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Hexabromocyclododecane Decreases the Lytic Function and ATP Levels of Human Natural Killer Cells

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Abstract

This study investigates the effect of Hexabromocyclododecane (HBCD) on the lytic function of human natural killer (NK) cells and on ATP levels in NK cells. NK cells are capable of lysing tumor cells, virally infected cells, and antibody-coated cells. HBCD is a, brominated cyclic alkane used primarily as an additive flame retardant. If HBCD interferes with NK cell function, this could increase risk of tumor development and/or viral infection. NK cells were exposed to various concentrations of HBCD for 24 h, 48 h, and 6 days before determining lytic function and ATP levels. ATP levels and lytic function were also determined in NK cells that were exposed to HBCD for 1 h followed by 24 h, 48 h, and 6 days in HBCD-free media. The results indicated that exposure of NK cells to 10 μM HBCD for 24 h causes a very significant decrease in both NK cell lytic function and ATP levels (93.5% and 90.5%, respectively). Exposure of NK cells to 10 μM HBCD for 1 h followed by 24 h in HBCD-free media showed a progressive and persistent loss of lytic function (89.3%) as well as a decrease in ATP levels (46.1%). The results indicate that HBCD exposures decreased lytic function as well as ATP levels. However, a decrease in lytic function was not necessarily accompanied by a similar decrease in ATP. Importantly, these results also indicate that a brief (1 h) exposure to HBCD causes a progressive loss of lytic function over a 6 d period.

Keywords

ATP; NK cells; Hexabromocyclododecane; lytic function

INTRODUCTION

Hexobromocyclododecanes (HBCDs) are non-aromatic, brominated cyclic alkanes used primarily as additive flame retardants (Birnbaun et al., 2004). HBCDs are used in foam and expanded polystyrene. End products include upholstered furniture, interior textiles, as well as building materials such as house walls, cellars, roofs and parking decks (de Wit, 2002). In 2001, 16,700 metric tons were produced worldwide, in which, 9500 metric tons were sold in the European Union.

Since HBCDs are not chemically bound to plastics or textiles, they can separate and leach from the surface of these products into the environment (de Wit, 2002). HBCDs are extremely hydrophobic/lipophilic compounds and thus have high potential for bioaccumulation (Germer et al., 2006). They have been detected in soil, sediment and sewage sludge (Hale et al., 2006), and also in dust particles in the air (Abdallah et al., 2008). HBCDs have also been found

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in several species of bird eggs (Bustnes et al., 2007; Polder et al.,2008), and marine animals (Peck et al., 2008), indicating accumulation/biomagnification in the food chain (Covaci et al., 2006). HBCDs are found in sewage sludge (de Boer et al., 2002; Sellstrom et al., 1999) and agricultural use of these sludges may distribute the HBCDs to the soil and thus into the food chain (Covaci et al. 2006). A Swedish study found elevated levels of HBCDs in air and sediments (Remberger et al., 2004). This same study also found atmospheric deposition of HBCDs at a remote site indicating that the persistence of HBCDs is such that they may undergo long-range transport (Remberger et al., 2004). HBCDs do not appear to be metabolized and in rainbow trout have been shown to decrease the cytochrome P4501A (CYP1A) protein levels and enzyme activity and to induce the enzyme, catalase (Ronisz et al., 2004).

Human exposure to HBCDs is believed to be through dust ingestion and dietary exposure (Abdallah et al., 2008) mainly through seafood (Knutsen et al., 2008; van Leeuwen et al., 2008). Moderate concentrations of HBCDs (100 pg/g of whole blood and 200 pg/g serum) have been found in human blood (Covaci et al., 2006; Knutsen et al., 2008), adipose tissue (Pulkrabova et al., 2009), and breast milk (Kakimoto et al., 2008; Thomsen et al., 2005; Thomsen et al., 2003). There are limited studies on the toxicological effects of HBCD on humans; however there have been studies on lab animals. van der Ven et al found when rats were exposed to HBCD, there was an increase in liver and pituitary weight, and also increased levels of cholesterol (van der Ven et al., 2006). HBCD has also been shown to have neurotoxic effects in mice (Eriksson et al., 2006) and to increase hepatic cytochrome P450 levels in rats (Germer et al., 2006).

