TOXICOLOGICAL SCIENCES **76,** 75–82 (2003)
DOI: 10.1093/toxsci/kfg225

Effect of Bromodichloromethane on Chorionic Gonadotrophin Secretion by Human Placental Trophoblast Cultures

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Received June 5, 2003; accepted August 14, 2003

Bromodichloromethane (BDCM) is a trihalomethane found in drinking water as a by-product of disinfection processes. BDCM is hepatotoxic and nephrotoxic in rodents and has been reported to cause strain-specific full-litter resorption in F344 rats during the luteinizing hormone-dependent phase of pregnancy. In humans, epidemiological studies suggest an association between exposure to BDCM in drinking water and increased risk of spontaneous abortion. To begin to address the mechanism(s) of BDCM-induced spontaneous abortion, we hypothesized that BDCM targets the placenta. Primary cultures of human term trophoblast cells were used as an *in vitro* model to test this hypothesis. Trophoblasts were allowed to differentiate into multinucleated syncytiotrophoblast-like colonies, after which they were incubated for 24 h with different concentrations of BDCM (20 nM to 2 mM). Culture media were collected and assayed for immunoreactive and bioactive chorionic gonadotropin (CG). Cultures exposed to BDCM showed a dose-dependent decrease in the secretion of immunoreactive CG as well as bioactive CG. The lowest effective BDCM concentration was 20 nM, approximately 35-times higher than the maximum concentration reported in human blood (0.57 nM). Trophoblast morphology and viability were similar in controls and cultures exposed to BDCM. We conclude that BDCM perturbs CG secretion by differentiated trophoblasts *in vitro*. This suggests that the placenta is a likely target of BDCM toxicity in the human and that this could be related to the adverse pregnancy outcomes associated with BDCM.

Key Words: **bioactivity; chlorination; trihalomethanes; pregnancy.**

Drinking water chlorination has been widely used around the world to prevent infectious disease. However, the disinfection by-products (DBPs) generated from chlorination have been reported to have a wide range of adverse effects on humans and laboratory animals (Boorman, 1999; Dunnick and Melnick, 1993; Swan *et al.*, 1998; Toussaint *et al.*, 2001). Trihalomethanes (THMs) are among the highest in concentration of the by-products. The bulk of the THMs is composed of four compounds: chloroform, bromodichloromethane (BDCM), chlorodibromomethane (CDBM), and bromoform, of which chloroform and BDCM are the most prevalent (Deinzer, 1978). It has been reported that concentrations of BDCM in chlorinated drinking water range from 1.9 to 183 mg/l, with an average of 16 mg/l (U.S. EPA, 1998; Krasner *et al.*, 1989). Currently, the maximum contaminant level for total trihalomethanes in drinking water in the United States is 80 mg/l (U.S. EPA, 1998). Humans are exposed to THMs through dermal absorption, inhalation, and ingestion. Most studies have focused on the carcinogenic effects of THMs, and there is an association between human exposure to total trihalomethane (TTHM) in drinking water and increased risk of cancer (Cantor *et al.*, 1999; Hildesheim *et al.*, 1998; King and Marrett, 1996; Villanueva *et al.*, 2001). BDCM is also hepatotoxic and nephrotoxic in laboratory animal models (Aida *et al.*, 1992; Chu *et al.*, 1982; Keegan *et al.*, 1998; Thornton-Manning *et al.*, 1994; Torti *et al.*, 2001).

An increased risk of small-for-gestational-age (SGA) births, stillbirths, spontaneous abortions, and low birth weight has been reported for women exposed to TTHM (Bove *et al.*, 1995; Dodds *et al.*, 1999; Gallagher *et al.*, 1998; Kramer *et al.*, 1992; Savitz *et al.*, 1995; Swan *et al.*, 1998; Waller *et al.*, 1998). Waller and colleagues reported an increased risk of spontaneous abortion among women who drank at least five glasses per day of cold tap water with ≥ 75 μg TTHM per liter. Stratified analysis further showed that only BDCM exposure was associated with spontaneous abortion (Waller *et al.*, 1998). In a Canadian study, increased risk of stillbirth was associated with THM consumption, and the strongest association was observed for BDCM exposure (King *et al.*, 2000); relative risk estimates

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associated with BDCM exposures were larger for asphyxia-related deaths than for unexplained deaths.

