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# Hexabromocyclododecane Decreases Tumor-cell-binding Capacity and Cell-Surface Protein Expression of Human Natural Killer Cells

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## Abstract

Hexabromocyclododecane (HBCD) is a flame retardant that decreases the lytic function of human natural killer (NK) cells. NK cells defend against tumor cells and virally infected cells. Thus, HBCD has the potential to increase cancer incidence and viral infections. NK cells must bind to their targets for lysis to occur. Thus, concentrations of HBCD that decrease lytic function were examined for their ability to alter NK binding to tumor targets. Levels of HBCD that caused a loss of binding function were examined for effects on expression of cell surface proteins needed for binding. NK cells exposed to HBCD for 24 h, 48 h, or 6 days or to HBCD for 1 h followed by 24 h, 48 h, or 6 days in HBCD-free media were examined for binding function and cell surface protein expression. The results indicated that exposure of NK cells to 10 μM HBCD for 24 h (which caused a greater than 90% loss of lytic function) caused a very significant decrease in NK cell binding function (70.9%), and in CD16 and CD56 cell-surface protein expression (57.8%, and 24.6% respectively). NK cells exposed to 10 μM HBCD for 1 h followed by 24 h in HBCD-free media (which caused a 89.3% loss of lytic function) showed decreased binding function (79.2%), and CD 16 expression (48.1%). Results indicate that HBCD exposures decreased binding function as well as cell-surface marker expression in NK cells and that these changes may explain the losses of lytic function induced by certain HBCD exposures.

### Keywords

hexabromocyclododecane; NK cells; CD16; CD56; binding function

## INTRODUCTION

Human natural killer (NK) cells are a primary immune defense against tumor cells and virally infected cells. NK cells prevent the development as well as the metastases of tumors (Lotzova, 1993; Vivier et al., 2004). They also play a central role in defending against viral infections. Thus, there is increased incidence of viral infections in individuals where the NK subset of lymphocytes is completely absent (Fleisher et al., 1982; Biron et al., 1989). 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). The NK cell must physically bind to a target cell (be it a tumor cell or virally infected cell) in order to destroy (lyse) the target. Both CD16 and CD56 may play a role in NK cell binding to target cells (Lotzova, 1993; Mandelboim et al.,

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1999). As these cells are the front line of immune response against tumor and virally infected cells (due to their ability to lyse target cells withour prior sensitization), anything that interferes with their function could lead to an increased incidence of tumors or viral infections.

Hexabromocyclododecane (HBCD) is a widely used brominated flame retardant. In 2001, the world-wide demand for HBCD was 16,700 metric tons (Covaci et al. 2006). HBCD is used in polystyrene foams that are used as thermal insulation in the building industry (Kajiwara et al. 2009). Additionally, it is applied to upholstery textiles that are used as furniture, drapery, and wall coverings (Kajiwara et al. 2009). During the manufacturing process of consumer products (such as upholstery fabrics), HBCD is usually sprayed on, or impregnated by immersing textiles in a solution mixed with HBCD (Kajiwara et al., 2009). Since it is not chemically bound to plastics or textiles, it can separate and leach from the surface of these products into the environment (de Wit, 2002). HBCD appears to bioaccumulate as it is a very hydrophobic/lipophilic compound (Germer et al., 2006). Due to its hydrophobic nature it binds to soil, sediments, and sewage sludge (Hale et al., 2006), and is found in dust particles in the air (Abdallah et al., 2008). HBCD has been detected in marine animals (Peck et al., 2008) and bird eggs (Bustnes et al., 2007; Janak et al., 2008; Polder et al., 2008). Its presence in eggs and animals indicates accumulation/biomagnification in the food chain (Covaci et al., 2006). Agricultural use of HBCD contaminated sewage sludge (de Boer et al., 2002; Sellstrom et al., 1999) may be one route to introducing HBCD into the soil and thus into the food chain (Covaci et al. 2006). One study also found atmospheric deposition of HBCD at a remote site indicating that the persistence of HBCD is such that it may undergo long-range transport (Remberger et al., 2004).

Dietary exposures as well as inhalation of contaminated dust particles appear to be major routes of HBCD exposure for humans (Abdallah et al., 2008). Some seafood contains measurable levels of HBCD and would thus be a source of dietary exposures (Knutsen et al., 2008; van Leeuwen et al., 2008). HBCD exposed rainbow trout have decreased cytochrome P4501A (CYP1A) protein levels and enzyme activity and show an induction of the enzyme, catalase (Ronisz et al., 2004), while a study of HBCD exposed rats showed increases in CYP2B1 and CYP2B2 (Germer et al., 2006). Additionally, rats have shown increased liver and pituitary weight and increased cholesterol in response to HBCD exposures (van der Ven et al., 2006) and it appears to be neurotoxic in mice (Eriksson et al., 2006). HBCD has been found in human blood (100 pg/g of whole blood and 200 pg/g serum) (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). Recently, we have shown that human NK cells exposed to HBCD exhibit significantly decreased ability to destroy tumor target cells (Hinkson and Whalen, 2009). Importantly, it was shown that the negative effects of an initial HBCD exposure maintain and/or develop following removal of the compound indicating a potentially greater risk from any exposure to the compound (Hinkson and Whalen, 2009).