Human Natural Killer (NK) cells are a subset of lymphocytes that are capable of killing tumor cells, virally infected cells, and antibody-coated cells. NK cells are defined by the absence of the T cell receptor/CD3 complex and by the presence of the CD56 and/or CD16 on the cell surface (Lotzova, 1993). NK cells play a central role in immune defense against viral infection and formation of primary tumors (Lotzova, 1993; Vivier et al., 2004). NK cells are responsible for limiting the spread of blood-borne metastases as well as limiting the development of primary tumors (Lotzova, 1993). NK cells also play a central role in immune defense against viral infection as evidenced by increased incidences of viral infection seen in individuals where the NK subset of lymphocytes is completely absent (Fleisher et al., 1982; Biron et al., 1989). These cells are the front line of immune response against tumor and virally infected cells due to their ability to lyse appropriate target cells without prior sensitization. Interference with NK-cell function by any compound could increase the risk of viral infection and tumor formation.

The goal of the current study was to examine whether HBCD interferes with the lytic function of NK cells. Effects of both chronic and acute (1 h followed by up to 6 d in HBCD-free media) exposures were examined. A progressive and/or persistent negative effect of a brief HBCD exposure indicates a potentially greater risk from the compound. Finally, the effects of HBCD on ATP levels in NK cells were studied, as decreases in ATP could account for a loss of lytic function.

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) or Lifeblood Biological Services (Memphis, TN) 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 (~ 25o 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 preparations used in the current study was >95% CD16+, and CD56+, 0% CD3+ by fluorescence microscopy and flow cytometry (Whalen et al., 2002).

Chemical preparation

HBCD was purchased from Fisher Scientific. HBCD was dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to give a 100 mM stock solution. Desired concentrations of HBCD were prepared by dilution of the stock into complete media containing 0.5% gelatin in place of the 10% bovine serum. Gelatin replaced BSA to avoid binding of HBCD to serum albumin, which could interfere with its delivery the cells. The final concentration of DMSO for HBCD exposures did not exceed 0.01%.

Cell Treatments

NK cells (at a concentration of 1.5 million cells/mL) were exposed to HBCD or Control for 24 h, 48 h or 6 days. Following Exposures, the cells were assayed for tumor-destroying function or ATP levels. Additionally, NK cells were exposed to HBCD for 1 h; following the 1 h exposure period, the HBCD-containing or control media was removed and replaced with fresh HBCD-free media and the cells were incubated in compound-free media for 24 h, 48 h or 6 days prior to being assayed for tumor-destroying function or ATP levels. The concentration range examined was 10 μM-0.5 μM HBCD.

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., 2003). 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 h 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.

ATP Assay

Following exposures of cells to the various concentrations of HBCD; 250,000 NK cells were added to 300 μL of somatic cell ATP releasing reagent (Sigma-Aldrich). To measure ATP,

100 μL of the lysed cell suspension was added to 100 μL of a Luciferin/Luciferase mixture (Sigma-Aldrich). The light emission was measured using a Fluoroskan Ascent FL luminescence plate reader (Thermo Electron Corp., Vantaa, Finland). ATP levels were determined from a standard curve (Dudimah et al., 2007).

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

Effects of exposures to HBCD for 24 h, 48 h, and 6 days on the Tumor-lysing function of NK Cells

Highly purified NK cells were exposed to 2.5–10 μM HBCD for 1 h, 0.5–10 μM HBCD for 24 h, 0.25–2.5 μM HBCD for 48 h, and 0.25–1 μM HBCD for 6 days. Exposure of NK cells to 10 μM for 1 h caused a very small but statistically significant decreases in lytic function (11.8% ± 7%, $p<0.01$). Exposures of NK cells to 2.5, 5 and 10 μM HBCD for 24 h caused very significant reductions in NK cell lytic function of 44.2±9.3%, 92.2±2.2%, and 94.9±2.7%, and respectively ($P<0.0001$) (Figure 1). There was no decrease of lytic function at 1 μM and 0.5 μM concentrations with a 24 h exposure. NK cells were exposed to concentrations of 0.5–2.5 μM HBCD for 48 h because higher concentrations caused decreased viability of the NK cells. Exposure of NK cells to 2.5 μM HBCD for 48 h decreased lytic function by 75.6±3.6% ($P<0.0001$) (Figure 1). Six day exposures of NK cells to HBCD were carried out at concentrations of 0.25 μM– 1 μM, as higher concentrations of HBCD decreased the viability of the NK cells. There was a decrease in tumor-lysing function with a 6 d exposure to 1 μM HBCD of 44±26.6% ($P<0.02$) (Figure 1).