Several animal studies have investigated halocarbon-associated spontaneous abortion. Oral administration of BDCM was shown to cause full-litter resorption (FLR) in F344 rats (Bielmeier *et al.*, 2001; Narotsky and Kavlock, 1995; Narotsky *et al.*, 1997). Bielmeier and colleagues (Bielmeier *et al.*, 2001) showed a 62% incidence of FLR in rats following aqueous gavage treatment with BDCM between gestational days 6 and 10. This encompasses a critical, luteinizing hormone (LH)-dependent period of pregnancy in rats. More recent work indicates that reduced LH secretion (Bielmeier *et al.*, 2002) and reduced luteal responsiveness to LH (Bielmeier *et al.*, 2003) may both contribute to BDCM-induced FLR in F344 rats. However, these effects are likely to be strain specific, as several investigators have failed to observe FLR in BDCM-exposed Sprague-Dawley (SD) rats (Bielmeier *et al.*, 2001; Christian *et al.*, 2001; Ruddick *et al.*, 1983). When similar doses (75 mg and 100 mg BDCM/kg day) were administered to SD rats under the same conditions as F344 rats, all of the SD rats maintained their pregnancies (Bielmeier *et al.*, 2001). Christian and colleagues conducted developmental toxicity studies with BDCM in Crl SD rats and rabbits. Instead of being administered by oral gavage, pregnant rats and rabbits were allowed to drink BDCM-containing water *ad libitum*. FLR was not observed, and no adverse effects on embryo-fetal viability were found at levels up to 900 ppm (Christian *et al.*, 2001). The authors postulated that the sensitivity difference might reflect the different reproductive performance and endocrine physiology of the species and strains or the difference in toxicokinetics resulting from the route of exposure.

While further investigation of differences in strain susceptibility could lead to a greater understanding of the toxic mechanism of BDCM, and while rodents provide a valuable model to study the potential adverse effects of environmental hazards, it is important to note both the differences and similarities in reproductive physiology between the rat and the human. In higher primates, including humans, it is the products of conception (placental trophoblast) that support pregnancy. Early pregnancy in higher primates is maintained by luteal secretion of progesterone, which is supported by chorionic gonadotropin (CG), an LH-like glycoprotein, produced by placental syncytiotrophoblast. In contrast, in the rat, luteal maintenance during midpregnancy depends on pituitary LH, which is under the control of the hypothalamus and higher brain centers. Therefore, the target tissues of BDCM-induced pregnancy failure in rats may differ from those in human pregnancy; however, it is possible that parallel mechanisms may exist for LH-mediated toxicity in rats and CG-mediated toxicity in humans.

The above observations suggest that a possible target of BDCM toxicity in higher primates is the placental syncytiotrophoblast. Specifically, it is possible that BDCM disrupts the synthesis and/or secretion of syncytiotrophoblast-derived CG. In the present study, we have tested the idea that BDCM targets trophoblasts by examining the effect of BDCM on CG secretion by primary cultures of human trophoblasts. The results show that BDCM reduces the secretion of immunoreactive and bioactive CG.

MATERIALS AND METHODS

Trophoblast isolation and primary culture. A detailed description of the procedure used to isolate cytotrophoblast cells from term human placentas has been given previously (Douglas and King, 1989). Briefly, the method described by Kliman (Kliman *et al.*, 1987) was modified by the substitution of a continuous Percoll gradient (yielding greater than 95% cytotrophoblast as assessed by intermediate filament immunocytochemistry) and the addition of a final step using immunomagnetic microspheres (Douglas and King, 1989) to remove the few remaining vimentin-positive and HLA-positive cells (yielding 100% cytotrophoblast). Absence of vimentin staining was confirmed for each batch of trophoblast cells.