In the current study the effects of HBCD exposures on the ability of NK cells to bind to target cells was examined. As mentioned earlier, binding is an essential step in the process by which NK cells lyse target cells. If HBCD exposures were to decrease the ability of NK cells to bind to targets, this could explain (at least in part) the HBCD-induced loss of lytic function. Certain cell surface proteins are needed for NK binding to target cells (Lotzova, 1993; Mandelboim et al., 1999). Effects of both chronic and acute exposures to HBCD were examined for their effects on binding and cell surface protein expression.

## 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) and Key Biologics, LLC (Memphis, TN) were used to prepare NK cells. Highly purified NK cells were obtained using a rosetting procedure. Buffy coats were mixed with 0.6–0.8 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). 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 30–40 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-inactivated bovine calf serum (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., 2002a).

### 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%. Appropriate DMSO controls were run.

### 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 binding capacity or cell-surface marker expression. 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 being assayed for binding function or cell-surface protein expression. The concentration range examined was 0.5–10 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.

### Conjugation Assay

The percentage of target cells with bound NK cells was determined at two effector to target ratios 12:1 and 6:1. The NK cells were treated as described above. Control and HBCD exposed NK cells were then suspended at a concentration of 240,000/50 μL (for the 12:1 ratio) and 120,000/50 μL (6:1 ratios). The target cell was the NK-susceptible K562 cell (human chronic myelogenous leukemia). Target cells were suspended at a concentration of 20,000/50 μL. Tumor cells (50 μL) were then placed in the wells of microwell plates containing 50 μL of control or HBCD exposed lymphocytes. Each condition was tested in triplicate. The plate was centrifuged at 300 g for 3 min and the cells were incubated at 37° C, air/$CO_2$, 19:1, for 10 min

and then placed on ice. Following the incubation period, the cells were gently resuspended with a micropipette, placed in a hemacytometer and viewed under a light microscope. The number of target cells with two or more lymphocytes bound to their surface was counted as well as the total number of targets to determine the percentage of tumor cells with lymphocytes bound. The minimum number of targets counted per determination was 50–100 (Whalen et al., 1992).

### Flow Cytometry

NK cells were exposed to the appropriate concentrations of HBCD for the appropriate length of time. Following exposure to HBCD, the cells were washed and prepared for analysis on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The cells were washed twice with ice-cold PBS and 100 μl of cell suspension (250,000–500,000 cells) was labeled with 10 μl of one of the following antibodies: anti-CD2, CD11a, CD11c, CD16, CD18, CD56, TNF-α, and Fas-L. (Pharmingen, San Diego, CA). Anti- CD2, CD11a, CD16, TNF-α, and Fas-L were FITC-conjugated antibodies. Anti- CD11c, CD18, and CD56 were phycoerythrin (PE)-conjugated. Appropriate FITC- and PE-conjugated isotype control antibodies were used. Each antibody was a monoclonal antibody (mouse IgGκ) that was specific for the human cell surface protein, such as CD16. Appropriate FITC- and PE-conjugated isotype control antibodies were used. The antibody-containing cell suspension was incubated for a minimum of 30 minutes on ice, in the dark. Following the incubation period the cells were washed twice with ice-cold PBS (1 mL) and suspended in 500 μl of ice-cold 1% paraformaldehyde in PBS. Samples were analyzed using the the FACSCalibur flow cytometer from Becton Dickinson Immunocytometry Systems, Inc (BDIS), San Jose, CA. Instrument performance was standardized weekly using Calibrite beads (BDIS) and the same instrument settings were used for all acquisitions. The assays were sufficiently uniform to use the same forward scatter (FSC), side scatter (SSC), and fluorescence (FL) settings. The sensitivity of the instrument was constant. The acquisition and analysis software for flow cytometry data was CELLQuest Pro from BDIS running on an Apple computer.

### 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, a p value of less than 0.05 was considered significant.

## RESULTS

### Effects of Exposures to HBCD for 24 h, 48 h and 6 day on the Binding Function of NK Cells

Highly purified NK cells were exposed to 2.5–10 μM HBCD for 24 h, 2.5μM HBCD for 48 h, and 1–2.5 μM HBCD for 6 days. Exposure to 10 μM HBCD for 24 h lead to a large decrease in NK cell binding function (70.9% ± 11.8%). At the 2.5 μM and 5 μM concentrations, there were moderate decreases in binding function (29.5% ± 17.3% and 41.9% ± 19.4% respectively) (Figure 1). A 48 h exposure to 2.5 μM HBCD caused a significant decrease (37.3% ± 14.6%) in tumor binding function (Figure 1). When NK cells were exposed to 1 and 2.5 μM of HBCD for 6 days, there was a significant decrease in tumor binding function (67.6% ± 16.9% and 76.4% ± 16.9% respectively) (Figure 1). To combine results from separate experiments (using cells from different donors) the tumor binding caused by treated cells was normalized to that of control cells in a given experiment.

