



Modulation of neurological related allergic reaction in mice exposed to low-level toluene

Tin-Tin-Win-Shwe, Shoji Yamamoto, Daisuke Nakajima, Akiko Furuyama, Atsushi Fukushima, Soheli Ahmed, Sumio Goto, Hidekazu Fujimaki *

Research Center for Environmental Risk, National Institute for Environmental Studies 16-2, Onogawa, Tsukuba, Ibaraki 305-8506, Japan

Received 1 February 2007; revised 14 March 2007; accepted 14 March 2007

Abstract

The contributing role of indoor air pollution to the development of allergic disease has become increasingly evident in public health problems. It has been reported that extensive communication exists between neurons and immune cells, and neurotrophins are molecules potentially responsible for regulating and controlling this neuroimmune crosstalk. The adverse effects of volatile organic compounds which are main indoor pollutants on induction or augmentation of neuroimmune interaction have not been fully characterized yet. To investigate the effects of low-level toluene inhalation on the airway inflammatory responses, male C3H mice were exposed to filtered air (control), 9 ppm, and 90 ppm toluene for 30 min by nose-only inhalation on Days 0, 1, 2, 7, 14, 21, and 28. Some groups of mice were injected with ovalbumin intraperitoneally before starting exposure schedule and these mice were then challenged with aerosolized ovalbumin as booster dose. For analysis of airway inflammation, bronchoalveolar lavage (BAL) fluid were collected to determine inflammatory cell influx and lung tissue and blood samples were collected to determine cytokine and neurotrophin mRNA and protein expressions and plasma antibody titers using real-time RT-PCR and ELISA methods respectively. Exposure of the ovalbumin-immunized mice to low-level toluene resulted in (1) increased inflammatory cells infiltration in BAL fluid; (2) increased IL-5 mRNA, decreased nerve growth factor receptor tropomyosin-related kinase A and brain-derived neurotrophic factor mRNAs in lung; and (3) increased IgE and IgG₁ antibodies and nerve growth factor content in the plasma. These findings suggest that low-level toluene exposure aggravates the airway inflammatory responses in ovalbumin-immunized mice by modulating neuroimmune crosstalk.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Airway; Toluene; Allergy; Mice; Cytokine; Neurotrophin

Introduction

An increasing trend in the prevalence of allergic diseases, such as asthma, rhinitis and eczema, has been reported from many industrialized countries (WHO regional Office for Europe, 2002). Outdoor and indoor air pollutants are environmental risk factors that have been shown to contribute to the development of respiratory infections and allergy. As for children who spend more time in homes than outside, indoor air pollution may play a role in the development of allergic diseases in concert with inhalant allergens (Eggleston et al., 1999).

Cigarette smoke and volatile organic compounds (VOCs) are potential chemical contributors to asthmatic airway inflamma-

tion in the home environment (Clark et al., 1999). While there appear to be no studies on the effects of inhalation of VOCs on the induction of respiratory diseases in houses and offices, it has been speculated that a positive relationship might exist between immunological alterations and inhalation of VOCs. The putative mechanisms underlying the aggravation of allergic diseases by VOCs and methods to identify susceptible subpopulations are still lacking.

However, there is a rapidly accumulating evidence to suggest that environmental agents may cause adverse health effects by disrupting the homeostatic regulatory mechanisms of the nervous and immune systems. Toluene is a toxic indoor air pollutant that can occur at relatively high concentrations in homes and buildings, and it has been speculated that chronic low-level exposure to this substance can impair neural and immune functions. In terms of the immunological effects, single

* Corresponding author.

E-mail address: fujimaki@nies.go.jp (H. Fujimaki).

exposures to toluene at 2.5, 100, 250 and 500 ppm have been shown to decrease the pulmonary bactericidal activity (Aranyi et al., 1985), and interleukin (IL)-2 produced by splenocytes in mice fed drinking water containing toluene (Hsieh et al., 1991). Hamsters inhaled a toluene/*n*-hexane mixture were shown to exhibit impaired host resistance to the *Mycobacterium* species (Palermo-Neto et al., 2001). Epidemiologically, occupational exposures to mixed organic solvents, such as toluene, ethyl acetate, isopropyl alcohol, and ethyl ketone, have been shown to decrease the number of T lymphocytes and increase the number of B lymphocytes in the peripheral blood (Tanigawa et al., 2001). Exposure to alkanes and aromatic compounds (toluene, *o*-xylene, *m*+*p*-xylene, 2-, 3- and 4-ethyl-toluene, chlorobenzene) have been shown to contribute to a skewed type 2 memory in response to allergen challenge (Lehmann et al., 2001). On the other hand, toluene inhalation has also been shown to lead to neurogenic disorders. Subacute exposure to 80 ppm toluene increased the number of dopamine D-2 receptors in rats and increased their locomotor activity (von Euler et al., 1991, 1993). An *in vivo* animal study reported that short-term exposure to 4000 ppm toluene protected against *N*-methyl-D-aspartic acid-induced neurotoxic effects (Cruz et al., 2003). From these findings, exposure to toluene may affect allergic inflammatory responses and also contribute to neural dysfunction.

The existence of interactions between the immune and the nervous systems, the “neuro-immune axis” has been controversial for many years. It has been now recognized that the nervous system and immune system speak a common biochemical language and communicate via a complete bidirectional circuit involving shared ligands such as neurotransmitters, neuroendocrine hormones, cytokines and the respective receptors (Blalock, 2005). Tracey (2002) has discovered the cholinergic anti-inflammatory pathway which is mediated primarily by nicotinic acetylcholine receptors on tissue macrophages, then leads to decreased production of proinflammatory cytokines. The identification of an immediate vagus-mediated regulation of pro-inflammatory cytokine production in tissue macrophages can have immense importance for development of novel anti-inflammatory therapeutic options (Andersson, 2005).

