



Differential immunogenic and neurogenic inflammatory responses in an allergic mouse model exposed to low levels of formaldehyde

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Abstract

It is suspected that exposure to low levels of formaldehyde induces or aggravates airway inflammation mediated by immunological and neurological reactions. To clarify the effect of this exposure on allergic inflammatory responses, we exposed female C3H/He mice to 0, 80, 400, or 2000 ppb formaldehyde for 12 weeks. When mice were immunized with ovalbumin (OVA) and then exposed to formaldehyde, the numbers of total bronchoalveolar lavage cells, macrophages, and eosinophils in the mice exposed to 2000 ppb formaldehyde were significantly increased compared to 0 ppb controls. However, the production of interleukin-1 β from bronchoalveolar lavage fluid of these mice decreased significantly. Immunization with OVA significantly increased the production of nerve growth factor, but exposure to 80 and 400 ppb formaldehyde significantly reduced the nerve growth factor levels in bronchoalveolar lavage fluid of the immunized mice. In vitro study, markedly increased lipopolysaccharide-stimulated interferon- γ production in culture supernatants of spleen cells from 2000 ppb formaldehyde-exposed, nonimmunized mice, and significantly increased OVA-stimulated monocyte chemoattractant protein-1 production in culture supernatants of spleen cells from 400 and 2000 ppb formaldehyde-exposed, immunized mice were observed. Exposure to 400 ppb formaldehyde induced significant decreases in anti-OVA IgG1 and IgG3 antibody productions in plasma, whereas anti-OVA IgE antibody production was not affected. In addition, the levels of nerve growth factor in plasma of 80 and 400 ppb formaldehyde-exposed, immunized mice significantly decreased compared to 0 ppb control, immunized mice. These results provide the first experimental evidence that low levels of long-term formaldehyde inhalation can induce differential immunogenic and neurogenic responses in allergic mice. © 2003 Elsevier Ireland Ltd. All rights reserved.

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1. Introduction

Formaldehyde (FA) is a toxic indoor air pollutant found at relatively high concentrations in homes and buildings in developed countries (Samet, 1990). The

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studies of unexplained symptoms observed in chemically sensitive subjects have been increased and it is suspected that exposure to low levels of indoor air pollutants modulates allergic and neurogenic inflammation (Ziem and McTamney, 1997; Rowat, 1998). Exposure to FA elicits a variety of allergic signs and symptoms and irritates the upper respiratory tract. For example, a positive relationship between exposure of workers to FA and asthma or asthmatic bronchitis has been described (Hendrick et al., 1982; Burge et al., 1985). Long-term exposure to FA significantly increased numbers of CD26-, IL-2-positive cells, B cells and autoantibodies in some patients with multiple organ symptoms involving central nervous system, upper- and lower-respiratory symptoms and gastroenteritis (Thrasher et al., 1990). In another group of workers exposed to FA, however, there was no indication of immunologically mediated respiratory disease, but some of the workers appeared to experience respiratory or ocular symptoms caused by an irritant mechanism (Grammer et al., 1990).

Exposure to low levels of FA affects various immune functions in animals. Swiecichowski et al. (1993) reported that exposure to 300 ppb FA for 8 h increased airway reactivity to acetylcholine in guinea pigs. Exposure to 250 ppb FA over five consecutive days significantly enhanced sensitization to inhaled OVA in guinea pigs, but exposure to 130 ppb FA gave no significant difference from the control group (Riedel et al., 1996). Exposure to 1600 ppb FA for 10 days increased OVA-specific IgE production in mice intranasally sensitized with OVA (Tarkowski and Gorski, 1995). Therefore, short-term FA exposure may directly enhance sensitization in airways and aggravate allergic inflammatory reactions. However, nothing is yet known about the long-term effects of low-level FA exposure on allergic inflammation.

Recently, the effect of repeated low-level FA on escape behavior or cocaine-induced behavior in mice was investigated and showed that 1000 ppb FA inhalation is aversive and produces behavioral changes (Wood and Coleman, 1995; Sorg et al., 2001). Formaldehyde inhalation induced the release of tachykinins from sensory nerve endings in rats (Ito et al., 1996). Dearman et al. (1999) showed that FA instillation on each shaved flank in mice stimulates the production of Th1-type cytokines such as IFN- γ at the local lymph nodes. These things suggest that FA

may cause sensory nerve irritation. Neurotrophins including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) play an important role in the development of airway inflammation and airway hyperresponsiveness (Braun et al., 2000) and induce the production of tachykinins such as substance P (Donnerer et al., 1992; Vedder et al., 1993), which is involved in allergic responses (Aloe et al., 1997). It is likely that exposure to FA stimulates vagus nerve endings to release neuropeptides which can activate immuno competent cells, and modulates allergic inflammation.

In this study, we evaluated the effect of long-term ambient levels of FA inhalation on immunogenic and neurogenic inflammatory responses in a mouse model of allergic inflammation.