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

NK cells were exposed to HBCD for 1 h after which the compound containing media was removed and the cells were washed two times with compound-free media. The NK cells were then suspended in HBCD-free media for 24 h, 48 h or 6 days before assaying for tumor binding function. Exposures of NK cells to 2.5 μM, 5 μM and 10 μM HBCD for 1 h followed by a 24 h incubation in HBCD-free media (Figure 2), caused significant decreases in binding function (43.0% ± 9.5%, 65.2% ± 18.2% and 79.2% ± 14.9%respectively). When NK cells were exposed to 2.5 μM, 5 μM, and 10 μM HBCD for 1 h followed by a 48 h period in HBCD-free media, there were also significant decreases in binding function (48.2% ± 19.9%, 48.1% ± 40.9% and 81.8% ± 15.3%, respectively) (Figure 2). 1 h exposures to 2.5 and 5 μM HBCD followed by a 6 day incubation in HBCD-free media resulted in large decreases (72.6% ± 13.5% and 88.8% ± 5.0%) in tumor biding function (Figure 2).

### Effects of Exposures to HBCD for 24 h, 48 h and 6 Days on Cell-Surface Protein Expression

Levels of key cell surface molecules involved in the NK cell interaction with target cells were monitored using flow cytometry. Control and HBCD-exposed cells were incubated with fluorescent-labeled antibodies for specific cell surface markers. The antibodies used were anti-CD2, CD11a, CD11c, CD16, CD18, CD56, TNF-α, and Fas-L. Of the cell surface proteins examined, only CD16 and CD56 expression were decreased by exposure of NK cells to HBCD. When NK cells were exposed to 10 μM HBCD for 24 h, there was a 57.8% ± 24.5% ($p < 0.05$) decrease in CD16 expression (as measured by mean fluorescence intensity) and a 24.6%±9.2% ($p<0.05$) decrease in CD56 expression. Figures 3A and B shows the shift in peak fluorescence intensity for a representative experiment. At the 5 μM concentration, there was only a significant decrease in CD16 expression (63.8±16.5%, $p<0.05$) (Figure 4, representative experiment). There was no significant decrease in CD16 or CD56 expression at the 2.5 μM concentration. When NK cells were exposed to 2.5 μM HBCD for 48 h or 1 or 2.5 μM HBCD for 6 days, there were no significant decreases in CD16 and CD56 expression.

### Effects of Exposure to HBCD for 1 h Followed by 24 h, 48 h, and 6 Days in HBCD-Free Media on Cell- Surface Protein Expression

Of the cell surface proteins examined, only CD16 expression was decreased. A 1 h exposure to 10 μM HBCD followed by 24 h in HBCD-free media caused a 48.2% ± 9.8%, ($p < 0.05$) decrease in CD16 expression (Figure 5, representative experiment), while a 1 h exposure to 5 μM and 2.5 μM HBCD followed by 24 h in HBCD-free media did not caused a significant decrease in CD16 expression. Additionally, 1 h exposures to HBCD followed by 48 h or 6 d incubations in HBCD-free media caused no statistically significant decreases in CD16 expression.

## DISCUSSION

In a recent study we found that exposure of human NK cells to the brominated flame retardant, HBCD, caused very significant decreases in the ability of NK cells to destroy tumor target cells (Hinkson and Whalen, 2009). This same study showed that a brief exposure to HBCD (1 h) could induce processes that lead to dramatic decreases in lytic function in the 24 h, 48 h, or 6 day period following the initial exposure. HBCD is an additive flame retardant and is known to leach from the products to which it is added into the environment (de Wit, 2002). It has been found in a variety of human tissues (Kakimoto et al., 2008; Pulkrabova et al., 2009; Thomsen et al., 2005,2003) including human blood (Covaci et al., 2006; Knutsen et al., 2008). The levels of HBCD found in human blood and serum (0.3 nM in serum) are lower than those where we see effects on NK lytic function (1 μM), however, there is a possibility of much higher accumulations in individuals who work in the application of HBCD to the various product in

which it is used. As we have shown that HBCD has negative effects on human NK immune function that persist even after its removal, it is important to address further the mechanism of HBCD-induced loss of NK lytic function. The purpose of the current study was to examine the effects of HBCD on the ability of NK cells to bind to their targets and to express cell surface markers, known to be involved in NK binding function, as possible causes of the HBCD-induced decreases in lytic function. NK cell binding to their targets is the first step of achieving target lysis. Thus, if HBCD were to interfere with binding, that could at least in part explain the HBCD-induced loss of lytic function. Since NK cells use specific cell surface proteins to recognize and bind to their targets, any observed decrease in binding could be explained by an accompanying decrease in specific cell surface protein expression.