Neurotrophins are molecules regulating and controlling crosstalk between the immune and peripheral nervous system during inflammatory lung diseases. They are constitutively expressed by resident lung cells and produced in increasing quantities by immune cells invading the airways under inflammatory conditions (Nockher and Renz, 2003). These neurotrophins may act as activation, differentiation and survival factors for cells of both the immune and nervous system (Nockher and Renz, 2003). Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) play important roles in neuroimmune crosstalk (Aloe et al., 1997; Stanisz and Stanisz, 2000) and they serve as a mediator linking allergic airway inflammation with neuronal changes (Braun et al., 1999; P  th et al., 2002). However, the effects of low-level exposure to VOCs on the interactions between allergic inflammation and neurotrophins remain unknown.

The aim of this study was to investigate the effects of low-level toluene exposure on the allergic airway inflammatory

responses and neurotrophin production in OVA-sensitized mice. For this purpose, we examined BAL fluid for inflammatory responses, plasma IgE, IgG₁ and IgG_{2a} for allergic responses, neurotrophins (NGF, BDNF and their receptors TrkA, TrkB and neurotrophin-3) and cytokines (IL-4, IL-5, IL-12, interferon (IFN)- γ) mRNA expressions in the lung for assessment of their role in neuro-immune cross talk.

Materials and methods

Animals. Male C3H/HeN mice (8-week-old) were purchased from Charles River Japan Inc.; 9-week-old mice were used for all the experiments. The animals were allowed access to food and water *ad libitum*. The study was conducted with the approval of the National Institute for Environmental Studies Ethics Committee for Experimental Animals.

Exposure to toluene. Toluene was generated in an organic solvent gas generator (Shibata Scientific Technology Ltd.), diluted with clean, filtered air to achieve the desired gas concentrations, and then introduced into an acrylic chamber (Fig. 1A). The atmospheric concentration of toluene in the chambers was measured by gas chromatography mass spectrometry (INFICON, NY). The average levels of control, 9 ppm and 90 ppm toluene were 0.01 ± 0.001 ppm, 9.1 ± 0.1 ppm, and 90.3 ± 0.6 ppm, respectively. The airflow rate through the chamber housing the experimental animals was $20 \text{ L} \times \text{min}^{-1}$. The concentrations of toluene were constant, irrespective of the sampling port location. Groups of mice ($n=6$) were exposed to 0, 9 and 90 ppm toluene for 3 consecutive days followed by once per week for 4 weeks in nose-only exposure chamber. These were Days 0, 1, 2, 7, 14, 21 and 28 of the study period. The allergic model groups of mice were given OVA immunization 3 times at Day 0, 14 and 28 approximately 1 h before toluene exposure. These mice were injected with 10 μg ovalbumin (OVA) plus 2 mg alum intraperitoneally on Day 0. Each of these mice was then challenged with nebulized OVA as a booster once every 2 weeks (Day 14 and 28) during the exposure period as described previously (Fujimaki et al., 2001) (Fig. 1).

Collection of specimens of bronchoalveolar lavage (BAL) fluid, lung, and blood. One day following the final toluene inhalation, the mice were euthanized under pentobarbital anesthesia, and BAL fluid and blood samples were collected. BAL fluid was collected by cannulating the trachea and lavaging the lungs twice with 1 ml of sterile saline. The recovered fluid about 1.8 ml in volume was pooled. The BAL fluid specimens were then centrifuged and the resultant cell pellet was diluted with sterile saline. Aliquots were analyzed by hemocytometer and trypan blue dye exclusion for determining the viable cell count and by light microscopy following Diff-Quik-staining (Dade, Brussels). In other experiments under the same experimental schedule, the lungs were collected under the anesthesia and frozen quickly in liquid nitrogen, and then stored at -80°C until total RNA of the lung was extracted.

Analysis of plasma neurotrophin and corticosterone levels. The concentrations of NGF and BDNF in plasma samples were measured by ELISA, in accordance with the manufacturer's directions (Promega, Madison, WI). Absorbance was measured with a microplate reader (Bio-Rad, Model 550) at wavelengths of 450 and 550 nm. The concentration of corticosterone in plasma samples was measured by RIA (SRL, Tokyo).

Quantification of lung mRNA expression levels. The expression levels of IL-4, IL-5, IL-12, interferon (IFN)- γ , NGF, BDNF, neurotrophin (NT)-3, tropomyosin-related kinase (Trk) A, Trk B and 18S mRNAs in the lung were estimated by quantitative real-time PCR analysis using ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc., USA) (Tin-Tin-Win-Shwe et al., 2005). Briefly, total RNA was isolated from the tissue samples using the BioRobot EZ 1 and EZ 1 RNA tissue minikit (Qiagen GmbH, Germany). First-strand cDNA was then synthesized from 400 ng of total RNA by means of SuperScript RNase H⁻ Reverse Transcriptase II (Invitrogen, USA) in accordance with the manufacturer's protocol. For use as standards, we diluted the lung cDNA samples serially to several folds. Briefly, we prepared reactions in a final volume of 25 μl containing 5 μl of each of the 5-fold diluted cDNA samples or each of the

