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The Induction of Phagocytic activation by Mixtures of the Water Chlorination By-Products, Dichloroacetate- and Trichloroacetate in Mice after Subchronic Exposure

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Abstract

In this study, groups of B6C3F1 male mice were treated with dichloroacetate (DCA), trichloroacetate (TCA), and mixtures of the compounds (Mix I, Mix II and Mix III) daily by gavage, for 13 weeks. The tested doses were 7.5, 15 and 30 mg DCA/kg/day and 12.5, 25 and 50 mg TCA/kg/day. The DCA: TCA ratios in Mix I, II and III were 7.5:12.5, 15:25 and 30:50 mg/kg/day, respectively. Peritoneal lavage cells were collected at the end of the treatment period and assayed for the biomarkers of phagocytic activation, including superoxide anion and tumor necrosis factor- α production, and myeloperoxidase activity. The mixtures produced non-linear effects on the biomarkers of phagocytic activation, with Mix I and II effects were found to be additive, but Mix III effects were found to be less than additive.

Keywords

Chloroacetates; Mixtures; Phagocytic activation

INTRODUCTION

The chlorinated drinking water contains different by-products, including the haloacetates that are formed from the reaction of chlorine with the organic material present in the surface water. Dichloroacetate (DCA) and trichloroacetate (TCA) are among these haloacetates and they are especially important because they can be also formed *in vivo* through the metabolism of trichloroethylene, a widely used industrial solvent and water contaminant [1-5]. Concerns about human risks arising from long term exposure to DCA and TCA through drinking chlorinated water have lead several investigators to study the compounds' long term toxic effects in rodents, where hepatotoxic- and hepatocarcinogenic effects were identified as their prominent outcomes [6-10]. Studies on the mechanisms of the compounds hepatotoxicity have found oxidative stress to be induced in the livers of mice after acute and long-term exposures [11-17]. Phagocytic activation indicated by the production of superoxide anion (O_2^-) (SA) and tumor necrosis factor alpha (TNF- α), and myeloperoxidase (MPO) activity in the peritoneal lavage cells (PLCs) of mice was found to be associated with the induction of hepatic oxidative stress and was suggested to be a protective mechanism against the compounds-induced hepatocarcinogenic effects [18].

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Humans are exposed to mixtures of environmental pollutants rather than to single compounds, where the outcome of toxic effects of the mixtures can be additive, greater than additive, or less than additive. Although some studies have been conducted on water samples that were assumed to contain mixtures of some water disinfection by-products, they were limited as for the definition of the composition of those mixtures and the toxic end points studied. Using Ames assay, a high correlation between mutagenicity and total organic halide concentrations was found in water samples collected from different drinking water treatment plants that used surface water from different locations [19]. Cytotoxicity and gene expression were found to be altered in cultured hepatocytes exposed to various dilutions of concentrates of chlorinated or ozonated/postchlorinated water that was originally collected from the Ohio River [20]. Different cytochrome P450 (CYP)-linked metabolizing enzymes were found to be either induced, or inactivated in fish exposed to water obtained from the Trasimene Lake in Italy and was disinfected with peracetic acid, sodium hypochlorite (NaClO), or chlorine oxide (ClO₂) [21]. The CYP-modulation in those fish was found to be associated with the production of reactive oxygen species and a significant increase in micronucleus frequency in the circulating erythrocytes [21].

While the fore mentioned studies have focused on the mutagenicity and toxicity of the water samples that were assumed to contain mixtures of different disinfection by-products, a number of studies have focused on the developmental toxicity of more defined mixtures. For example, increased resorption rates and eye malformation were observed in surviving litters of rats that were exposed during pregnancy to mixtures of five haloacetates [22]. Andrews et al. [23] have reported additive dysmorphogenic effects in embryos of rats exposed *in vitro* to mixtures of dichloroacetate, dibromoacetate and bromochloroacetate, and have indicated the adequacy of the dose-additivity model for the prediction of the observed toxicity of the compounds.

In this study, we tested the outcome of long-term exposure of mice to simple defined mixtures of two important haloacetates present in the chlorinated drinking water on phagocytic activation.

MATERIALS AND METHODS

Chemicals

Quantikine mouse TNF- α Immunoassay kit was purchased from R & D Systems (Minneapolis, MN). All other chemicals and reagents used for this study were purchased from Sigma-Aldrich®, USA.

Animals and treatments

Experiments in this study were conducted according to an animal protocol that was approved by the Institutional Animal Care and Handling Committee at the University of Toledo. B6C3F1 male mice were used for this study because they were previously found to be sensitive to the acute and long term effects of DCA and TCA [6-10, 12-18]. The animals were purchased from Harlan Teklad (Indianapolis, Indiana) at 6 weeks of age and weighed approximately 20 g. They were caged in a room at 21° C with a 12 hr light/dark cycle and had free access to food and water. After they were allowed to acclimate for 3 days, the animals were randomly divided into 10 groups (6 animals/ group). DCA and TCA solutions were prepared in distilled water (pH adjusted to 7.0 with NaOH), and were administered to groups of mice, by gavage at daily doses of 7.5, 15 and 30 mg DCA/kg, and 12.5, 25, and 50 mg TCA/kg for 13 weeks. The doses of DCA and TCA were estimated to correspond to those producing 15%, 25% and 35% of the previously reported maximal doses for the

induction of the biomarkers of phagocytic activation in the subchronic studies in B6C3F1 mice [18].