Trophoblast cells do not replicate in culture and were plated in either 24-well cluster dishes or LabTek chamber slides at a density of 400,000 cells/cm^2. In order to induce morphological differentiation and produce syncytiotrophoblast-like colonies, the cytotrophoblast cells were cultured in Keratinocyte Growth Medium (KGM; Clonetics Corporation, San Diego, CA) containing 10% fetal calf serum (FCS). We have previously shown that this medium supports both morphological and biochemical differentiation of cytotrophoblast cells (Douglas and King, 1990, 1993; Ho *et al.*, 1997a). Typically, after 48 h in KGM more than 90% of the culture consists of multinucleated syncytiotrophoblast-like colonies as determined by desmosomal and nuclear staining (Douglas and King, 1993). The cells also secrete bioactive CG (Ho *et al.*, 1997a).

Incubation of syncytiotrophoblast cultures with BDCM. BDCM (>98% purity, stabilized with potassium carbonate, Aldrich Chemical Co. Milwaukee, WI) was prepared in KGM containing 10% FCS and stored at 4°C in amber vials with Teflon-silicon lined caps. (Fisher Scientific, Pittsburgh, PA).

Cytotrophoblast cells were cultured in KGM for 48 h to induce morphologic differentiation and produce syncytiotrophoblast-like colonies (see above). The culture medium was removed and fresh KGM with or without BDCM (20 nM, 20 μM, or 2 mM) was added. The plates were sealed with plate seals and incubated at 37°C for an additional 24 h. At the end of the experiment, the culture medium was removed and stored at –20°C until used for determination of immunoreactive and bioactive CG levels. The adherent cells were processed for protein determination using a modified Lowry method (Bennett, 1982). Other identically treated trophoblast cultures were fixed and processed for morphological evaluation (see below). Additional cultures were exposed to BDCM as above and used for determination of lactate dehydrogenase (LDH) levels (see below).

CG immunoassay. Levels of immunoreactive CG in culture media were determined by ELISA as previously described (O'Connor *et al.*, 1988; Taylor *et al.*, 1992). The primary capture antibody was clone B 109, which reacts with intact hCG, and the second antibody was clone B 108, which reacts with β-hCG (Canfield *et al.*, 1987; O'Connor *et al.*, 1988). Immunoreactive CG was expressed as ng/mg cellular protein.

CG bioassay. Bioactive CG was measured by *in vitro* bioassay as described previously (Chen *et al.*, 2003; Jia *et al.*, 1991). Briefly, clonal human fetal kidney cells (cell line 293) were cotransfected with the LH/CG receptor and a luciferase reporter gene. Fifty-μl hCG CR 127 standards (provided by R. Canfield, Columbia University, NY), internal controls, and experimental samples, were then added. At the end of the incubation (16 h), the cells were lysed, and the lysates used for estimation of luciferase activity. LH/CG bioactivity was calculated by reference to a CR 127 standard curve and was expressed as ng/mg cellular protein.

LDH assay. LDH activity in culture supernatants was determined using the fluorescence-based CytoTox-ONE kit obtained from Promega Corporation, Madison, WI 53711. LDH activity is expressed in arbitrary fluorescence units.

Assessment of trophoblast morphology. Syncytiotrophoblast cultures (in 8-chamber LabTek slides) were established as described above and then incubated with or without BDCM for an additional 24 h. The cells were fixed and permeabilized using ice-cold methanol, after which they were incubated in PBS/gelatin. Cell-cell borders and nuclei were revealed by staining cultures simultaneously with an anti-desmosomal protein antibody (Douglas and King, 1990) and 7-aminoactinomycin D (7-AAD) (10 μg/ml) (Scholz *et al.*, 1996). The primary anti-desmosomal protein antibody was detected using an Oregon Green-labeled secondary antibody. The slides were mounted using glycerol vinyl alcohol (GVA) aqueous mounting medium and examined using a Nikon Eclipse E800 epifluorescence microscope. Images were captured using an Optronics DEI750 CCD camera and Adobe Photoshop software. Identical exposure and brightness level settings were used for test and control samples.