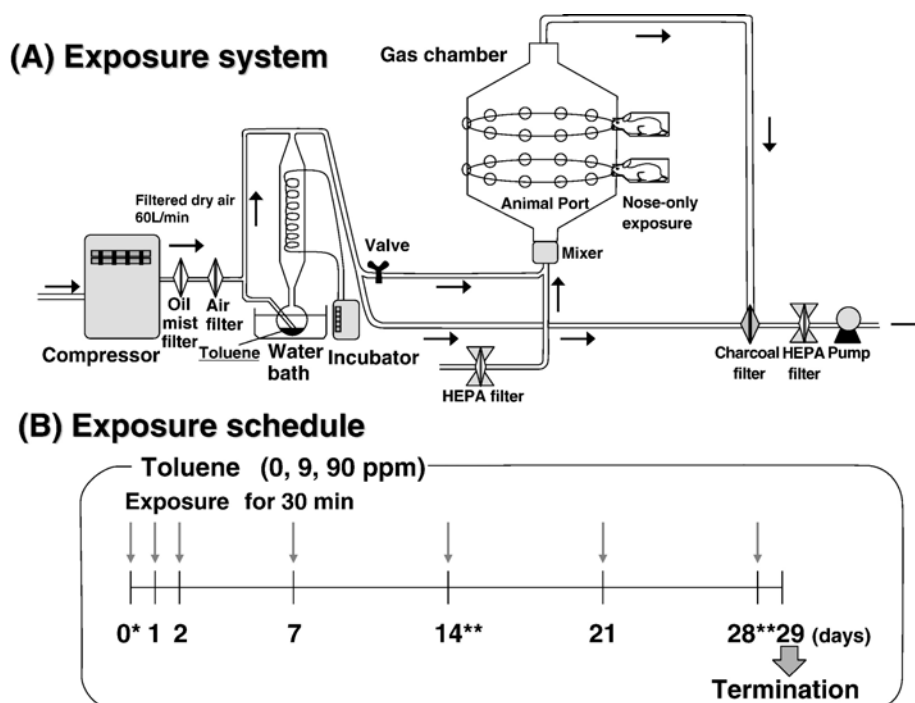


Fig. 1. Upper panel shows nose-only exposure chamber of toluene for mouse and lower panel shows experimental schedule for the present study. Asterisks indicate the ovalbumin immunization. One asterisk indicates intraperitoneal injection and two asterisks indicate aerosol sensitization.

diluted standards, 12.5 μ l of 2 $\times$ Master Mix (Quantitect Probe PCR Kit or Quantitect SYBR Green PCR Kit), 2.5 μ l of 10 μ M primer-mix of NT-3 or each of 1.5 μ l of 10 μ M primer-mix for other genes and 5 μ l or 6 μ l of RNase free H₂O. The SYBR Green I based PCR was performed using a dissociation protocol and the conditions were as follows: initial 1 cycle of enzyme activation for 15 min at 95 $^{\circ}$ C, followed by 20 to 35 cycles of amplification (20 cycles for the 18 S rRNA primer pair and 35 cycles for the others) comprising initial denaturation at 94 $^{\circ}$ C for 15 s, annealing at 59 $^{\circ}$ C or 60 $^{\circ}$ C for 30 s and elongation at 72 $^{\circ}$ C for 30 s. Tissue 18 S rRNA level was examined for use as the internal control. Data were analyzed by the comparative threshold cycle method. Then, we normalized the relative expression level of each mRNA individually by the 18 S rRNA content in the respective samples and expressed as mRNA signals per 18 S rRNA.

Measuring total and anti-OVA IgE, IgG₁, IgG_{2a} titers in plasma. Plasma total IgG₁ and IgG_{2a} antibodies were measured by ELISA as previously described (Fujimaki et al., 2001, 2004). The OD at 490 and 595 nm was determined by using a microplate reader (Bio-Rad Laboratories). Total IgE antibodies in plasma were measured by using mouse ELISA kits (Seikagaku Co., Tokyo). Plasma titers of antigen-specific IgE, IgG₁ and IgG_{2a} antibodies were also measured by ELISA.

Statistical analysis. All the data were expressed as the mean $\pm$ standard error (S.E.). Statistical analysis was performed using the StatMate II statistical analysis system for Microsoft Excel, Version 5.0 (Nankodo Inc., Tokyo, Japan). Dose–response data were analyzed by one-way analysis of variance with a *post hoc* analysis using Dunnett's multiple comparisons. * P < 0.05, ** P < 0.01.

Results

Analysis of airway inflammatory response following low-level toluene inhalation

To analyze the local inflammatory responses in the mice following low-level exposure to toluene, we investigated the accumulation of inflammatory cells in the lungs. In the

nonimmunized mice, the total BAL cell count increased in a dose-dependent manner, although the increase was not statistically significant (Fig. 2A), while only the number of macrophages in the BAL fluid showed significant increase in the toluene-exposed mice, with no remarkable changes in the number of neutrophils and lymphocytes (Fig. 2B). In the OVA-immunized mice, on the other hand, exposure to 9 ppm toluene resulted in a significant increase in the number of macrophages in the BAL fluid, while exposure to 90 ppm toluene resulted in a significant decrease in the number of macrophages in the BAL fluid. There were no significant changes in the number of other inflammatory cells in the BAL fluid following inhalation of toluene in the mice (Figs. 2C, D, E).

Next, we analyzed changes in levels of cytokines related to allergic inflammation in the lung of immunized mice using quantitative real-time PCR method.

Exposure to 90 ppm toluene significantly decreased the expression of IFN- γ mRNA in the lung (Fig. 3C). Although exposure to 9 ppm toluene significantly increased the IL-5 mRNA expression in the lung, no change was observed following 90 ppm toluene exposure in the immunized mice (Fig. 3D). There were no significant differences in the expression of IL-4 and IL-12 mRNAs in the lung following toluene exposure (Figs. 3A, D).

Analysis of systemic allergic response following low-level toluene inhalation

To investigate the systemic effects of toluene exposure, the antibody levels in the plasma were measured by ELISA. The total IgG₁ and IgE titers in the plasma in the nonimmunized