Effects of Exposures to HBCD for 1 h Followed by 24 h, 48 h, and 6 Days in HBCD-Free Media on the Tumor-Lysing Function of NK Cells

NK cells were exposed to 10 μM HBCD for 1 h showed only a 12% decrease in lytic function and those exposed to 5 or 2.5 μM HBCD showed no loss of lytic function (Figure 1). However, it is possible that an initial exposure to HBCD for 1 h could cause changes in NK cell lytic function that would manifest (and/or intensify) at later time points. To address this possibility, NK cells were exposed to 2.5 – 10 μM HBCD for 1 h which was then removed and the NK cells were then suspended in HBCD-free media for 24 h, 48 h or 6 days before assaying for lytic function. When NK cells were exposed to 2.5–10 μM, HBCD for 1 h and then incubated in HBCD-free media for 24 h prior to assaying for lytic function, decrease in lytic function of 24.9±13.5% (2.5 μM), 69.2±28.4% (5 μM), and 89.3±10.6% (10 μM) were seen ($P<0.0001$) (Figure 2). NK cells exposed to these same concentrations of HBCD and incubated for 48 h in HBCD-free media prior to assay, showed reductions in lytic function of 22.1±20.7% (2.5 μM), 52.4±16.9% (5 μM), and 88.1±11.2% (10 μM) ($P<0.02$). Exposure of NK cells to these same HBCD concentrations for 1 h, followed by a 6 day period in HBCD-free media decreased lytic function 39.7±32.2% (2.5 μM), 94.8±4.9% (5 μM),, and 89.9±2.15% (10 μM), ($P<0.0001$).

Effects of Exposures to HBCD for 24 h, 48 h, and 6 Days on ATP Levels in NK Cells

To determine the effects of varying concentrations of HBCD on the ATP levels in human NK cells, the cells were exposed to HBCD or control for the indicated lengths of time and the amount of ATP in control cells and treated cells were calculated from a standard curve. To combine results from separate experiments (using cells from different donors) the levels of ATP were normalized to the ATP level from the control cells in a given experiment (percentage

of control). NK cells exposed to 2.5 μM, 5 μM, and 10 μM HBCD for 24 h showed significant decreases in ATP levels of 52.4±15.9%, 78.4±2.8%, and 90.5±3.1%, respectively ($P<0.0001$) (Figure 3). A reduction in ATP levels of 50.2±34.3% was seen with a 48 h exposure to 2.5 μM HBCD ($P<0.0001$) (Figure 3). A 6 day exposure of NK cells to 0.5 μM and 1 μM HBCD lead to very small decreases in ATP levels of 26.7±32.2% and 13.8 ±19.0%, respectively ($P<0.02$) (Figure 3).

Effects of Exposures to HBCD for 1 h Followed by, 24 h, 48 h, and 6 Days in HBCD-Free Media on ATP Levels in NK Cells

As with lytic function, it is possible that an initial exposure to HBCD for 1 h could cause changes in NK cell ATP levels that would manifest later, even thought the compound has been removed. Thus, we examined if the effects of exposures to HBCD persisted and/or intensified following removal of the compound. To address this possibility, NK cells were exposed to 2.5 – 10 μM HBCD for 1 h which was then removed and the NK cells were then suspended in HBCD-free media for 24 h, 48 h or 6 days before assaying for ATP levels. NK cells exposed to HBCD for 1 h and then incubated for 24 h in HBCD-free media prior to measuring ATP levels showed decreases of 23.2±10.8% (2.5 μM), 39±15% (5 μM), and 46.2±30.1% (10 μM), ($P<0.001$) (Figure 4). NK cells were exposed to 5 and 10 μM HBCD for 1h and given a 48 h period in HBCD-free media before assay showed decreases in ATP levels of 37.5±52.1% and 55.1±46.8%, respectively ($P<0.05$) (Figure 4). A 1 h exposure of NK cells to 2.5 μM HBCD followed by 6 d in HBCD-free media lead to a decrease in ATP of 50.3±39.6% ($P<0.01$) (Figure 4).