Figure 6 plots the loss of lytic function seen in a previous study (Hinkson and Whalen, 2009) with the loss of binding function seen in this study for ease of comparison. As shown in Figure 6A, loss of lytic function after 24 h exposures to HBCD was somewhat greater at some concentrations of HBCD than the loss of binding function. A 48 h exposure to 2.5 μM HBCD caused NK cells to lose 75.6% of lytic function and 37.3% of binding function (Figure 6B). 6 day exposure HBCD resulted in a large decrease in both lytic and tumor binding function (Figure 6C). Thus, it appears that the lack of ability of NK cells to bind to their target may be a significant part of the reason for the loss of lytic function but probably does not explain the extent of loss of lytic function that has been seen with 24 h and 48 h continuous exposures to HBCD. Lytic function (Hinkson and Whalen, 2009) and binding function of NK cells exposed to HBCD for 1 h and then given 24 h, 48 h, or 6 d in HBCD-free media prior to measuring function are compared in Figure 7. The loss of binding function and loss of lytic function were similar in extent in these cells indicating that loss of binding function may significantly account for the loss of lytic function seen under these conditions.

Loss of binding function could be due to loss of specific cell surface proteins utilized by NK cells to recognize and bind to their targets. Therefore, those HBCD exposures that caused loss of binding function were examined for their capacity to alter expression of several cell surface proteins. Certain cell surface markers are known to be involved in NK binding/lytic function. CD11a, CD18, CD16, and CD56 have all been shown to be important in binding to target cells (Lotzova, 1993: Mandelboim et al., 1999). The results indicated that there was a very significant loss of the cell surface proteins CD16 and CD56 in NK cells exposed to 10 μM HBCD for 24 h. Exposure to 5 μM HBCD for 24 h caused a significant decrease in CD16. Following a 1 h exposure to 10 μM HBCD there was a significant loss of CD16 after 24 h. Although there were losses of binding function seen after 24 h and 48 exposures to 2.5 μM HBCD and 6 d exposures to 1μM HBCD, there were no significant decreases in CD16 expression. Thus, decreases in CD16 expression can but does not necessarily accompany a loss of binding function in the HBCD exposed NK cells.

Studies examining the relationship between loss of NK binding function accompanying loss of lytic function have been carried out using other environmental contaminants, including butyltins, ziram, and another brominated flame retardant, tetrabromobisphenol A (Whalen et al., 2002a,b; Odman-Ghazi et al., 2003; Taylor et al., 2009; Kibakaya et al., 2009). These studies, like the current study, have shown that loss of binding function can, but does not necessarily, accompany a loss of lytic function. The effects of butyltins and ziram on the expression of cell surface proteins have also shown that there were very significant decreases in CD16 in NK cells under conditions where binding was decreased (Whalen et al., 2002b; Odman-Ghazi et al., 2003; Taylor et al., 2009). This is similar to what was seen with HBCD exposures. Thus loss of CD16 may be at least in part responsible for the loss of binding seen with HBCD as well as other environmental contaminants that have been shown to interfere with NK lytic and binding function. Since loss of binding was not always accompanied by statically significant decreases in CD16 it is likely that other aspects of the NK-target binding

process are being interfered with upon exposure to HBCD. Previously, it was shown that HBCD exposures decreased ATP levels in NK cells (Hinkson and Whalen, 2009) and there may be a role for diminished ATP levels in the decrease in binding as certain process involved in formation of the synapse between NK cells and target cells are ATP dependent (Andzelm et al., 2007).

In summary the current study shows: 1.) Exposure of NK cells to HBCD interferes with ability of NK cells to bind to tumor target cells; 2.) HBCD-induced loss of binding function does not appear to explain the extent of the loss of NK cell ability to lyse tumor cells under some conditions; 3.) HBCD-induced loss of binding function can be accompanied by a significant decrease in the NK cell surface protein CD16, which has a role in NK binding to certain target cells.

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Whalen MM, Ghazi S, Loganathan BG, Hatcher F. Expression of CD16, CD18 and CD56 in tributyltin-exposed human natural killer cells. Chemico-Biol Interactions 2002;139:159–176.