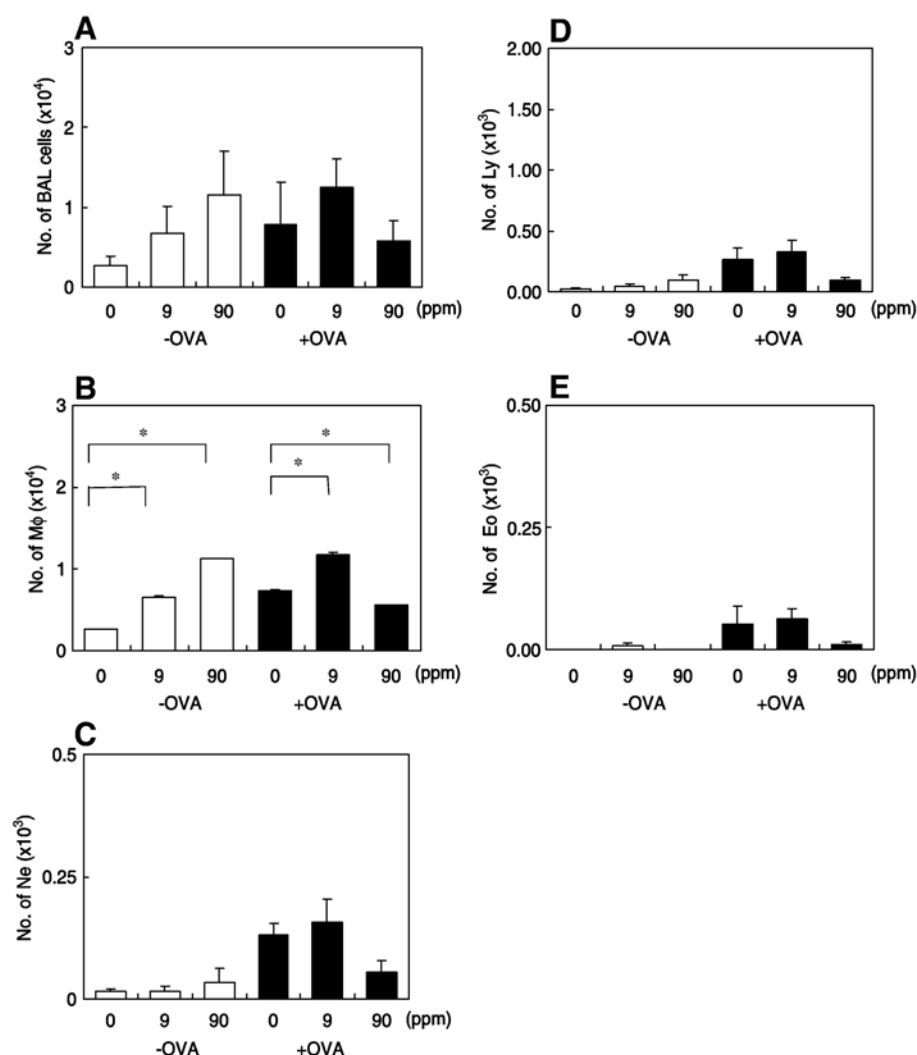


Fig. 2. The number of inflammatory cells in BAL fluid from nonimmunized (–OVA) and OVA-immunized (+OVA) mice exposed to 0, 9, and 90 ppm toluene ($n=6$). No. of total BAL cells ($\times 10^4$), (B) No. of macrophages (Mφ) ($\times 10^4$), (C) No. of neutrophils (Ne) ($\times 10^3$), (D) No. of lymphocytes (Ly) ($\times 10^3$), (E) No. of eosinophils (Eo) ($\times 10^3$), * $P < 0.05$.

mice following exposure to toluene observed no significant differences compared with those of control mice (data not shown). In the mice subjected to OVA sensitization and challenges, however, the total levels of IgE antibody in the plasma increased following exposure to 90 ppm toluene (Fig. 4A). Although the total plasma level of IgG₁ was significantly increased in both of 9 and 90 ppm toluene-exposed, immunized mice (Fig. 4B), no significant changes in the total IgG_{2a} levels were observed (Fig. 4C). Both anti-OVA IgE and IgG₁ antibody levels in the plasma were significantly increased following 9 ppm toluene inhalation, but not following 90 ppm toluene inhalation (Figs. 4D, E). There were no significant differences in the plasma anti-OVA IgG_{2a} antibody levels between the control mice and toluene-exposed mice (Fig. 4F).

It has been demonstrated that NGF protein levels in serum increased significantly in a mouse model of allergic inflammation (Braun et al., 1998). When we examined the NGF content in the plasma, the production of NGF from nonimmunized mice

showed no significant changes by toluene inhalation, but in OVA immunized mice 9 ppm toluene inhalation significantly increased the content of NGF in the plasma (Fig. 5). There was no increase in BDNF content in the plasma from toluene-exposed, immunized mice (data not shown). The corticosterone concentrations in the plasma from toluene-exposed, immunized mice were 219.2 ± 34.4 (ng/ml) for 0 ppm control group, 175.6 ± 28.0 for the 9 ppm toluene-exposed group, and 134.4 ± 30 for the 90 ppm toluene-exposed group. These results indicate that low-level toluene inhalation can stimulate allergic inflammatory response to OVA and the increase of NGF concentration in the plasma from toluene-exposed, immunized mice showed no correlation with plasma corticosterone levels.

Analysis of lung neurotrophins following low-level toluene inhalation

Neurotrophins have been shown not only to mediate inflammatory signals among neurons, immune and lung tissue

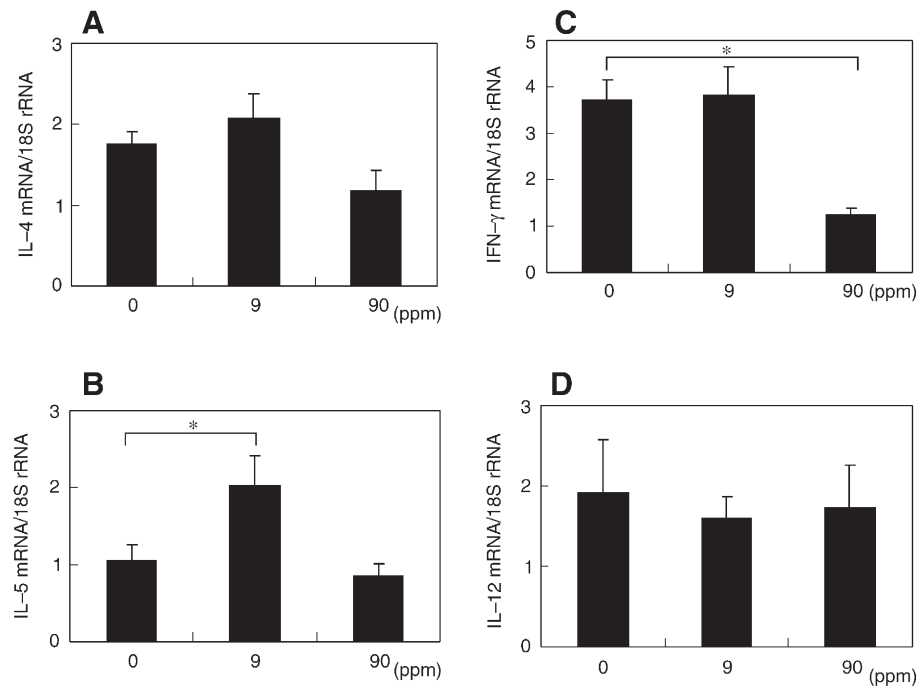


Fig. 3. Expression of cytokine mRNAs in lung from immunized mice exposed to 0, 9, and 90 ppm toluene ($n=6$). (A) IL-4, (B) IL-5, (C) IFN- γ , (D) IL-12, $*P < 0.05$.