DISCUSSION

The ability of NK cells to lyse tumor cells is an essential immune function that if compromised could leave an individual with an increased susceptibility to developing cancer (Lotzova, 1993). HBCD has been found in bird eggs (Bustnes et al., 2007; Polder et al.,2008), and marine animals (Peck et al., 2008), indicating that it is accumulating and biomagnifiying in the food chain (Covaci et al., 2006). HBCD is found in sewage sludge (de Boer et al., 2002; Sellstrom et al., 1999) and agricultural use of these sludges may distribute the HBCDs to the soil and thus into the food chain (Covaci et al. 2006). It appears that there may be emission of HBCD from the products in which it is used and that it may undergo long-range transport (Remberger et al., 2004). HBCD does not appear to be metabolized (Ronisz et al., 2004). As HBCD is a very prevalent environmental contaminant that is found in human tissues (Covaci et al., 2006; Kakimoto et al., 2008; Knutsen et al., 2008; Pulkrabova et al., 2008; Thomsen et al., 2005; Thomsen et al., 2003) and does not appear to be metabolized ((Ronisz et al., 2004), it is important to examine its capacity to interefere with human NK cell function. This study examined whether HBCD exposure decreases the lytic function of NK cells. Additionally, this study examined if the effects of exposures to HBCD persisted and/or intensified following removal of the compound. If HBCD is able to cause continued disruption of NK function even after its removal this has implications for the level of hazard that even a limited exposure could present. Finally, in order to begin to address the mechanism of HBCD-induced decreases in lytic function, the effects of HBCD on ATP levels in NK cells were also examined, as the process by which NK cells lyse tumor cells is ATP-dependent (Andzelm et al., 2007).

Exposure of NK cells to10 μM HBCD for 24 h caused a very significant decrease in the ability of NK cells to lyse tumors cells, and also caused very significant decreases in levels of ATP. The decreases in ATP levels were roughly comparable to those of lytic function for each of the concentrations tested at 24 h. These results suggested that HBCD-induced decreases in ATP may in part cause the decreases in the lytic function seen after a 24 hour exposure to HBCD (Figure 5A). After a 48 h exposure to HBCD the decrease in ATP levels was about

76% and the decrease in ATP was about 50% with exposure to 2.5 μM HBCD (Figure 5B). A 6 day exposure of NK cells to 2.5 μM HBCD decreased lytic function by greater than 90% and decreased ATP levels by about 47% (Figure 5C). The decreases in ATP levels seen with 2.5 μM HBCD after 48 h and 6 d of exposure were both about 50%. This may suggest that a decrease in ATP of 50% is sufficient to decrease lytic function, which as mentioned above is an ATP-dependent process (Andzelm et al., 2007). However, exposure to 1 μM HBCD for 6 d caused a 44% decrease in lytic function but decreased ATP levels by only 13%. Previous studies using rotenone and oligomycin have shown that 13% decrease in ATP is not sufficient to cause any decrease in NK lytic function (Dudimah et al., 2007). Thus, the loss of lytic function seen with this exposure cannot be explained by ATP decreases.

As mentioned above, if loss of lytic function seen with HBCD exposures were to persist and/or intensify after the removal of the compound, this would indicate a potentially greater danger due to even a brief exposure to low levels of HBCD. The levels reported to be found in human serum average near 0.3 nM (Covaci et al., 2006). The results of studies examining the effects of a 1 h exposure followed by 24 h, 48 h, and 6 d in HBCD-free media on tumor lysing function were similar to those seen when NK cells were continuously exposed to HBCD for 24 h, 48 h, or 6 days. A 1 h exposure to 10 μM HBCD caused almost no decreases in lytic function (11.8%). However, in the 24 h period following this 1 h exposure the loss of lytic function was dramatically increased to about 90%. Importantly, the results show that a concentration such as 5 μM HBCD (which has no effect on NK lytic function after a 1 h exposure) causes a 70% loss of lytic function within the 24 h period following that exposure. This loss escalates to greater than 90% by 6 d following the initial 1 h exposure. Thus, there is a progressive impairment of NK cell function following a brief initial exposure to HBCD. In the periods following 1 h exposures to HBCD there were also decreases in ATP levels, which may possibly account for some of the loss of lytic function.