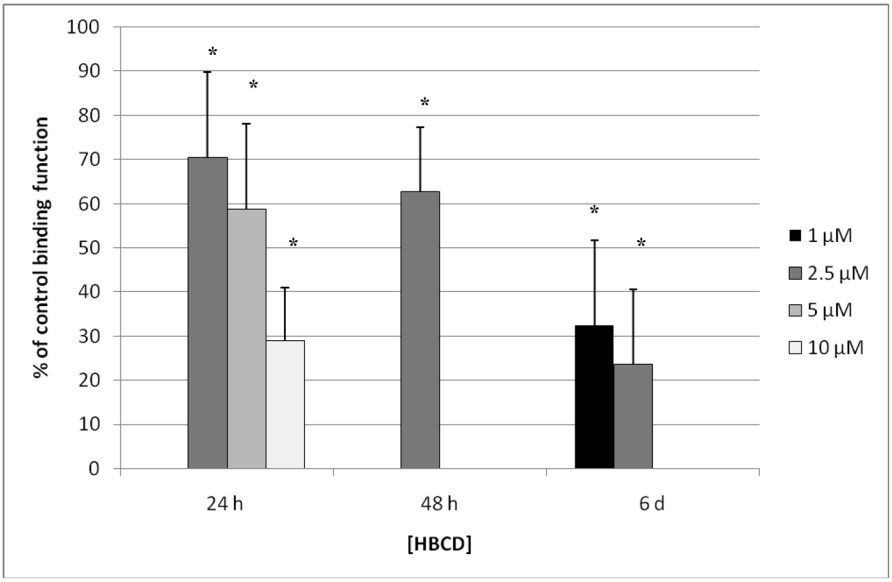



**Figure 1.**

Effects of 24 h, 48 h and 6 day continuous exposures to HBCD on the ability of NK cells to bind K562 tumor cells. Light gray bars = NK cells exposed to 1 μM HBCD; Medium gray bars = NK cells exposed to 2.5 μM HBCD; Dark gray bar = NK cells exposed to 5 μM HBCD; Black bars = NK cells exposed to 10 μM HBCD. Results are mean±S.D. (n=9 for 24 h and 48 h exposures and n=13 for 6 day exposures). * indicates that the decrease in binding was statistically significant ($p<0.01$).

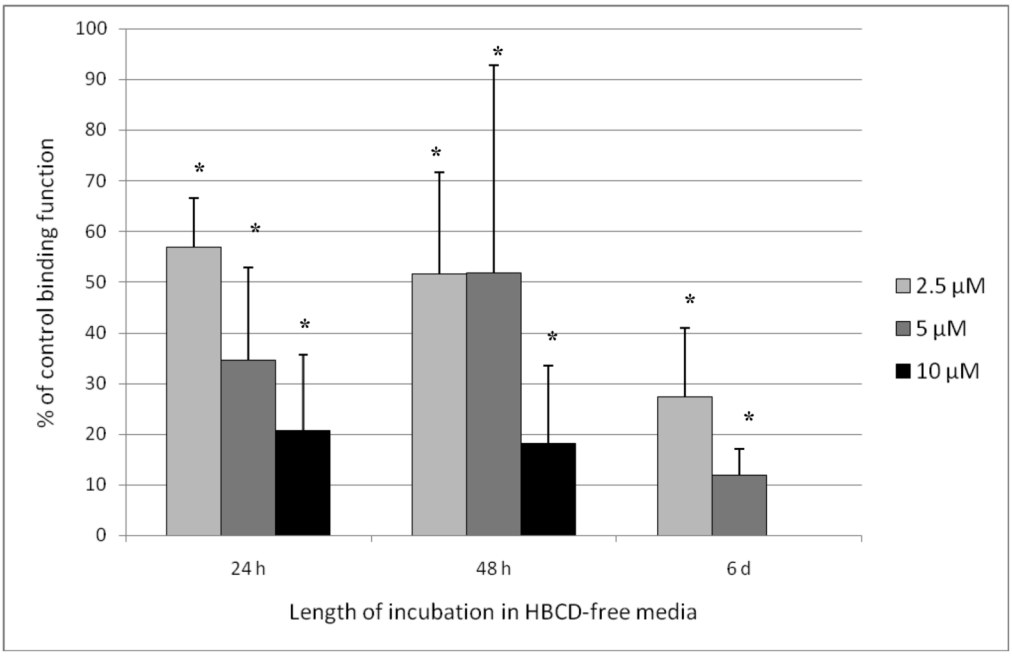
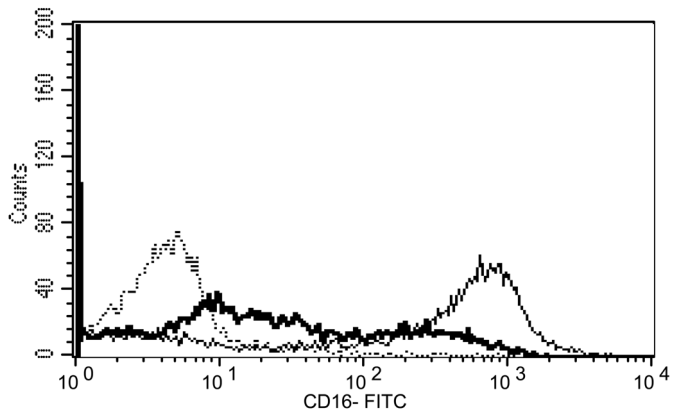



**Figure 2.**

Effects of a 1h exposures to HBCD followed by 24 h, 48 h and 6 day in HBCD-free media on the ability of NK cells to bind to K562 tumor cells. Medium gray bars = NK cells exposed to 2.5 μM HBCD; Dark gray bar = NK cells exposed to 5 μM HBCD; Black bars = NK cells exposed to 10 μM HBCD. Results are mean±S.D. (n=9 for all lengths of exposure). * indicates that the decrease in binding was statistically significant ($p<0.01$).