Three different mixtures (Mix I, Mix II and Mix III) of DCA and TCA were also prepared. The DCA: TCA ratios in Mix I, II and III corresponded to 7.5:12.5, 15:25, and 30:50 mg/kg/day, respectively. The mixtures were prepared in distilled water (pH was adjusted to 7.0 with NaOH), and were administered to groups of mice, by gavage, daily for 13 weeks. Animals in the control group were administered distilled water (pH adjusted to 7.0 with NaOH) daily, by gavage, for 13 weeks. The animals were monitored on daily basis and their body weights were taken. No signs of toxicity or any unusual behaviors were observed in any of the treatment groups. Also, no significant differences were observed in the body weight gain of any of the treatment groups when compared with the control group.

Collection of the peritoneal lavage cells (PLCs)

The mice in the different treatment groups were euthanized at the end of the treatment period using CO₂ anesthesia followed by cervical dislocation. A cut was made in the abdominal skin of the euthanized animals to expose the peritoneum that was inspected for fluid accumulation/ sepsis as signs of inflammation. However, no signs of inflammation were observed in any of the treatment groups. Three ml of buffer, containing 140 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES, and 2 mM CaCl₂ (pH 7.3) was injected into the peritoneal cavity of each animal, and the cavity was massaged for 30 seconds to release the PLCs that are commonly composed of macrophages, monocytes and neutrophils. The fluid was then withdrawn from the peritoneal cavity by a syringe and was centrifuged at 1700 × g for 10 min. After centrifugation, the supernatant of each sample was removed and the cellular pellet was re-suspended in 2 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing L-methionine, 200 mM L-glutamine, penicillin-streptomycin solution (10,000 U penicillin + 10,000 µg streptomycin)/ ml, 1 M HEPES buffer, 10 mM Minimum Essential Medium-Non Essential Amino Acids (MEM-NEAA,) 100 mM sodium pyruvate solution, and 10% fetal bovine serum (FBS). The viabilities of the PLCs were confirmed microscopically by the Trypan blue exclusion method and all the cells from the different treatment groups and the control were found to be 98-99% viable. Cell suspensions were used for the determination of the production of tumor necrosis factor (TNF)-α and superoxide anion (SA), and myeloperoxidase (MPO) activity.

Determination of TNF-α

Aliquots of 200 µl of cellular suspensions were plated on 96-well plates, and the plates were incubated at 37° C in a humidified atmosphere containing 5% CO₂ for 24 h. The media were collected and were assayed for TNF-α levels using the Quantikine mouse TNF-α Immunoassay, according to the procedure described in the kit. Absorbances of the samples in each plate were determined at 450 nm, using a microplate reader. TNF-α levels were determined according to a standard curve prepared with serial dilutions of recombinant murine TNF-α standard that was provided with the kit.

Determination of superoxide anion (SA)

Cellular suspensions were assayed for SA, using the cytochrome *c* reduction assay of Babior et al. [24], with modifications. The reaction tubes contained aliquots of 50 µl of PLC suspensions and 1.95 ml of 0.05 mM cytochrome *c* oxidase in phosphate buffered saline (PBS), pH 7.2. The tubes were incubated at 37 ° C for 15 minutes, and the reactions were then terminated by placing the tubes in crushed ice. The tubes were centrifuged at 3000 rpm for 15 minutes and absorbances of the supernatants were recorded at 550 nm, using a Spectronic 20 spectrophotometer. Absorbance values were converted to nmoles of cytochrome *c* reduced/min, using the extinction coefficient $2.1 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$.

Determination of MPO activity

Approximately, 500 μ l of cellular suspension of each sample was freeze-thawed 3 times and then centrifuged at 4,000 g for 15 min. Supernatants were collected and used for the determination of MPO activity according to the method of Bradley et al. [25], where 0.1 ml of the supernatant of each sample was mixed with 2.9 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The absorbances of the reaction mixtures were recorded every 30 seconds for 5 minutes at 460 nm, using a Spectronic 20 spectrophotometer. The slopes of the reactions were converted into units of MPO activity, where one unit is defined as that degrading one micromole of hydrogen peroxide per minute at 25° C. A molar extinction coefficient of 11300 M⁻¹ cm⁻¹ for the oxidized o-dianisidine was used.

Determination of protein

The amounts of proteins in different cellular suspensions were determined according to the method of Lowry et al. [26], using bovine serum albumin as a standard. Data for various biomarkers were expressed per mg protein.