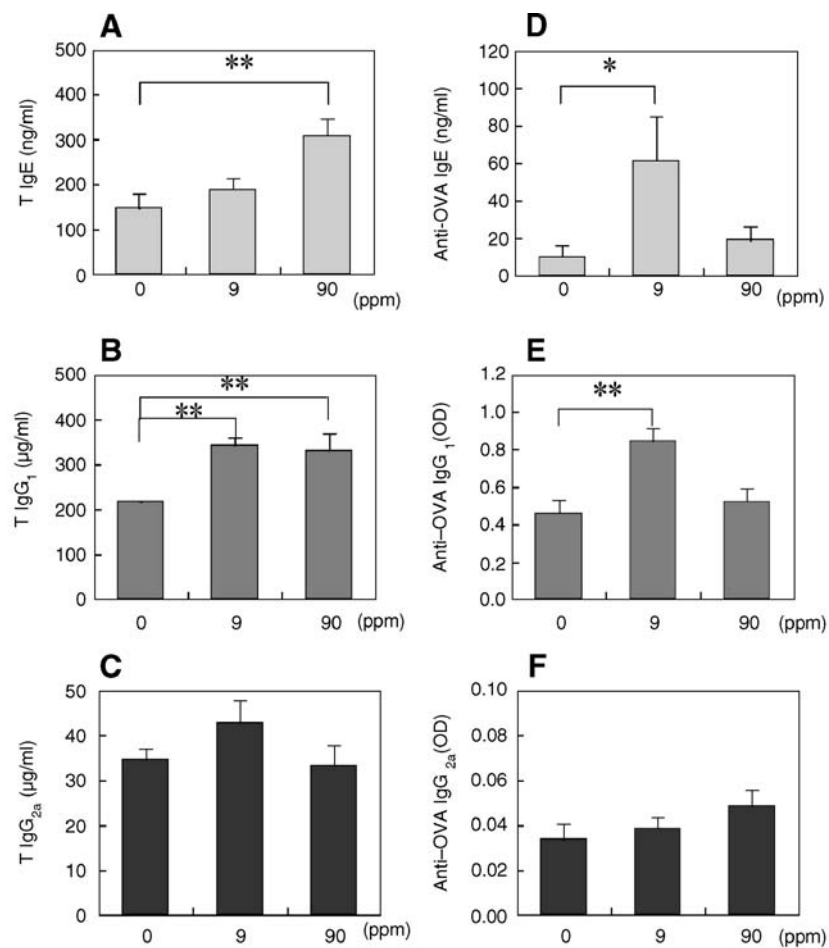


Fig. 4. Plasma levels of total Ig and anti-OVA Ig in immunized mice ($n=6$). (A) Total IgE antibody levels, (B) total IgG₁ antibody levels, (C) total IgG_{2a} antibody levels, (D) anti-OVA IgE antibody levels, (E) anti-OVA IgG₁ titers, (F) anti-OVA IgG_{2a} titers, $*P < 0.05$, $**P < 0.01$.

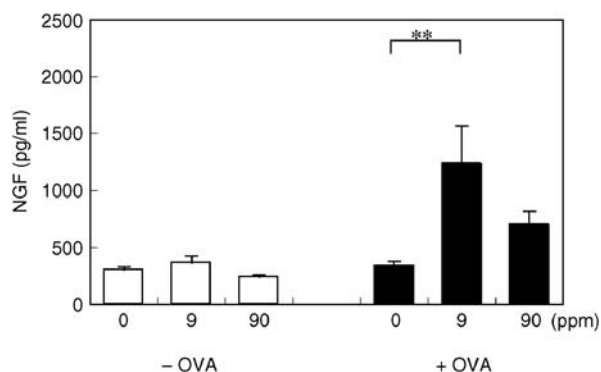


Fig. 5. Plasma levels of NGF following toluene exposure in nonimmunized (-OVA) and OVA-immunized mice ($n=6$), $**P<0.01$.

cells but also to promote the survival of eosinophils (Braun et al., 1998; Hahn et al., 2006). No significant differences in the expression of NGF and NT-3 mRNAs in the lung were found between mice exposed to toluene and control mice (Figs. 6A, C). The expression of BDNF mRNA in the lung decreased significantly in immunized mice following exposure to 9 and 90 ppm toluene (Fig. 6B). On the contrary, NGF receptor Trk A mRNA expression was significantly reduced in both the 9 and

90 ppm toluene-exposed, immunized mice (Fig. 6D). There was no significant difference in the expression of BDNF receptor Trk B mRNA in the lung between toluene-exposed and control mice (Fig. 6E).

Discussion

In this study, we examined the effects of low-level toluene exposure on the induction of allergic inflammatory responses in mice lung and found that low-level toluene inhalation enhanced antigen-specific inflammatory responses only in mice that had been sensitized and challenged with ovalbumin. These results suggest that low-level toluene in indoor air may exacerbate the inflammatory response in asthmatic population.