These results suggest that HBCD induces processes that lead to the persistent inhibition of the lytic process. Irreversible inhibitory binding of HBCD to a component(s) of the NK cell that is(are) needed for lytic function, may be one possible explanation. HBCD is a very lipophilic compound (Germer et al., 2006) and thus it may to some extent associate with cell membranes and thus alter the function of membrane proteins needed for binding and or recognition of target cells by NK cells. There are many possible target proteins in the NK cell where a hydrophobic compound such as HBCD might bind. The active sites of most cellular enzymes are usually hydrophobic pockets created from the protein structure (Copeland and Anderson, 2002) and would thus be ideal locations for HBCD to bind depending on whether the overall shape of the active site could accommodate the size of the HBCD. Enzymes critical to the NK lytic process, such as the mitogen activated protein kinase (MAPK) pathway (Trotta et al, 1998; Trotta et al., 2000; Wei et al., 1998) or the myosin IIA (Andzelm et al., 2007) needed for release of cytotoxic granules are just a few of the many possible targets. Previously we have shown that the activity of MAPK pathway enzymes was altered by another hydrophobic environmental contaminant, tributyltin (TBT), when NK cells were exposed for as little as 10 minutes (Aluoch and Whalen, 2005, Aluoch et al., 2006, Aluoch et al., 2007). Additionally, HBCD may bind in mitochondrial membranes either by associating in the lipid portion or by binding to specific proteins and this may lead to interference with electron transport/oxidative phosphorylation and decreased ATP levels. Again, TBT has been shown to bind to the Fo portion of F-ATP synthase in bacteria, which is very similar to mitochondrial ATP synthase (von Ballmoos et al., 2004). Further studies are needed to examine these potential areas of HBCD interaction with NK cells. However, it is clear from the current study that HBCD interferes with the ability of NK cells to carry out their essential function of lysing tumor cells. Thus, it has the potential for increasing the risk of cancer development in exposed individuals.

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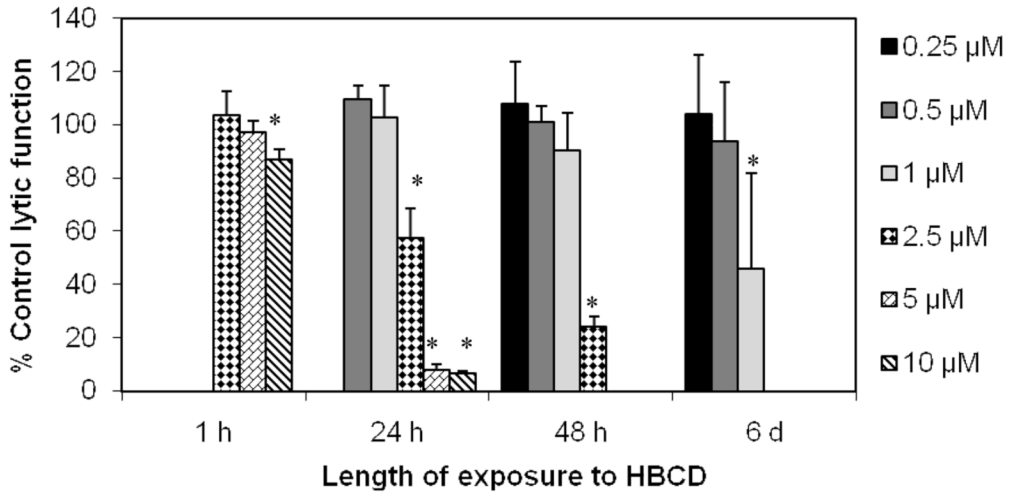


Figure 1.

Effects of HBCD exposures on the ability of NK cells to lyse tumor cells. NK cells were exposed to 10–0.25 μM HBCD for 1 h, 24 h, 48 h, or 6 days. To combine results from separate experiments (using cells from different donors) the levels of lysis were normalized as the percentage of the lytic function of the control cells in a given experiment. Results were from three separate experiments using different donors (triplicate determinations for each experiment, n = 9, mean±S.D.). * indicates statistically significant change ($p<0.05$)

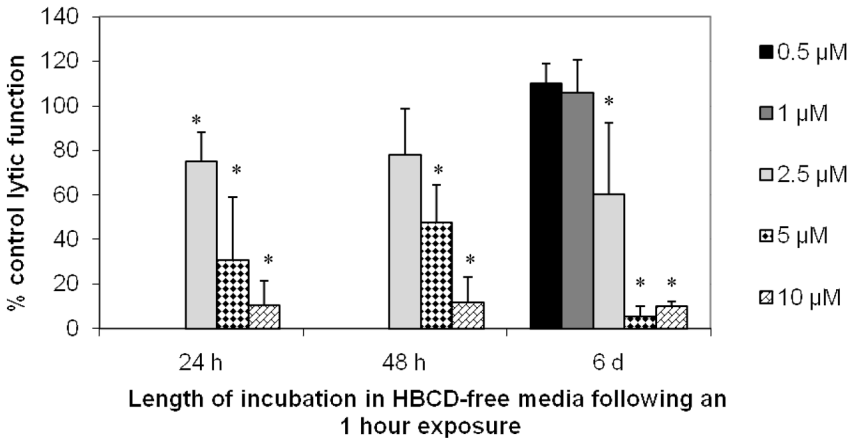


Figure 2.