STATISTICAL METHODS

Data are expressed as means of 6 samples (animals) $\pm$ S.D. A one-way Analysis of Variance (ANOVA) was used to determine the statistical differences between groups, with Scheffe's S method used as a post hoc test. Values (columns) with non-identical superscripts in figures 1A, 2A, 3A are significantly different, as compared with each other within each of the DCA, TCA and Mix groups, and also with the control. Student's t-test was used to compare between the observed and the predicted effects of the mixtures. A significance level of $p < 0.05$ was employed in all of the statistical analyses.

RESULTS

Figures 1 A, 1B and 1 C, demonstrate SA production, MPO activity and TNF- α levels, respectively, in the PLCs of mice treated with different doses of DCA and TCA, and the three mixtures (Mix I, II and III) of DCA and TCA. Treatment with the individual compounds resulted in significant and dose-dependent increases in the three tested biomarkers. Treatment with mixtures of the compounds also resulted in significant increases in those biomarkers when compared with their corresponding controls. However, the effects of Mix II on the three biomarkers were found to be significantly higher than those of Mix I, but Mix II effects were not significantly different from those produced by Mix III.

Figures 2A, 2B and 2C demonstrate comparisons between the observed and the predicted effects of the three mixtures on SA production, MPO activity, and TNF- α level, respectively. The observed effects refer to the experimentally determined effects of the mixtures on the three tested biomarkers (Figures 1A, 1B and 1C), after subtracting the average control value from each sample. The predicted effects indicate the mathematical sum of the experimentally determined effects of the individual compounds in each mixture after subtracting the average control value from the effect of each compound in that mixture. While no significant differences were found between the observed and the predicted effects of Mix I and Mix II on any of the tested biomarkers, significantly lower effects were reported for the observed, as compared with the predicted effects of Mix III on those biomarkers.

DISCUSSION

A subchronic dose-response study on DCA and TCA effects on hepatic SA and TNF- α levels, and MPO activity was previously conducted in B6C3F1 mice, where 77 mg of DCA or TCA/kg/day was found to produce maximal effects [18]. Based on that, the doses that corresponded to 15%, 25% and 35% of maximal effects were estimated to be 7.5, 15 and 30 mg DCA/kg/day, respectively, and 12.5, 25, and 50 mg TCA/kg/day, respectively. The differences in the TCA and DCA doses noted here are attributed to the sharper and greater increases that were previously observed with DCA effects of different doses on those biomarkers, as compared with those of TCA [18].

Assuming additivity, the 15%, 25% and 35% of maximal effects of each compound would be expected to produce 30%, 50% and 70% of maximal responses effects, respectively, by Mix I, II and III for this study. This design while allowing the observation of any additive effects, it also allows observing any greater than additive (> 70%) effects. The non-significant differences between the observed and predicted effects of Mix I and II on SA, MPO and TNF- α indicate production of additive effects by those specific mixtures on the induction of phagocytic activation. This can be supported by the findings of Andrews et al. [23] who reported production of additive dysmorphogenic effects in the embryos of rats exposed *in vitro* to different haloacetates mixtures. Phagocytic activation induced by DCA and TCA was previously suggested to provide protection against the long- term effects of the compounds [18]. Therefore, the additive effects resulted from exposure to Mix I and II are expected to be associated with protection against those long-term effects. Although the observed effects of Mix III are less than additive when compared with the predicted values, they were not significantly different from the observed effects of Mix II. Hence, protection through phagocytic activation similar to that provided by Mix II is expected to be provided against Mix III long-term toxicity. These suggestions can be supported by the studies of Hooth et al. [27] that found the carcinogenic responses to mixtures of some water disinfection by-products to be less than additive in rats.

Although the reason for the change of the mixtures effects from additive to less than additive with increasing concentrations is not known at this point, it may be attributed to a possible interference with the toxicokinetics of the two compounds. For example, DCA- and TCA-induced oxidative stress was suggested to be due to the free radicals that are transiently generated through their metabolism, where approximately 50% of any single dose of TCA, but more than 98% of any dose of DCA is expected to be metabolized [13]. However, we suggest when the two compounds administered as mixtures, generation of higher free radicals concentration in response to DCA in the mixtures of higher compound concentrations may alter the balance of TCA metabolism, favoring higher production of reactive metabolites by TCA. Further studies are being designed to confirm this suggestion.

In summary, the results of this study indicate the production of additive effects on phagocytic activation by DCA and TCA mixture concentrations that produce < 70% of maximal responses, but less than additive effects are produced by mixture concentrations that produce 70% of maximal responses.

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Figure 1A, 1B and 1 C.

SA production (A), MPO activity (B), and TNF- α levels (C) determined in PLCs of mice treated with different doses of DCA, TCA, or DCA and TCA mixtures (Mix I, II and III). Non- identical superscript of columns within each of the DCA, TCA and the different Mix groups indicate statistically significant differences ($p < 0.05$) when compared with each other within that group, as well as with the control.

Figure 2 A, 2B and 2C.

The observed and the predicted effects of mixtures (Mix) I, II and III on SA production (A), MPO activity (B) and TNF- α level (C) in the PLC of mice. * indicates significant difference between the observed and the predicted effects ($p < 0.05$), by Student's t-test.