Immunocytochemical staining for CG. Cultured adherent cells were fixed in methanol and then stained with a polyclonal antibody against the β subunit of human CG (N1534; DAKO Corporation, Carpenteria, CA). The monoclonal antibody used for the CG ELISA (see above) was not used for immunocytochemistry because it was not reactive with fixed trophoblast cells. The primary antibody was detected using Oregon Green-labeled goat anti-rabbit Ig secondary antibodies. As a control, other cultures were incubated with a matched rabbit immunoglobulin instead of the primary antibody. Images were captured directly into Photoshop using identical exposure and brightness/contrast settings. Digitized images were analyzed using Image Pro software (Media Cybernetics, Silver Spring, MD). Fluorescence intensity was measured by reference to a fluorescence standard curve obtained using fluorescence calibration beads (Molecular Probes, Eugene, OR). Images from at least six random fields viewed using a 20× objective were analyzed from each of four experiments.

Statistical analysis. All experiments were performed six times, and each experiment used cells obtained from different placentas. Cells from different placentas were not pooled. Within an experiment, means were obtained from duplicate determinations. The immunoreactivity and bioactivity of hCG under different treatment conditions was compared using ANOVA with repeated measures followed by student-Newman-Keuls comparison and linear trend post-tests with a significance level of 0.05. ANOVA with repeated measures on rank was performed if the normality assumption was not met. The data are presented as mean ± SEM. The analyses were performed using SPSS software and Prism Software.

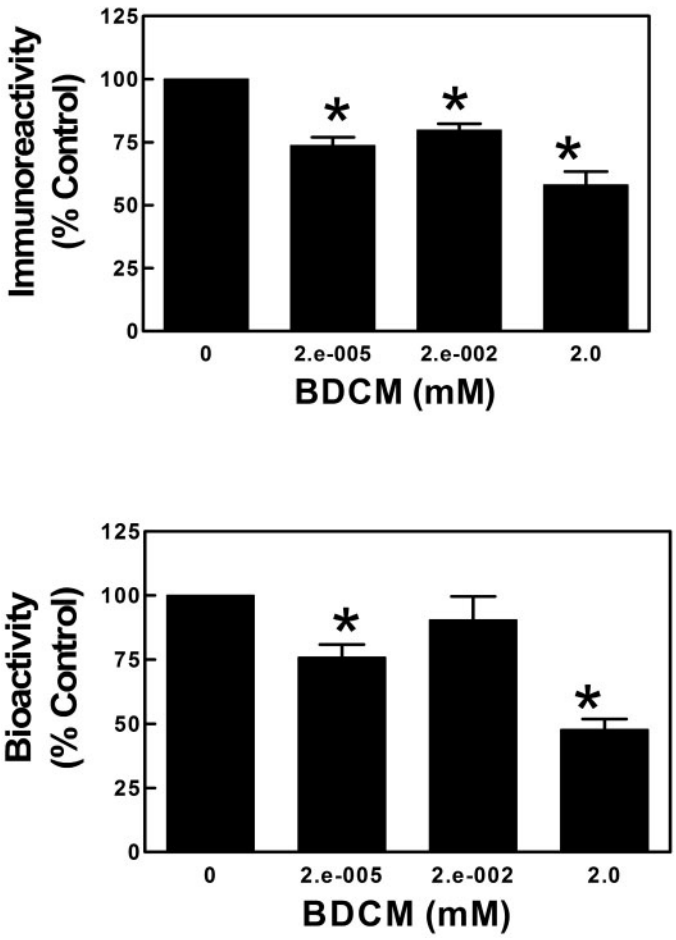

FIG. 1. Effect of BDCM on the secretion of immunoreactive and bioactive CG. Trophoblasts were cultured for 48 h under differentiation-inducing conditions, after which they were incubated in the presence or absence of BDCM for an additional 24 h. The culture media were collected and analyzed for immunoreactive (top panel) and bioactive (bottom panel) CG as described in the Methods section. Results are means ± SEM from six separate experiments (6 different placentas). The asterisks indicate values that are significantly different ($p < 0.05$) from the control. ANOVA with trend analysis showed a significant ($p < 0.05$) dose-dependent decrease in CG secretion.