A

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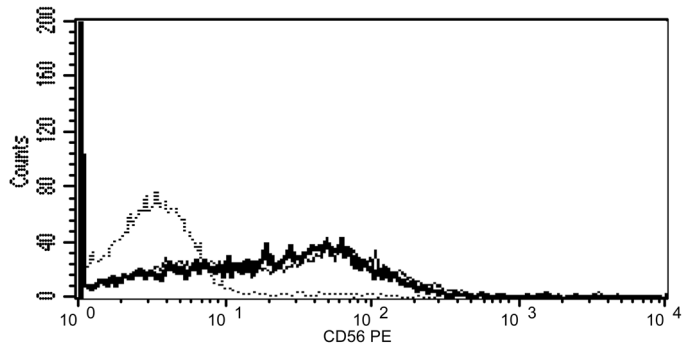


**Figure 3.**

Histograms from representative experiments showing effects of 24 h exposure to 10 μM HBCD on CD16 and CD 56 expression in NK cells. A.) 24 h exposure to 10 μM HBCD: Dashed line = IgG control; thin solid line = control NK cells stained with anti-CD16 antibody; bold line = HBCD-exposed cells stained with anti-CD16 antibody; y axis = cell number; x axis = fluorescence intensity. B.) 24 h exposure to 10 μM HBCD: Dashed line = IgG control; thin solid line = control NK cells stained with anti-CD56 antibody; bold line = HBCD-exposed cells stained with anti-CD56 antibody; y axis = cell number; x axis = fluorescence intensity.

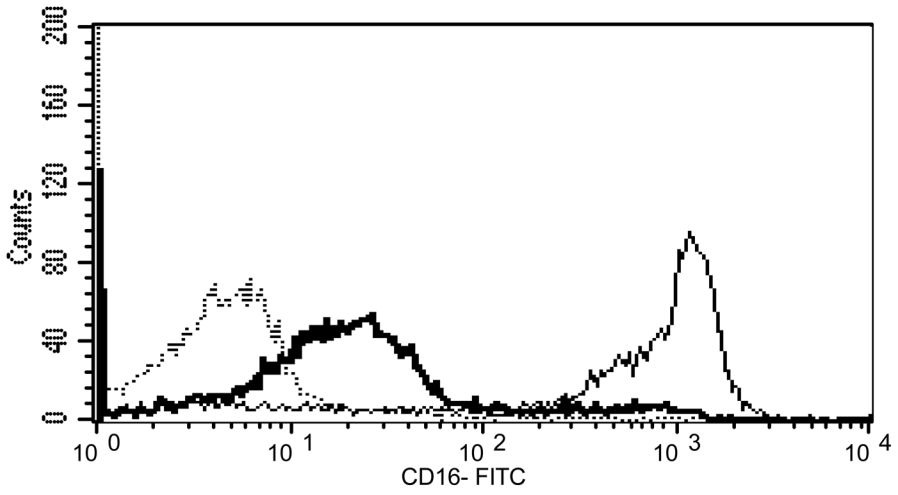


**Figure 4.**

Histogram from a representative experiment showing effects of 24 h exposure to 5 μM HBCD on CD16 expression in NK cells. Dashed line = IgG control; thin solid line = control NK cells stained with anti-CD16 antibody; bold line = HBCD-exposed cells stained with anti-CD16 antibody; y axis = cell number; x axis = fluorescence intensity. y axis = cell number; x axis = fluorescence intensity.