Our findings show that exposure to high-level toluene significantly increased the number of alveolar macrophages in the BAL fluid in non-immunized mice. However, in the OVA-immunized mice, while exposure to low-level toluene caused a significant increase in the number of alveolar macrophages, high-level toluene did not cause any such increase. Exposure of immunized mice to low-level toluene significantly increased the expression of IL-5 mRNA in the lung. Moreover, anti-OVA IgE and IgG₁ production in the plasma were significantly increased

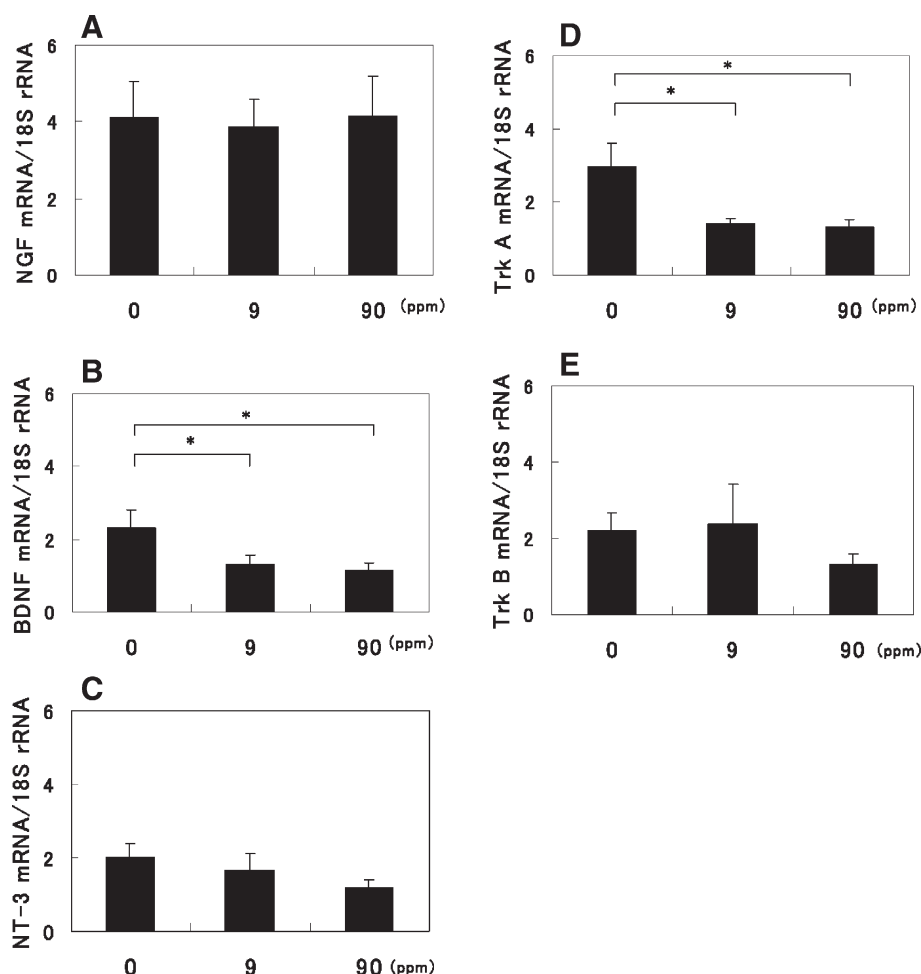


Fig. 6. Expression of neurotrophins and their receptor mRNAs in lung from immunized mice exposed to 0, 9, and 90 ppm toluene ($n=6$). (A) NGF, (B) BDNF, (C) NT-3, (D) Trk A, (E) Trk B, $*P<0.05$.

following exposure to low-level toluene. These findings indicate the adjuvant effects of low-level toluene on the manifestation of allergic response to OVA. However, it has not been shown dose-dependency in the adjuvant activity. No mechanism has been proposed to the above adjuvant effect, but one possibility is that toluene inhalation contributes to neural dysfunction related to sensory stimulation and homeostatic mechanisms compensate for toxicant-induced changes. Actually, in OVA immunized mice 9 ppm toluene inhalation significantly increased the content of NGF in the plasma.

In histological examination, exposure of immunized mice to low-level toluene resulted in no significant changes in the epithelial cells or mast cells of the nasal mucosa (data not shown). Thus, low-level toluene exposure does not appear to exert any significant histological effects in the nasal mucosa. Chronic low-level exposure to toluene has been suspected to cause impairment of neural and immune function. It is speculated that adaptive and homeostatic mechanisms in mice exposed to low-level toluene compensate for the toxicant-induced changes to maintain a relatively constant internal environment. Continuous or frequent exposures to a given stimulus favor the development of tolerance, whereas intermittent exposures favor sensitization (Post, 1980). In the present experiment, we found that exposure of immunized mice to 9 ppm toluene increased NGF contents in the plasma and enhanced Th2-type immune response to OVA. Our present data indicate that NGF levels in the plasma of low-level toluene-exposed mice are correlated with OVA-specific IgE and total IgG₁ antibody titers. NGF levels in serum are related to total IgE titers in patients with allergic asthma (Bonini et al., 1996). However, anti-NGF treatment induced no differences in serum IgE titers in allergic early-phase reaction (Päth et al., 2002). While neither toluene alone nor OVA immunization was able to induce significant NGF production, the combination of low-level toluene and OVA stimulation induced a strong production.

Recently, the neurotrophins have been identified as important mediators of inflammatory signals regarding the bronchial nervous system and they play a role in bidirectional signaling between cells of the immune and nervous systems. Under physiologic conditions, the local production of neurotrophins is low in both human and animal studies (Nockher and Renz, 2006). Increased levels of BDNF and NGF were observed during allergic diseases and these may regulate immune functions as well as neuroimmune interactions (Bonini et al., 1999; Braun et al., 2000; Vega et al., 2003). Neurotrophins act as autocrine as well as paracrine signaling (Nockher and Renz, 2006). In a murine model of allergic asthma, macrophages in BAL fluid strongly produce NGF and BDNF after allergen challenge (Braun et al., 1999; 2004). Our present results indicate that low-level toluene exposure increased the number of macrophages in BAL fluid, but decreased the expression of Trk A and BDNF mRNAs in the lung tissue. BDNF partially mediated the development of allergen-induced neuronal hyperactivity, and infiltrating T lymphocyte subsets, macrophages and airway epithelial cells constitute the cellular sources of BDNF (Braun et al., 1999; 2004). Down-regulation of the