RESULTS

Effect of BDCM on Secretion of Immunoreative and Bioactive CG

Before exposure to BDCM, cytotrophoblast cells were incubated for 48 h in differentiation-inducing medium to yield multinucleated syncytiotrophoblast-like colonies (Douglas and King, 1990, 1993; Ho *et al.*, 1997a). The formation of multinucleated colonies was confirmed by desmoplakin immunocytochemistry for each batch of cells (Douglas and King, 1990). Different concentrations of BDCM were then added, and the culture supernatants were removed 24 h later for assay of immunoreactive and bioactive CG. We have previously reported in detail the time course of immunoreactive and bioactive CG secretion from human trophoblast cultures (Ho *et al.*, 1997a). The results in Figure 1 show that exposure to BDCM caused a significant dose-dependent decrease in the secretion of immunoreactive CG. At the highest concentration tested (2 mM), BDCM reduced the secretion of immunoreactive CG by 37%. BDCM also reduced the levels of bioactive CG in culture supernatants from differentiated trophoblast in a dose-dependent manner. At the highest concentration tested (2 mM), BDCM reduced the secretion of bioactive CG by 53%. When the ratio of bioactive to immunoreactive CG (B/I ratio) was calculated, no effect of BDCM on this parameter was found.

Effect of BDCM on Trophoblast Viability and Morphology

Primary trophoblast cultures do not replicate, so cellular protein levels were determined to assess potential BDCM-mediated cell loss/detachment. Levels of cellular protein showed no significant differences between BDCM-treated and

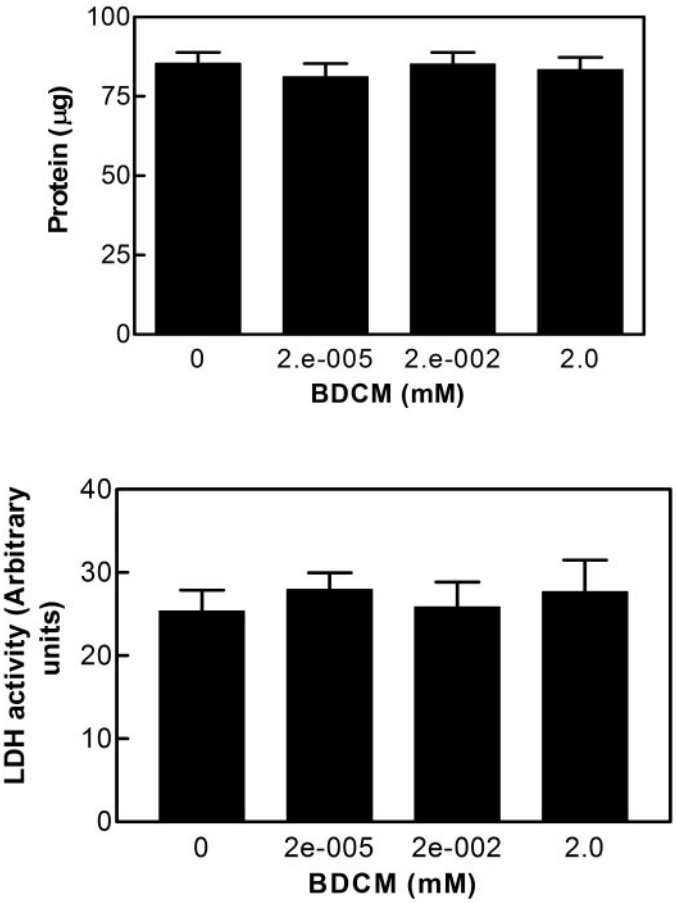

FIG. 2. Effect of BDCM on trophoblast viability. Trophoblasts were cultured for 48 h under differentiation-inducing conditions, after which they were incubated in the presence or absence of BDCM for an additional 24 h. After removal of culture supernatants, the protein content of the adherent cells was measured (top panel). Culture supernatants were assayed for LDH activity (bottom panel) as described in Methods. Results are means ± SEM from three separate experiments. ANOVA showed no significant difference between means.

untreated cultures (Fig. 2, top panel). Potential cytotoxic effects of BDCM were also tested by measuring levels of LDH in culture supernatants following exposure to different concentrations of BDCM. The data in Figure 2 (bottom panel) show that LDH activity in culture supernatants was unchanged following exposure of cells to BDCM for 24 h.