2. Materials and methods

2.1. Animals

Female C3H/He mice (8 weeks old) were purchased from Charles River Japan Inc. (Tokyo, Japan); 10-week-old mice were used in all experiments. Food and water were given ad libitum. The experiments were conducted under the control of the Ethics Committee of Animal Care and Experimentation in accordance with The Guiding Principle for Animal Care Experimentation, University of Occupational and Environmental Health, Japan, and the Japanese Law for Animal Welfare and Care (No. 221).

2.2. Formaldehyde exposure

FA was generated from paraformaldehyde (Hori and Arashidani, 1997). It was diluted with clean, filtered air to achieve the desired gas concentrations, and introduced into 400 l stainless steel and glass chambers. FA in the chambers was sampled in a Sep-Pak DNPH-Silica cartridge (Waters Co., Milford, MA) and determined by high-performance liquid chromatography. It was also continuously monitored with a Formaldemeter 400 (PPM Technology Ltd., Wales, UK). All groups of five mice were exposed to 0 (control), 82 ± 6 ppb (mean $\pm$ S.D.), 393 ± 34 ppb, or 1874 ± 240 ppb for 16 h a day (from 17:30 to 9:30), 5 days a week, for 12 weeks (Fig. 1, Group A).

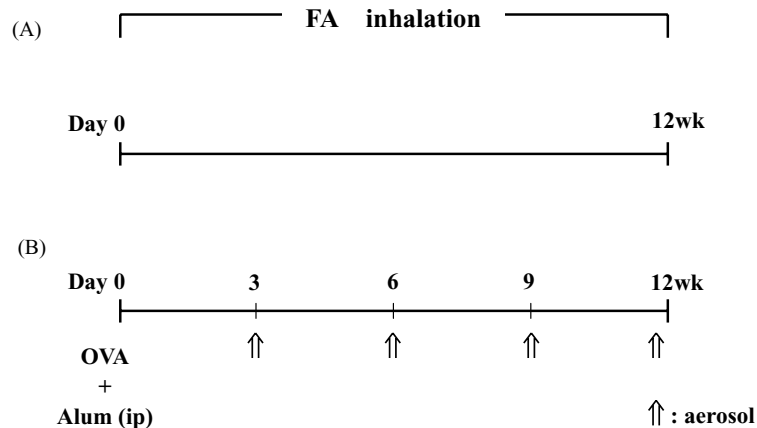


Fig. 1. Experimental design for exposure of mice to low levels of FA inhalation with or without OVA immunization. In Group A, each group of five mice was exposed to 0 (control), 80, 400, or 2000 ppb for 16 h a day, 5 days a week, for 12 weeks. In Group B, some groups of six mice were injected intraperitoneally with 10 μ g ovalbumin (OVA) plus 2 mg alum before their exposure to FA. On weeks 3, 6, 9, and 11 of the exposure period, each of these mice received at the same day of each week aerosolized OVA as a booster. All data shown are representative of two or three separate experiments.

2.3. Schedule for immunization

Some groups of six mice were injected intraperitoneally with 10 μ g ovalbumin (OVA) plus 2 mg aluminum hydroxide before their exposure to FA (Fig. 1, Group B). On weeks 3, 6, 9, and 11 of the exposure period, each mouse received aerosolized OVA for 6 min as a booster according to the methods previously described (Imai et al., 1983).

2.4. Collection of samples and culture of spleen cells

The day after the final FA inhalation, mice were sacrificed under sodium pentobarbital anesthesia, and spleens and blood samples were collected. For the collection of bronchoalveolar lavage (BAL) fluid, the lungs were lavaged twice with 1 ml of sterile PBS, and this procedure was repeated; approximately 90% of the instilled 2 ml was recovered. The combined BAL fluid was then centrifuged and stored at -70°C until analysis. For cellular profile of BAL fluid, the numbers of macrophages, neutrophils, lymphocytes and eosinophils were calculated by total cell count using a hemocytometer and smears of lavaged cells stained with Diff-Quik (International Reagents Co., Ltd., Kobe, Japan).

Spleens were forced through a stainless steel mesh, and the cells were counted in RPMI-1640 culture medium containing 10% fetal bovine serum, 5 mM HEPES, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cell proliferation of spleen cells (2×10^5 cells per well per 200 μ l) stimulated with mitogens (lipopolysaccharide (LPS; 0.5 μ g per well); phytohemagglutinin A (PHA; 5 μ g per well)) or OVA was measured by using a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; Roche Diagnostics GmbH, Mannheim, Germany). After 72 h, the optical densities at 450 and 630 nm were measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA).

For cytokine production, spleen cells (1×10^6 cells per well per 200 μ l) from nonimmunized mice were cultured with LPS or PHA, and spleen cells from immunized mice were cultured with OVA. Culture supernatants were collected at 48 h for the assay of cytokine, centrifuged, and frozen at -70°C until assayed. For chemokine production, spleen cells from immunized mice were cultured with or without OVA. Culture supernatants were collected at 24 h for the assay of chemokine after OVA stimu-

lation, centrifuged, and frozen at -70°C until assayed.