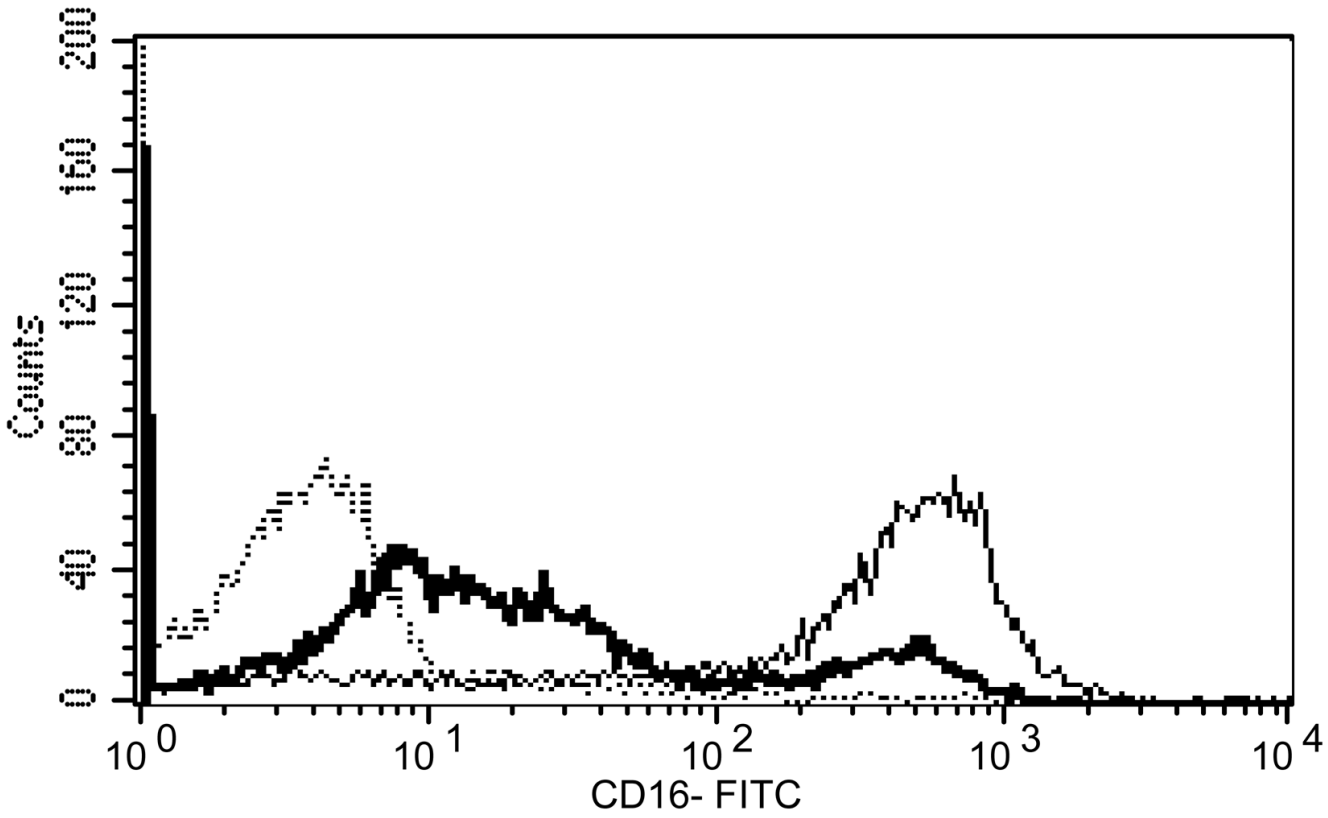


**Figure 5.**
Histogram from representative experiment showing effects of a 1h exposure to 10 μM HBCD followed by 24 h in HBCD media on CD16 expression in NK cells. Dashed line = IgG control; thin solid line = control NK cells stained with anti-CD16 antibody; bold line = HBCD-exposed cells stained with anti-CD16 antibody; y axis = cell number; x axis = fluorescence intensity; y axis = cell number; x axis = fluorescence intensity.