We also assessed the morphological appearance of trophoblast cultures exposed to BDCM. Cultures were stained with an antibody specific for desmosomal proteins and with 7-AAD, which stains nuclei (Fig. 3). As expected, the control culture (Fig. 3A) showed very little desmosomal protein staining, with multiple nuclei within a common cytoplasm, consistent with multinucleated syncyctiotrophoblast. Large multinucleated syncytiotrophoblast-like colonies were also seen in cultures exposed to 2 mM BDCM. No cell loss or alteration in colony size was apparent for cultures incubated in the presence of BDCM.

Immunocytochemical Staining for CG in Trophoblasts Treated with BDCM

Syncytiotrophoblast-like cultures were incubated in the presence or absence of 2 mM BDCM for 24 h. The cells were fixed and stained with a polyclonal antibody against the β subunit of human CG. This antibody reacts with intact CG and free βb subunit. Cultures incubated in the absence of BDCM showed a typical bright diffuse staining pattern (Fig. 4A). Cultures treated with BDCM also showed a diffuse staining pattern (Fig. 4B). No reduction in fluorescence intensity was detected either visually or following computerized image analysis of fluorescence micrographs (results not shown).

DISCUSSION

Thus far, all human pregnancy outcome data regarding THMs result from epidemiological studies. Of these studies, only a few have investigated the association of individual THM compounds, as well as THMs, with pregnancy outcome (King *et al.*, 2000; Waller *et al.*, 1998). One potential weakness of all epidemiological studies is the misclassification of exposures. This is particularly challenging when volatile DBPs such as THMs are investigated. Other issues, such as mobility during pregnancy, lack of individual exposure data, the temporal and spatial variation of total THMs and individual THMs during

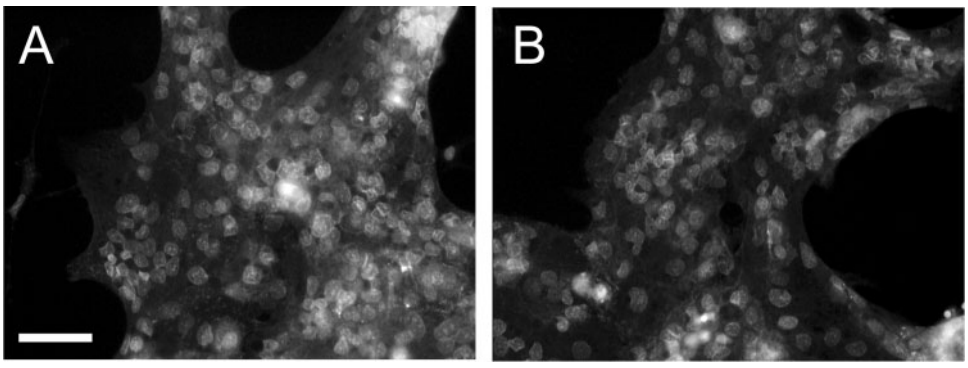

FIG. 3. Effect of BDCM on trophoblast morphology. Syncytiotrophoblast cultures were incubated in the absence (A) or presence (B) of BDCM (2 mM) for 24 h and then stained to reveal nuclei (red fluorescence) and desmosomal proteins (green fluorescence) as described in Methods. Note the absence of intercellular desmosomal protein staining in both A and B. The bar represents 50 μm. This experiment was performed four times, using cells from different placentas, with identical results.