expression of Trk A mRNA, but not Trk B mRNA, in the lung may suggest an increase in the NGF content in the plasma. Therefore, our data showing exposure to 9 ppm toluene increased the production of NGF in the plasma are compatible. During allergic inflammation, NT-3 was only produced by cells of the inflammatory infiltrate but not by bronchial epithelial cells (Hahn et al., 2006). In the present study, no remarkable changes in the number of inflammatory cells except macrophages in BAL fluid and expression of NT-3 mRNA were shown. Thus, dysregulation of neurotrophins such as BDNF and NGF appear to play a role in the modulation of allergic inflammatory responses following toluene exposure in the immunized mice. Activation of the sympathetic nervous system has predominantly anti-inflammatory effects that are mediated through direct nerve to immune cell interaction or through the adrenal neuro-endocrine axis (Andersson, 2005). Since no significant differences in the concentration of corticosterone in plasma were found between toluene-exposed and control mice, the increase in the production of NGF in plasma is independent upon the adrenal neuro-endocrine axis.

Finally, we found that low-level toluene exposure induced allergic airway inflammatory responses and increased circulating NGF level in OVA sensitized mice. However, no alteration of NGF level and significantly decreased BDNF level in the local lung tissues suggested that dysregulation of neuroimmune cross talk in low-level toluene exposed immunized mice. However, neurogenic inflammation by release of neuropeptides such as substance P and calcitonin gene-related peptide may play some important roles in initiation of airway inflammatory response (Barnes, 2001). Further studies addressing the activation of sensory nerves in the lung by toluene inhalation are needed.

Characterization of these compensatory processes is particularly important at low, environmentally relevant doses, while the risk of toxic effects is typically only inferred from the effects observed at much higher doses. Concentrations of 40 ppm (in European Union) and 32 ppm (in USA) have been proposed as “no observed adverse effects” level (Andersen et al., 1983; Zavalic et al., 1998). In Japan, the occupational exposure limit for toluene proposed by the Japan Society for Occupational Health is 50 ppm (Japan Society for occupational health, 1994). Recent studies in Japan indicate that the concentrations of toluene in homes range from <0.01 to 1.63 (ppm), with mean concentration of 0.023 ppm toluene throughout a year (Ministry of Land infrastructure and Transport in Japan, 2002). Therefore, we selected reliable toluene concentrations between occupational exposure level and in door air level and found enhancement of allergic inflammatory responses in immunized mice.

To evaluate the environmental risk of various VOCs in the induction or exacerbation of allergic inflammatory response, it is necessary to develop new tools. The implications of our present findings are significant, not only for the dose-responses in terms of toxicology, but also in terms of the reliability of the animal model, and the risk assessment applications. Further studies will be required to determine whether low-level exposure to other VOCs in indoor air pollutants might also

have the ability to exacerbate allergic inflammation. Taken together, our exposure system is a useful tool for evaluating the development of Th2-type immune responses following low-level VOCs stimulation.

In conclusion, low-level exposure of immunized mice to toluene can aggravate allergic inflammatory responses and may disturb neuroimmune crosstalk in the relationship between neurotrophin and allergic inflammations.

Acknowledgments

We thank Drs. N. Kunugita, H. Hori, and K. Arashidani (University of Occupational and Environmental Health) for their helpful discussions and comments. We also thank Mrs. S. Murakami, K. Ohnishi and S. Shimizu for their technical assistance. This research was partly supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