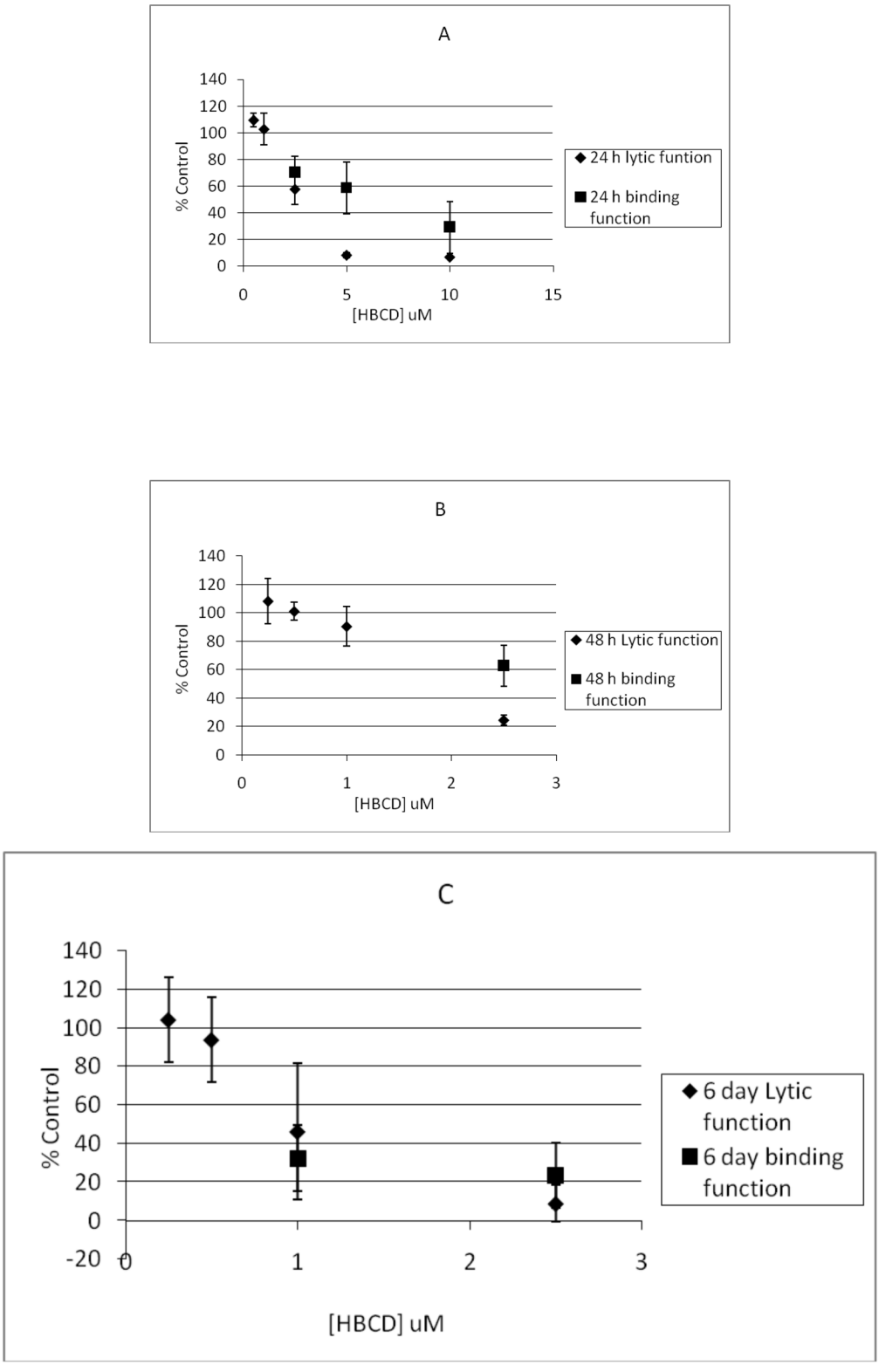


**Figure 6.**

Effects of HBCD exposure on lytic function (◆) and binding function (■) (plotted on the same graph). (A) 24 h exposure to HBCD, (B) 48 h exposure to HBCD, (C) 6 days exposure to HBCD.

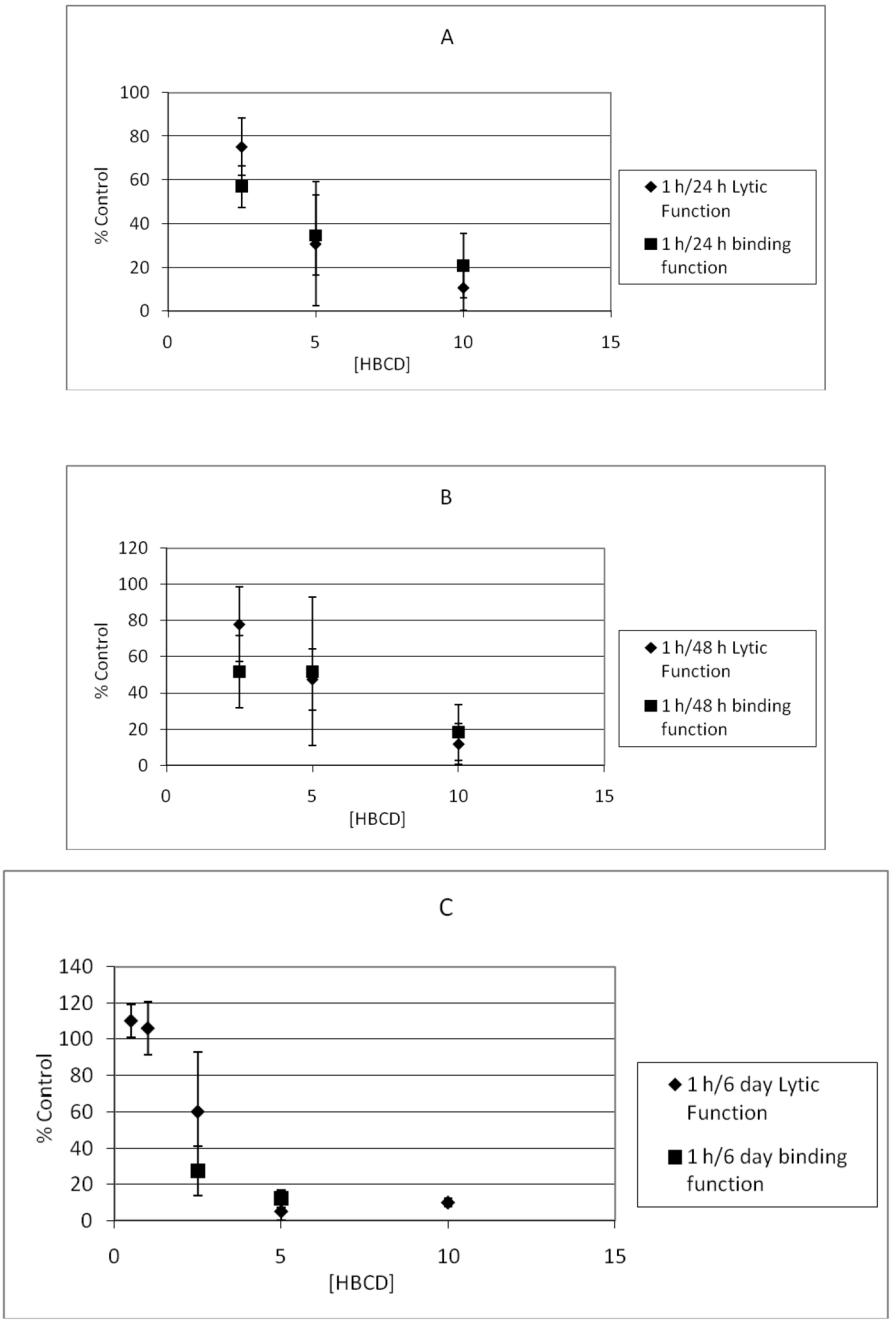


**Figure 7.**

Effects of 1 h exposures to HBCD followed by varying periods in HBCD-free media on lytic function (◆) and binding function (■) (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.