Original Article

Enhancement of immediate allergic reactions by trichloroethylene ingestion via drinking water in mice

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ABSTRACT — The prevalence of allergic disorders is increasing in industrial areas and countries. Recent reports suggest that some environmental pollutants are related to the increase in allergic diseases, and we reported that trichloroethylene (TCE) is a candidate chemical for causing the increase of allergic diseases, as TCE ingestion is associated with allergic reaction enhancement. TCE is widely used in many industries, and it is commonly detected as an environmental contaminant. This study aimed to clarify the immunotoxicity of TCE in detail. BALB/c mice were treated with TCE dissolved in drinking water for 2 and 4 weeks, and the mice were immunized with ovalbumin (OVA)/aluminum hydroxide (alum) twice. On the final day of the TCE exposure period, we measured the active cutaneous anaphylaxis (ACA) reaction and the antigen-specific IgE level in serum as well as the histamine level at the allergic reaction site and assayed the proliferation rates of splenocytes collected from the animals. The ACA reaction was enhanced by TCE ingestion. The OVA specific IgE level in mice was enhanced by TCE exposure for 4 weeks. The proliferation rate of the splenocytes was enhanced by TCE ingestion for 2 and 4 weeks. The enhancement of the ACA reaction by TCE ingestion via drinking water may be related to the increase in splenocyte proliferation. On the other hand, it may be weakly related to antigen-specific IgE production.

Key words: Immediate allergic reaction, Trichloroethylene, T-cells

INTRODUCTION

The number of allergic patients has been increasing rapidly in industrialized countries in the last 2 or 3 decades. Also, while there are fewer patients in unindustrialized countries, there are more patients in urban areas (Nicolai, 1997; Yemaneberhan *et al.*, 2004). Although genetic factors such as family disease history are important in the development of allergic diseases, the rapid increase in patients is not likely to have been caused by changing genetic factors and has strong relationships with changing environmental factors (Sears, 1997; Yazdanbakhsh *et al.*, 2002). There are obvious risk factors such as increasing exposure to indoor allergens, environmental pollutants, or changes in diet (Lau *et al.*, 2000), but other factors to consider including environmental pollutants such as diesel exhaust particles (DEP) and formaldehyde (Dong *et*

al., 2005a, 2005b; Sadakane *et al.*, 2002; Takano *et al.*, 1997).

We concentrated on chloride organic chemicals as an environmental factor and reported that organic chemical exposure affects immediate phase reactions such as the passive cutaneous anaphylaxis (PCA) reaction and antigen-stimulated degranulation from mast cells, and trichloroethylene (TCE) had the strongest effect among the chemicals (Seo *et al.*, 2008). TCE is a synthetic solvent that is used in dry cleaning as a cleaner and in various industries to remove oils and greases from metal. It is also used to extract fats and waxes. It has also become a social problem in that the groundwater in some areas has been contaminated by TCE because of its high persistency and pervasiveness. Although the Japanese standard value for drinking water of TCE is the concentration of 0.03 mg/l, higher concentrations of TCE than the standard value was

detected from 0.2% of groundwater as the survey of the Japanese Ministry of Environment in 2007. It is reported that TCE is toxic for the nervous system, liver, and kidneys and increases the risk of leukemia (Gist and Burg, 1995; Barton and Clewell, 2000), in addition it is anticipated carcinogenesis (Scott and Chiu, 2006). There are a few studies about the immunotoxicity of TCE (Kaneko *et al.*, 2000; Peden-Adams *et al.*, 2006; Sanders *et al.*, 1982), however there is no study about the effect of TCE on immediate allergic reaction besides our previous paper (Seo *et al.*, 2008).

In the current study, to investigate the toxic effects of TCE ingestion on the immune system, we used the active cutaneous anaphylaxis (ACA) reaction and found that TCE ingestion enhances the ACA reaction. We also investigated the mechanisms of the enhancement of ACA reaction.