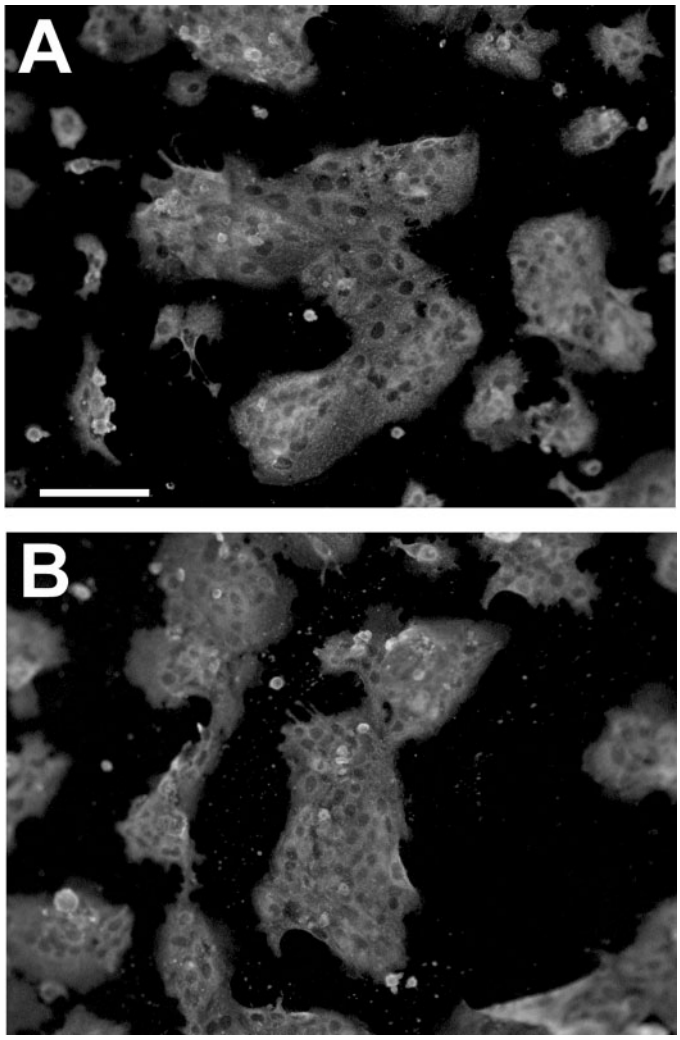

FIG. 4. Immunofluorescence localization of CG in trophoblasts exposed to BDCM. Syncytiotrophoblast cultures were incubated in the absence (A) or presence (B) of BDCM (2 mM) for 24 h. The cultures were then stained using an anti-CG antibody as described in the Methods section. The horizontal bar in A represents 50 μm. This experiment was performed four times, using cells from different placentas, with identical results.

pregnancy, the consumption of tap water versus bottled water, and cold water versus hot water are difficult to control in population-based field studies. Humans are also exposed to THMs through inhalation and skin contact (Backer *et al.*, 2000; Lynberg *et al.*, 2001; Miles *et al.*, 2002; Nieuwenhuijsen *et al.*, 2000). Population-based studies are also complicated by the presence of other DBPs in drinking water. These difficulties in assessing exposures may result in exposure misclassification biases that could produce substantial underestimates of risk as well as attenuated exposure-response trends (Swan *et al.*, 1998). Therefore, the reproductive toxicity of individual THMs needs to be evaluated and characterized under well-designed laboratory conditions with a controlled exposure route.

The most important finding from this study is that BDCM, a major by-product resulting from disinfection of drinking water, appears to target human placental trophoblasts. This conclusion is based on the BDCM dose-dependent reduction in the secretion of immunoreactive and bioactive CG from viable, primary cultures of human placental trophoblasts. These cells are the sole source of CG during normal human pregnancy and play a major role in the maintenance of the conceptus. Given the vital role of CG in maintaining pregnancy, a decrease in the amount of bioactive hormone that is produced by placental trophoblasts could have adverse effects on pregnancy outcome. The lowest effective target concentration of BDCM found in the present study (24 h exposure) was 20 nM, which is 100-fold higher than maximum baseline blood levels and approximately 35-fold higher than the maximum peak level reported in blood after showering (Miles *et al.*, 2002). Compared to median values, the lowest effective concentration in this study was approximately 500- and 80-fold higher than baseline and after-shower blood concentrations, respectively. Blood BDCM concentrations decrease significantly from 10 to 30 min after showering, but concentrations at the latter time point were elevated compared to baseline values (Backer *et al.*, 2000). While every precaution was taken to minimize loss of BDCM *in vitro* by volatilization, it is likely that actual concentrations in culture media were lower than the target dose. Clearly, caution must be exercised in extrapolating these *in vitro* results to the *in vivo* situation, but nonetheless, they indicate that the placenta could be a target for BDCM. A placental target for BDCM is consistent with epidemiological studies showing increased incidence of spontaneous abortion in women exposed to BDCM. *In vivo* studies using a nonhuman primate model are needed in order to better understand the physiological consequences of these *in vitro* observations.

