statistically significant difference from cells exposed to TBT in the absence of GSH ($P<0.00001$). C.) NK cells were exposed to 1 μM or 0.50 μM DBT for 48 h in the absence or presence of GSH, as described for the 1 h exposure. * indicates a statistically

significant difference from cells exposed to TBT in the absence of GSH ($P<0.01$). D.) Shows the results of exposures to 1 μM DBT in the absence and presence of 1 mM GSH (no preincubation) as a function of the length of exposure. The concentrations of DBT tested at each length of exposure were based on prior studies (Dudimah et al. 2007a) and the fact that these concentrations do not decrease the viability of NK cells at those lengths of exposure. Data are the mean±S.D. for a minimum of 9 determinations from a minimum of three individual donors.



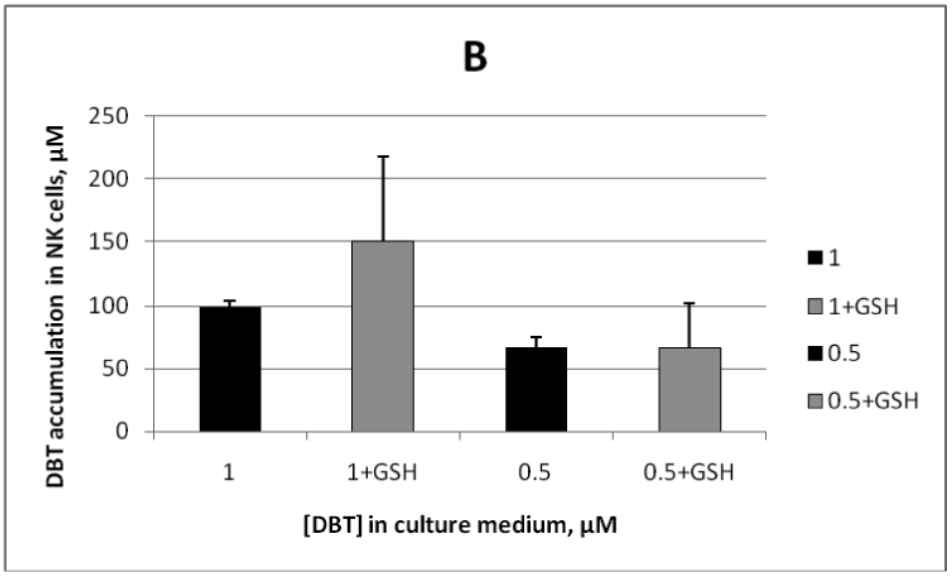


Figure 3.

Effect of 1 mM GSH on the accumulation of DBT by NK cells. A.) NK cells were exposed to either 10 µM or 5 µM DBT for 1 h in the absence (black bars) or presence (gray bars) of 1 mM GSH, which was added immediately prior the addition of the DBT. Uptake was determined using ^{14}C-labelled DBT as described in Whalen et al. 2008. B.) NK cells were exposed to either 1 µM or 0.5 µM DBT for 24 h in the absence (black bars) or presence (gray bars) of 1 mM GSH, which was added immediately prior the addition of the DBT. Data are the mean±S.D. for 9 determinations at 1 h and 6 determinations at 24 h from a minimum of three individual donors.