MATERIALS AND METHODS

Animals

Male BALB/c mice (7-9 weeks olds at the start of TCE exposure) were obtained from Japan SLC, Inc. (Shizuoka, Japan) and rested for a week after arrival. They were maintained under a 12-hr light/dark cycle at $25 \pm 2^\circ\text{C}$ and 40-60% relative humidity, with free access to food and drinking water. The experiments were undertaken following the guidelines for the care and use of experimental animals written by the Japanese Association for Labora-

tory Animal Science in 1987. This study was approved by the ethics committee of Gifu Pharmaceutical University.

Reagents and medium

Ovalbumin (OVA; Albumin from chicken egg white, grade V) was purchased from SIGMA-ALDRICH Inc. (St. Louis, MO, USA). Concanavalin A (ConA) and diphenhydramine hydrochloride were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). [^3H] Thymidine (methyl- ^3H) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). TCE and dimethylsulfoxide (DMSO) 2-mercaptoethanol (2-ME) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). RPMI 1640, penicillin-streptomycin, and horse serum were purchased from Invitrogen (Grand Island, NY, USA). RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), 2-ME (50 μM), and horse serum (10 %; v/v) was used as the culture medium.

TCE exposure and immunization

The schedule of this experiment shows in Fig. 1. In detail, 300 mg/l TCE stock solution were prepared in DMSO. The stock solution was diluted with distilled water, and 0.03 and 3 mg/l TCE solutions were prepared and used as drinking water. The animals were divided into groups of 6 and received TCE in their drinking water at concentrations of 0, 0.03, or 3 mg/l for 2 or 4 weeks. The water was changed every other day to ensure dose

Fig. 1. Time schedule of TCE ingestion and OVA/Alum immunization. Mice were treated with TCE for 2 weeks (A) or 4 weeks (B) and immunized with 1 μg OVA / 5 μg Alum by i.p. injection twice.

maintenance.

The mice were immunized with 1 µg OVA/5 µg aluminum hydroxide (alum) in 0.2 ml phosphate buffered solution (PBS) by intraperitoneal (i.p.) injection. As a control group, mice were treated with 0.2 ml PBS without OVA and alum.

ACA

On the final day of the TCE exposure period, the mice were challenged with 50 µg/ml OVA in 0.02 ml PBS by subcutaneous (s.c.) injection into the ear and were immediately injected with 0.5% Evan's blue (Nacalai Tesque, Inc., Kyoto, Japan) solution into the tail vein. Thirty minutes after the challenge, the mice were sacrificed and their ears were collected to measure the extravasated dye. To determine the amount of non-specifically extravasated dye, only PBS was injected into the other ear of the mice.

Extraction and quantification of the extravasated dye was performed using the method (Inagaki *et al.*, 1984). The amount of dye was measured colorimetrically at 620 nm.

Histamine levels in the mouse ear

Ears were collected from each mouse at 30 min after the OVA challenge. The histamine levels in the ears were measured by HPLC with the postcolumn derivatization methods reported by (Itoh *et al.*, 1992; Yamatodani *et al.*, 1985).

OVA-specific IgE levels in mice serum

On the final day of the exposure period, to determine Ag-specific IgE levels, blood samples were collected from each mouse, and the OVA-specific IgE levels in serum were measured by a mouse OVA-specific IgE ELISA kit (Dainihon Sumitomo, Osaka, Japan).

Splenocyte proliferation assay

On the final day of the exposure period, we collected mice spleen. Splenocytes from mice were cultured in 96-well flat-bottom plates (5×10^5 cells/well). OVA or ConA was added at a concentration of 100 µg OVA/ml or 10 µg ConA/ml in culture medium. After 96 hr of incubation at 37°C, the cultures received 740 kBq [³H] thymidine/ml, and after 16 hr the cells in the wells were collected. The amount of [³H] thymidine incorporated into the cells was measured by a liquid scintillation counter (Packard BioScience Co., Meriden, CT, USA).