Effects of 1 h exposures to HBCD followed by 24 h, 48 h, or 6 days in HBCD-free media on the ability of NK cells to lyse tumor cells. NK cells were exposed to 10–0.5 μM HBCD for 1 h. Results were from at least three separate experiments using different donors (triplicate determinations for each experiment, n =9, mean±S.D.), as described in Figure 1. * indicates statistically significant change ($p<0.05$)

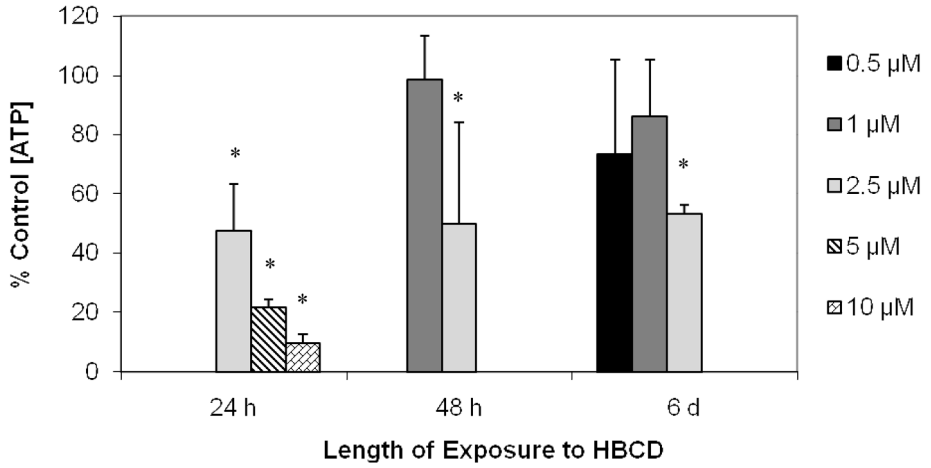


Figure 3.

Effects of HBCD exposures on ATP levels NK cells. NK cells were exposed to 10–0.5 µM HBCD for 24 h, 48 h, or 6 days. To combine results from separate experiments (using cells from different donors) the levels of ATP were normalized as the percentage of the ATP level from the control cells in a given experiment. Results were from three separate experiments using different donors (triplicate determinations for each experiment, n = 9, mean±S.D.). * indicates statistically significant change ($p<0.05$)

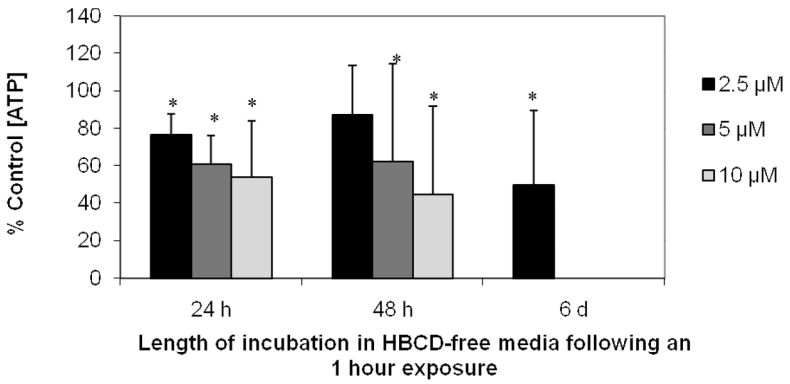
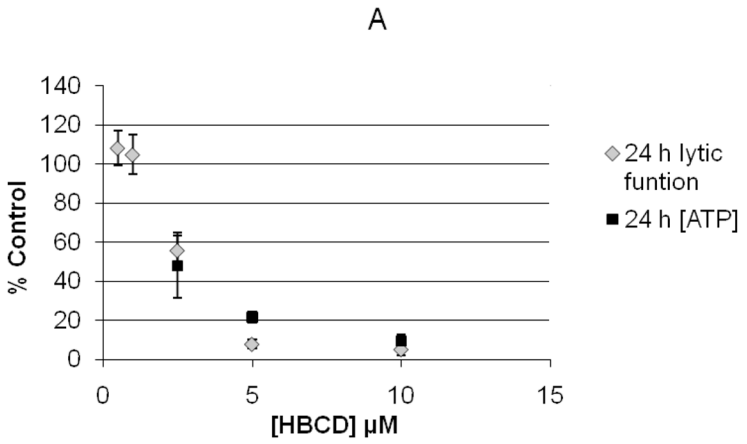
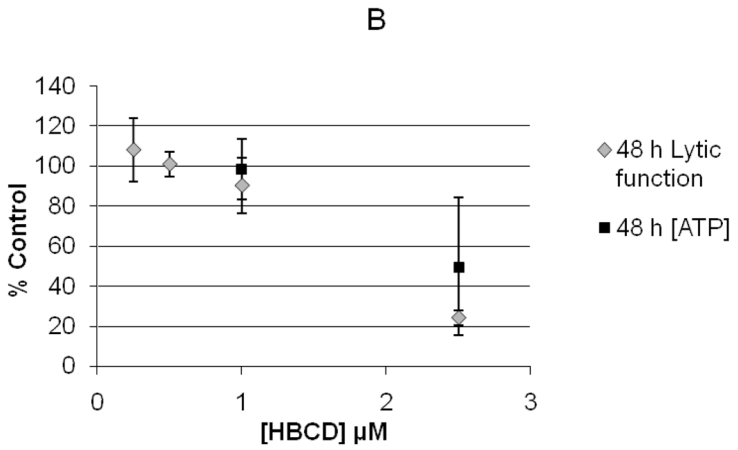