- Aloe, L., Bracci-Laudiero, L., Bonini, S., Manni, L., 1997. The expanding role of nerve growth factor: from neurotrophic activity to immunologic diseases. *Allergy* 52, 883–994.
- Andersen, I., Lundqvist, G.R., Molhave, L., Pedersen, O.F., Proctor, D.F., Vaeth, M., Wyon, D.P., 1983. Human response to controlled levels of toluene in six-hour exposures. *Scand. J. Work, Environ. Health* 9, 405–418.
- Andersson, J., 2005. The inflammatory reflex—introduction. *J. Int. Med.* 257, 122–125.
- Aranyi, C., O'Shea, W.J., Sherwood, R.L., Graham, J.A., Miller, F.J., 1985. Effects of toluene inhalation on pulmonary host defenses of mice. *Toxicol. Lett.* 25, 103–110.
- Barnes, P.J., 2001. Neurogenic inflammation in the airways. *Respir. Physiol.* 125, 145–154.
- Blalock, J.E., 2005. The immune system as the sixth sense. *J. Int. Med.* 257, 126–138.
- Bonini, S., Lambiase, A., Bonini, S., Angelucci, F., Magrini, L., Manni, L., Aloe, L., 1996. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. *Proc. Natl. Acad. Sci. U.S.A.* 93, 10955–10960.
- Bonini, S., Lambiase, A., Bonini, S., Levi-Schaffer, F., Aloe, L., 1999. Nerve growth factor: an important molecule in allergic inflammation and tissue remodeling. *Int. Arch. Allergy Immunol.* 118, 159–162.
- Braun, A., Appel, E., Baruch, R., Herz, U., Botchkarev, V., Paus, R., Brodie, C., Renz, H., 1998. Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma. *Eur. J. Immunol.* 28, 3240–3251.
- Braun, A., Lommatzsch, M., Mannsfeldt, A., Neuhaus-Steinmetz, U., Fischer, A., Schnoy, N., Lewin, G.R., Renz, H., 1999. Cellular sources of enhanced brain-derived neurotrophic factor production in a mouse model of allergic inflammation. *Am. J. Respir. Cell Biol.* 21, 537–546.
- Braun, A., Lommatzsch, M., Renz, H., 2000. The role of neurotrophins in allergic bronchial asthma. *Clin. Exp. Allergy* 30, 178–186.
- Braun, A., Lommatzsch, M., Neuhaus-Steinmetz, U., Quarcoo, D., Glaab, T., McGregor, G.P., Fischer, A., Renz, H., 2004. Brain-derived neurotrophic factor (BDNF) contributes to neuronal dysfunction in a model of allergic airway inflammation. *Br. J. Pharmacol.* 141, 431–440.
- Clark, N.M., Brown, R.W., Parke, R.E., Robins, T.G., Philbert, M.A., Keeler, G.J., Israel, B.A., 1999. Childhood asthma. *Environ. Health Perspect.* 107, 421–429.
- Cruz, S.L., Gauthereau, M.Y., Camacho-Muñoz, C., López-Rubalcava, C., Balster, R.L., 2003. Effects of inhaled toluene and 1,1,1-trichloroethane on seizures and death produced by *N*-methyl-D-aspartic acid in mice. *Behav. Brain Res.* 140, 195–202.
- Eggleston, P.A., Buckley, T.J., Breysse, P.N., Wills-Karp, M., Kleeberger, S.R., Jaakkola, J.J.K., 1999. The environment and asthma in U.S. inner cities. *Environ. Health Perspect.* 1107, 439–450.
- Fujimaki, H., Ui, N., Endo, T., 2001. Induction of inflammatory response of mice exposed to diesel exhaust is modulated by CD4⁺ and CD8⁺ T cells. *Am. J. Respir. Crit. Care Med.* 164, 1867–1873.
- Fujimaki, H., Kurokawa, Y., Kunugita, N., Kikuchi, M., Sato, F., Arashidani, K., 2004. Differential immunologic and neurogenic inflammatory responses in an allergic mouse model exposed to low levels of formaldehyde. *Toxicology* 197, 1–13.
- Hahn, C., Islamian, A.P., Renz, H., Nockher, W.A., 2006. Airway epithelial cells produce neurotrophins and promote the survival of eosinophils during allergic airway inflammation. *J. Allergy Clin. Immunol.* 117, 787–794.
- Hsieh, G.C., Sharma, R.P., Parker, R.D.R., 1991. Hypothalamo–pituitary–adrenocortical axis activity and immune function after oral exposure to benzene and toluene. *Immunopharmacology* 21, 23–32.
- Japan Society for Occupational Health, 1994. Toluene. *Ind. Med.* 36, 267–273.
- Lehmann, I., Rehwagen, M., Diez, U., Seiffart, A., Rolle-Kampczyk, U., Richter, M., Wetzig, H., Borte, M., Herbarth, O., 2001. Enhanced in vivo IgE production and T cell polarization toward the type 2 phenotype in association with indoor exposure to VOC: results of the LARS study. *Int. J. Hyg. Environ. Health* 204, 211–221.
- Ministry of Land Infrastructure and Transport in Japan, 2002. Annual Reports.
- Nockher, W.A., Renz, H., 2003. Neurotrophins in inflammatory lung diseases: modulators of cell differentiation and neuroimmune interactions. *Cytokine Growth Factor Rev.* 14, 559–578.
- Nockher, W.A., Renz, H., 2006. Neurotrophins in allergic diseases: from neuronal growth factors to intercellular signaling molecules. *J. Allergy Clin. Immunol.* 117, 583–589.
- Palermo-Neto, J., Santos, F.A.M., Guerra, J.L., Santos, G.O., Pinheiro, S.R., 2001. Glue solvent inhalation impairs host resistance to *Mycobacterium bovis*-induced infection in hamsters. *Vet. Hum. Toxicol.* 43, 1–5.
- Post, R.M., 1980. Intermittent versus continuous stimulation: effect of the interval on the development of sensitization or tolerance. *Life Sci.* 126, 1275–1282.
- Päth, G., Braun, A., Meents, N., Kerzel, S., Quarcoo, D., Raap, U., Hoyle, G.W., Nockher, W.A., Renz, H., 2002. Augmentation of allergic early-phase reaction by nerve growth factor. *Am. J. Respir. Crit. Care Med.* 166, 818–826.
- Stanisz, A.M., Stanis, J.A., 2000. Nerve growth factor and neuroimmune interactions in inflammatory diseases. In: Conti, A., Maestroni, G.J.M., McCann, S.M., Sternberg, E.M., Lipton, J.M., Smith, C.C. (Eds.), *Neuroimmunomodulation Perspectives at the New Millennium*. Annals of The New York Academy of Sciences, vol. 917, pp. 268–272.
- Tanigawa, T., Araki, S., Nakata, A., Yokoyama, K., Sakai, T., Sakurai, S., 2001. Decreases of natural killer cells and T-lymphocyte subpopulations and increases of B lymphocytes following a 5-day occupational exposure to mixed organic solvents. *Arch. Environ. Health* 56, 443–448.
- Tin-Tin-Win-Shwe, Yamamoto, S., Kakeyama, M., Kobayashi, T., Fujimaki, H., 2005. Effect of intratracheal instillation of ultrafine carbon black on proinflammatory cytokine and chemokine release and mRNA expression in lung and lymph nodes of mice. *Toxicol. Appl. Pharmacol.* 209, 51–61.
- Tracey, K.J., 2002. The inflammatory reflex. *Nature* 420, 853–857.
- Vega, J.A., García-Suárez, O., Hannevad, J., Pérez-Pérez, M., Germanà, A., 2003. Neurotrophins and the immune system. *J. Anat.* 203, 1–19.
- von Euler, G., Ögren, S.-O., Bondy, S.C., McKee, M., Warner, M., Gustafsson, J., Eneroth, J.-Å., Fuxe, P., 1991. Subacute exposure to low concentrations of toluene affects dopamine-mediated locomotor activity in the rat. *Toxicology* 67, 333–349.
- von Euler, G., Ögren, S.-O., Li, X.M., Fuxe, K., Gustafsson, J.-Å., 1993. Persistent effects of subchronic toluene exposure on spatial learning and memory, dopamine-mediated locomotor activity and dopamine D2 agonist binding in the rat. *Toxicology* 77, 223–232.
- WHO Regional Office for Europe, 2002. In: Tamburini, G., von Ehrenstein, O.S., Bertollini, R. (Eds.), *Children's Health and Environment: A Review of Evidence*. European Environment Agency.
- Zavalic, M., Mandic, Z., Turk, R., Bogadi-Sara, A., Plavec, D., 1998. Quantitative assessment of color vision impairment in workers exposed to toluene. *Am. J. Ind. Med.* 33, 297–304.