Cytokine quantification

The quantification of IL-3, IL-4, IFN-γ and TNF-α levels in the cultured medium collected from splenocytes

after stimulated 96 hr in OVA immunization group were measured by enzyme-linked immunosorbent assay (ELISA). These cytokine levels were measured with cytokine ELISA kit (Biosource International, Inc., Camarillo, CA, USA). All protocols were followed manufacture's methods.

Statistical analysis

Data are shown as the mean ± S.D. Statistical analyses were performed with one-way ANOVA followed by Bonferroni's post-hoc procedure or two-way ANOVA. P values less than 0.05 were considered to be statistically significant.

RESULTS

Daily drinking volume and increase rates of mice body weight

The average volume of drinking water in the TCE exposure period for consumed by the TCE treated groups on each days was not significantly different from that consumed by 0 mg/l TCE exposure group (data not shown). Each mouse in all groups consumed 4.3 ml/day as an average volume of drinking water on each days. These doses were calculated to given an average dose of 0.12 µg/day (0.03 mg/l TCE exposure group) and 12 µg/day (3 mg/l TCE exposure group), respectively. There was no significantly difference of the increase rates of mice body weight among the TCE exposure groups and control groups (data not shown).

TCE ingestion alters immediate phase allergic reactions

We previously reported that TCE ingestion enhances the PCA reaction (Seo *et al.*, 2008). In the current study, we assessed the effect of TCE ingestion on the ACA reaction. The intensity of dye extravasation in mouse ears was significantly increased in the 3 mg/l TCE exposure group compared to the 0 mg/l TCE exposure group (Figs. 2A and B). The ACA reaction was enhanced by TCE exposure, and the enhancement of the ACA reaction disappeared after pretreatment with diphenhydramine (anti-histamine drug) (Fig. 3). Next, we measured the histamine content of the mouse ears and found that the histamine content was significantly increased in the 3 mg/l TCE exposure group (1.64 ± 0.27 µg/ml) compared to the 0 mg/l group (0.91 ± 0.35 µg/ml) (Fig. 4). These results suggest that the enhancement of ACA reaction is caused by increased histamine release induced by TCE ingestion.

Fig. 2. The effect of TCE ingestion via drinking water on the ACA reaction. Mice were treated with TCE for 2 weeks (A) or 4 weeks (B) and immunized with 1 μ g OVA / 5 μ g Alum by i.p. injection twice. On the final day of the TCE exposure period, the mice were challenged by s.c. injection into their ear with OVA. The amount of extravasated dye was measured colorimetrically at 620 nm. The data are expressed as means $\pm$ S.D. (n = 3~5). Statistical differences for 0 mg/l TCE exposure group were calculated by Two- factor repeated measures ANOVA. * P < 0.05.

Fig. 3. The effect of diphenhydramine on the ACA reaction exacerbated by TCE ingestion via drinking water. Mice were treated with TCE for 2 weeks and immunized with 1 μ g OVA/5 μ g Alum i.p. injection twice. The mice were treated with diphenhydramine 30 min before the challenge. The data are expressed as means $\pm$ S.D. (n = 3~5). Statistical differences were for 0 mg/l TCE exposure group calculated by Bonferroni test after One-way ANOVA. * P < 0.05 ** P < 0.01.

Enhancement of allergic reactions by trichloroethylene ingestion

Fig. 4. The effect of TCE ingestion via drinking water on histamine levels in the mouse ear. Mice were treated with TCE for 2 weeks and immunized with 1 μ g OVA/5 μ g Alum i.p. injection twice. The mice were challenged by s.c. injection into their ear with OVA. The histamine levels in the mouse ear were measured by HPLC. The data are expressed as a ratio v.s. 0 mg/l group. Statistical differences were calculated by Bonferroni test after One-way ANOVA. * $P < 0.05$.