Although the current findings indicate that BDCM may target the placenta in humans, the F344 rat model of BDCM-induced pregnancy loss appears to involve other target tissues. Initially, reduced serum progesterone levels, together with an inability to detect a reduction in serum LH, suggested that BDCM may affect the luteal response to LH during the LH-dependent period of gestation in F344 rats (Bielmeier *et al.*, 2001). However, a more sensitive assay revealed BDCM-induced reductions in serum LH, indicating an effect on the hypothalamic-pituitary axis resulting in reduced LH secretion (Bielmeier *et al.*, 2001, 2002). More recently, a contributory role of the corpora lutea has also been implicated; corpora lutea exposed to BDCM *in vitro* showed reduced responsiveness to CG (Bielmeier *et al.*, 2003).

Our previous studies have shown that early pregnancy loss in macaques and humans is associated with changes in the serum levels of immunoreactive and bioactive CG (Guo *et al.*, 1999; Ho *et al.*, 1997b). In these latter studies, early pregnancy loss was associated with a decrease in the secretion of bioactive CG and an increase in the secretion of immunoreactive CG (i.e., a net decrease in the ratio of bioactive [B] to immunoreactive [I] CG). These observations led to the suggestion that a

decrease in the secretion of bioactive CG (and a decrease in the B/I ratio) may provide a marker for impending pregnancy failure. In a previous *in vitro* study we showed that the environmental toxicant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), caused an increase in the secretion of immunoreactive CG and no change in the secretion of bioactive CG in human trophoblast cultures (Chen *et al.*, 2003). The net effect was still a decrease in the B/I ratio. In the present *in vitro* study, both bioactivity and immunoreactivity were decreased to a similar extent such that the B/I ratio was unchanged in cultures exposed to BDCM. If similar findings are obtained from *in vivo* studies and such studies are associated with early pregnancy loss, then this would suggest that a decrease in the B/I ratio is not the only marker for impending pregnancy failure. Until *in vivo* studies are carried out with nonhuman primates, further speculation on the significance of the B/I ratio is premature.

Although we were able to detect an effect of BDCM on the secretion of immunoreactive CG, we were not able to show an effect on intracellular CG levels in syncytiotrophoblast cultures based on immunocytochemical staining. This could be related to the use of different antibodies, the lower resolution of the image analysis procedure, and to the fact that the effects on immunoreactive CG secretion, while significant, were modest.

The mechanism by which BDCM perturbs CG secretion is unknown at present. Reduced CG secretion could result from disruption of CG synthesis at the translational or posttranslational level. Since it has been demonstrated that the biological activity of hCG is largely determined by the degree of glycosylation, it is possible that BDCM affects the glycosylation of CG subunits. Another possibility is that BDCM affects subunit dimerization. This could be the result of perturbations in protein folding during processing (Bedows *et al.*, 1993; Furuhashi and Suganuma, 2000; Ruddon *et al.*, 1996; Tominga *et al.*, 1991; Wang *et al.*, 1988, 1989). It is also possible that BDCM could affect CG secretion indirectly through disruption of gonadotropin releasing hormone (GnRH) activity. GnRH is thought to be a major regulator of placental CG synthesis and secretion (Barnea *et al.*, 1991; Currie *et al.*, 1992; Kikkawa *et al.*, 2002; Siler-Khodr *et al.*, 1987), and human trophoblasts express a GnRH receptor and secrete GnRH (Cheng *et al.*, 2000; Lin *et al.*, 1995; Wolfahrt *et al.*, 1998). If an effect of BDCM on GnRH is substantiated, this would parallel the proposed mechanism of BDCM-induced pregnancy loss in rodents. Further experiments will be required to determine if the effects of BDCM are due to alterations in the degree of CG glycosylation and/or subunit dimerization, or whether other mechanisms (perhaps involving GnRH) are involved.

ACKNOWLEDGMENTS

This work was supported by NIH Grants ES06198, ES05707, and P42ES04699. Placental tissue was made available to us through the help and cooperation of the medical and nursing staff at Sutter Memorial Hospital, Sacramento, CA. The information in this document has been funded in part by the U.S. Environmental Protection Agency. It has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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