Figure 4.

Effects of 1 h exposures to HBCD followed by 24 h, 48 h, or 6 days in HBCD-free media on the ATP levels in NK cells. NK cells were exposed to 10–2.5 μM HBCD for 1 h. Results were from at least three separate experiments using different donors (triplicate determinations for each experiment, n=9, mean±S.D.), as described in Figure 3. * indicates statistically significant change ($p<0.05$)





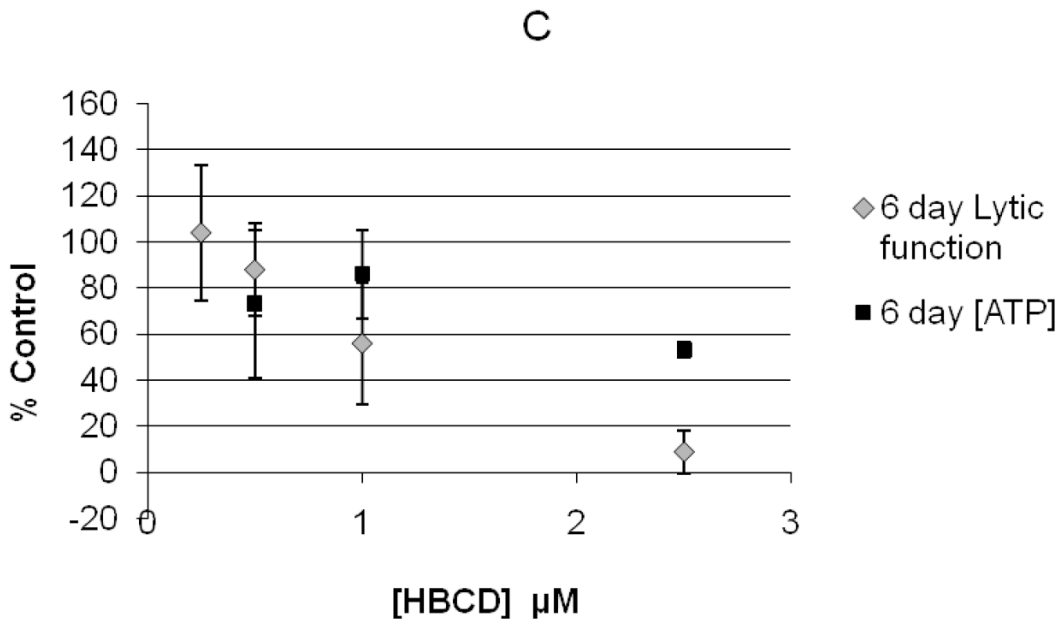


Figure 5.
Effects of HBCD exposure on lytic function and ATP levels (plotted on the same graph). (A) 1 h exposure to HBCD, (B) 24 h exposure to HBCD, (C) 48 h exposure to HBCD, (D) 6 days exposure to HBCD.