Enhancement of allergic reactions by TCE ingestion is less related to antigen-specific IgE production

To investigate what caused the enhancement of immediate phase allergic reactions, we measured antigen-specific IgE levels in mouse serum. The OVA-specific IgE levels in serum significantly increased in the group exposed to 0.03 mg/l TCE for 4 weeks, but those for the group exposed to 3 mg/l TCE for 4 weeks did not increase (Fig. 5B), and when the mice were exposed to TCE for 2 weeks, no differences appeared in any group (Fig. 5A). These results suggest that TCE ingestion increases antigen-specific IgE levels; however, the increases in IgE levels did not occur in parallel with those of the ACA reaction. The increases in antigen-specific IgE levels were less related to the enhancement of allergic reactions caused by TCE ingestion.

Splenocyte proliferation was activated by TCE ingestion

The proliferation rates of the OVA- and ConA-stimulated splenocytes were significantly increased in the OVA

immunization group that received 3 mg/l TCE exposure for 2 weeks, but there were no differences in the 0.03 mg/l TCE exposure group compared to the 0 mg/l TCE exposure group with or without immunization (Fig. 6A). With immunization, OVA- and ConA-stimulated cell proliferation was significantly increased by 0.03 and 3 mg/l TCE exposure for 4 weeks (Fig. 6B). These results show that the activation of splenocyte proliferation is strongly related to the enhancement of allergic reactions induced by TCE ingestion.

TCE ingestion change cytokine production from splenocyte

IFN- γ and IL-4 productions from splenocytes by OVA stimulation were significantly increased in 0.03 mg/l TCE exposure group (Figs. 7A and B). IL-3 and TNF- α productions from splenocytes were also significantly increased in 0.03 and 3 mg/l TCE exposure group (Figs. 7C and D). These results show that productions of some cytokines from splenocytes are enhanced by TCE ingestion.

Fig. 5. The effect of TCE ingestion via drinking water on antigen specific IgE production. Mice were treated with TCE for 2 weeks (A) or 4 weeks (B) and immunized with 1 μ g OVA / 5 μ g alum twice. To determine Ag-specific IgE levels, we collected a blood sample from each mouse, and the OVA-specific IgE levels in serum were measured by mice OVA-specific IgE ELISA. The data are expressed as means $\pm$ S.D. (n = 4~5). Statistical differences for 0 mg/l TCE exposure group were calculated by Bonferroni test after One-way ANOVA. * P < 0.05.

Fig. 6. The effect of TCE ingestion via drinking water on splenocyte proliferation. Mice were treated with TCE for 2 weeks (A) or 4 weeks (B) and immunized with 1 μ g OVA / 5 μ g alum twice. 5×10^5 cells /well of splenocytes from treated mice were seeded after being incubated with OVA and ConA for 96 hr. The wells were pulsed with [³H] thymidine for the final 16 hr of culture. The amount of [³H] thymidine incorporated into the cells was measured by liquid scintillation. The data are expressed as means $\pm$ S.D. (n = 3, individually). Statistical differences for 0 mg/l TCE exposure group in each cultured group (Control, OVA and ConA) were calculated by Bonferroni test after One-way ANOVA. * P < 0.05, **P < 0.01.

Enhancement of allergic reactions by trichloroethylene ingestion

Fig. 7. The effect of TCE ingestion via drinking water on cytokine production from splenocytes. Mice were treated with TCE for 2 weeks and immunized with 1 μ g OVA / 5 μ g alum twice. 5×10^5 cells /well of splenocytes from treated mice were seeded after being incubated with OVA. The quantification of IFN- γ (A), IL-4 (B), IL-3 (C) and TNF- α (D) levels in the cultured medium collected from splenocytes after stimulated 96 hr in OVA immunization group were measured by ELISA. The data are expressed as means $\pm$ S.D. (n = 3, individually). Statistical differences for 0 mg/l TCE exposure group were calculated by One-Way ANOVA. **P < 0.01.

DISCUSSION

In the current study, the ACA reaction was significantly enhanced by TCE ingestion via drinking water, but the enhancement disappeared after pretreatment with an anti-histamine drug (diphenhydramine). In addition, an increase in histamine content at the allergic reaction site was observed after TCE ingestion. Thus, TCE ingestion

may enhance immediate phase reactions by activating mast cells and increasing the release of histamine from mast cells. In the previous study we reported TCE exposure affects the passive cutaneous anaphylaxis (PCA) reaction and antigen-stimulated degranulation from mast cells (Seo *et al.*, 2008). The current results are consistent with the previous report.