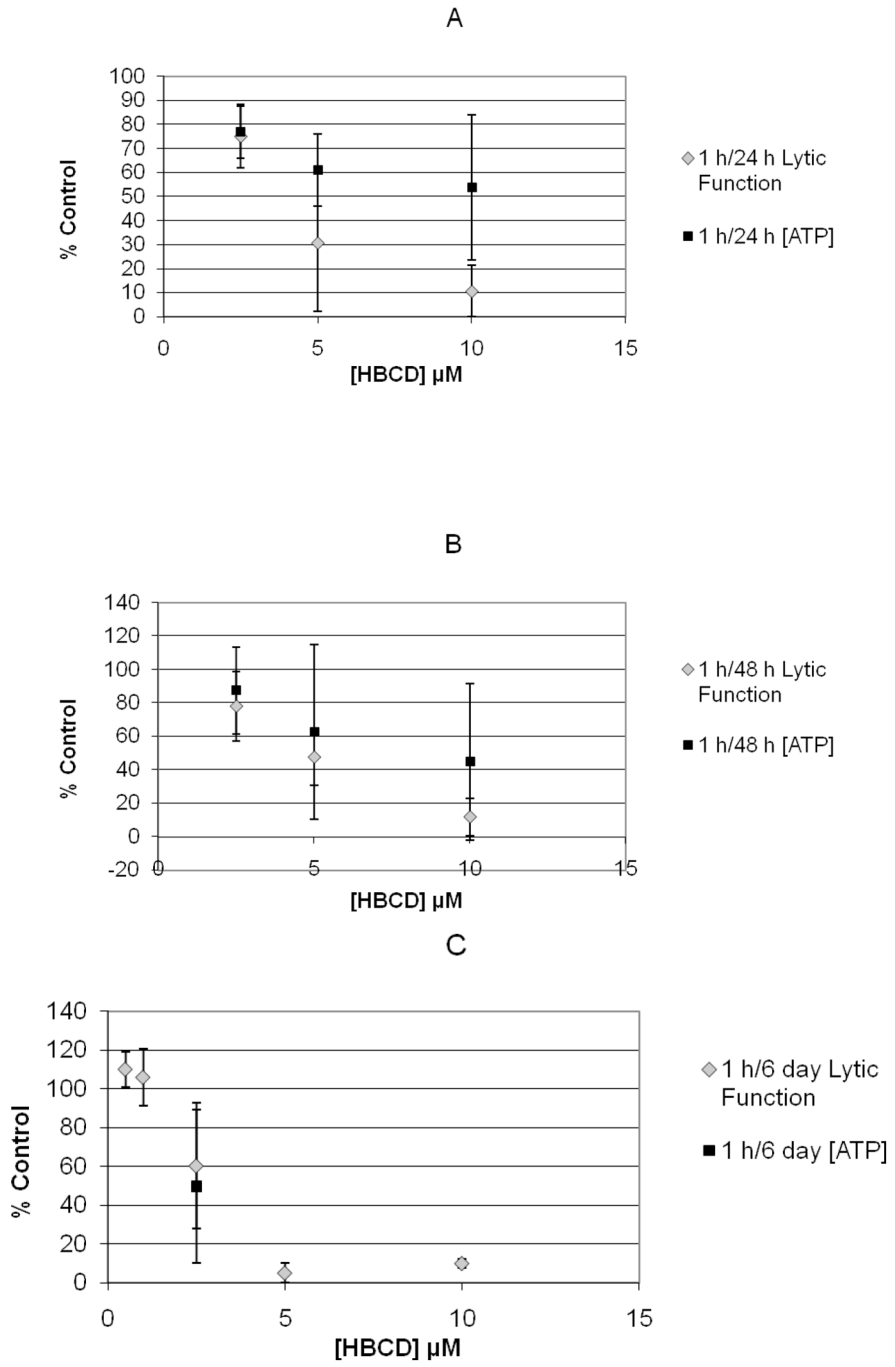


Figure 6.
Effects of 1 h exposures to HBCD followed by varying periods in HBCD-free media on lytic function and ATP levels (plotted on the same graph). (A) 1 h exposure to HBCD followed by 24 h in HBCD-free media, (B) 1 h exposure to HBCD followed by 48 h in HBCD-free media, (C) 1 h exposure to HBCD followed by 6 days in HBCD-free media.