It is well known that degranulation is one of the major

immediate phase allergic reactions that occurs after the interaction of IgE and antigen. The cross-linking of IgE with FcεRI on mast cells triggers the release of vasoactive mediators, synthesis of prostaglandins and leukotrienes, and the transcription of cytokines (Gould *et al.*, 2003). To elucidate the mechanism of the enhancement of degranulation, we investigated antigen-specific IgE levels in serum and splenocyte proliferation.

Antigen-specific IgE production was increased by TCE ingestion at the lower dose (0.03 mg/l), but the increase in IgE levels did not parallel the enhancement of the ACA reaction, so the antigen-specific IgE levels are considered to be less related with the enhancement. Some papers reported that antigen-specific IgE levels were poorly associated with skin sensitivity and basophil degranulation (Conroy *et al.*, 1977; Dembo and Goldstein, 1978; Malveaux *et al.*, 1978; Purohit *et al.*, 2005), and mast cells were activated by IgE-independent pathways (Tatemoto *et al.*, 2006), these results support current results.

Antigen-stimulated and ConA-stimulated splenocyte proliferation was activated by TCE ingestion, and the activation paralleled the enhancement of the ACA reaction. It was reported previously that TCE ingestion affects Th1 immune responses and the T-cell subset, but does not affect mitogen-induced splenic cells or T or B cell proliferation without immunization (Kaneko *et al.*, 2000; Peden-Adams *et al.*, 2006). However, our results show that TCE ingestion affects antigen and ConA stimulated splenocyte proliferation, splenocyte T cell proliferation is altered by TCE ingestion and immunization.

Productions of cytokines from splenocytes were increased by TCE ingestion. IL-4 and IFN-γ productions were increased only at the concentration of 0.03 mg/l TCE ingestion, so the enhancement of allergic reaction by TCE ingestion might be somewhat related with Th1/Th2 balance. TNF-α production was also increased by TCE ingestion, it might be relate with the enhancement of inflammation, because TNF-α is well known as a pro-inflammation cytokine. It was reported that IL-3 derived from T cells are required for the development and activation of the mast cells (Murakami *et al.*, 1995; Lantz *et al.*, 1998). IL-3 production was increased, and mast cells development and activation be enhanced by TCE ingestion.

In addition, mast cells and T cells clearly have bidirectional influence on each other. It was reported that this relationship can be attributed to the biological effects of T cell derived mediators on mast cell function (Alam *et al.*, 1992; Grant *et al.*, 1991; Levi-Schaffer *et al.*, 1991), and also mast cell-T cell interactions involving the binding of cell surface molecules have been linked to the biologic

effects exerted by cytokines and other mediators secreted by these two cell populations (Baram *et al.*, 2001; Bhattacharyya *et al.*, 1998; Inamura *et al.*, 1998; Mekori and Metcalfe, 1999; Brill *et al.*, 2004). We previously reported that organic chemical exposure enhanced antigen-stimulated degranulation from mast cells (Seo *et al.*, 2008), additionally the enhancement of antigen-stimulated T cell proliferation is possibly attributable to the alteration of degranulation by mast cells from current study.

In conclusion, TCE ingestion alters antigen-specific T cell proliferation; moreover, TCE ingestion may affect antigen-specific IgE production and cytokine production from antigen-specific T cells and affect mast cell development by some cytokines derived from T cells. In addition, the histamine level of the reaction site was enhanced, and a stronger response to antigen was induced by TCE ingestion. The detailed mechanisms of these effects are unclear, but the dose of TCE in this study might be realistically encountered during the normal course of daily life, so TCE ingestion from drinking water may be associated with the causation of allergic diseases.

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Enhancement of allergic reactions by trichloroethylene ingestion

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