2.5. Assay for cytokine, chemokine and neuropeptide production

We quantitated cytokine production in supernatants from cultured spleen cells by using mouse interleukin (IL)-4, IL-5 (Endogen Inc., MA), interferon (IFN)- γ (Amersham International plc, England), macrophage inflammatory protein (MIP)-1 α , and monocyte chemoattractant protein (MCP)-1 (R&D systems, Minneapolis, MN) ELISA kits. For the BAL fluid, we used mouse ELISA kits to quantify production of tumor necrosis (TNF)- α , IL-1 β , IL-5, IL-6, granulocyte/macrophage colony-stimulating factor (GM-CSF) (Amersham International), eotaxin, MIP-1 α , MCP-1 (R&D systems, Minneapolis, MN) and neuropeptides of NGF, BDNF (Promega, Madison, WI) and substance P (Assay Designs, Inc., Ann Arbor, MI). Absorbance at 450 and 550 nm or at 405 and 570 nm was measured using a microplate reader.

2.6. Assay for OVA-specific IgE, IgG1, IgG2a, IgG3 and total IgE in plasma

We performed ELISA to quantify anti-OVA IgE in plasma. Briefly, 96-well microimmunoplates (Nunc, Roskilde, Denmark) were incubated with 15 $\mu\text{g}/\text{ml}$ rat monoclonal anti-mouse IgE (Seikagaku Co., Tokyo, Japan) overnight, washed and blocked with Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), and then incubated with 100 μl aliquots of diluted samples (1:10) or standards for 2 h at room temperature. After six washes, 100 μl biotin-conjugated OVA was added to each well at room temperature for 1 h. Each well was incubated with 100 μl streptavidin–horseradish peroxidase conjugate for 1 h at room temperature. After eight washes, substrate buffer containing *o*-phenylenediamine and H_2O_2 was added to each well; 30 min later, the reaction was terminated. Absorbance was measured at 490 and 595 nm with a microplate reader. Total IgE antibodies in plasma were measured by using mouse ELISA kits (Seikagaku Co., Tokyo, Japan).

Anti-OVA IgG1, IgG2a and IgG3 antibodies in plasma were measured by ELISA. Briefly, after each well of a microtiter plate had been coated with OVA

overnight, the plate was washed three times in $0.1\times$ Block Ace containing 0.05% Tween 20. Each well was incubated as follows, with six washes between each step: 100 μl diluted samples (1:100); with 100 μl diluted (1/1200) horseradish peroxidase-labeled anti-mouse IgG1, IgG2a, or IgG3 (Southern Biotechnology, Birmingham, AL); and with 100 μl of substrate buffer containing *o*-phenylenediamine-2HCl and H_2O_2 . Absorbance was read at 490 and 595 nm with a microplate reader.

2.7. Flow cytometric analysis

We analyzed the immunophenotypes of spleen cells and blood lymphocytes from each group of mice by flow cytometry. We purchased the following monoclonal antibodies from Pharmingen (San Diego, CA): phycoerythrin (PE)-labeled rat IgG anti-CD4 (clone GK1.5), fluorescein isothiocyanate (FITC)-labeled rat IgG anti-CD8a (clone 53-6.7), Cy-Chrome-labeled hamster IgG anti-CD3 (clone 145-2C11), FITC-labeled rat IgG anti-CD19 (clone RA3-6B2), and corresponding isotype-matched controls. Stained cells were resuspended in buffered salt solution containing 0.1% sodium azide. Cell samples were analyzed on a EPICS XL-MCL System II (Beckman Coulter, Inc., Pasadena, CA). For each sample, 10 000 events were collected, and data were analyzed using System II software.

2.8. Histological analysis

Lungs and tracheal rings were fixed with 4% paraformaldehyde solution and embedded in paraffin. Embedded blocks were sectioned at a thickness of 4 μm and stained with hematoxylin and eosin and analyzed under light microscopy. Mast cells were stained with 1% toluidine blue aqueous solution.

2.9. Statistical analysis

All data are presented as the mean $\pm$ standard error (S.E.), which is indicated by bars in the figures. Statistical analysis was performed with the StatMate II Statistical analysis system for Microsoft Excel Version 5.0 (Nankodo Inc., Tokyo, Japan). Differences between group means were analyzed using ANOVA with a post hoc test (Bonferroni/Dunn). $P < 0.05$ was considered to be significant.

Table 1
Effect of FA on body weight and organ weights in C3H mice^a

Group (ppb)	Ag	BW (g)	Thymus weight (mg)	Splenic weight (mg)
0	–	28.0 ± 1.2	27.9 ± 1.7	152.5 ± 11.4
	+	28.2 ± 1.0	28.9 ± 1.1	159.2 ± 15.5
80	–	27.4 ± 0.6	28.4 ± 1.3	128.3 ± 4.6
	+	28.2 ± 0.7	29.5 ± 1.6	137.6 ± 4.8
400	–	27.7 ± 0.8	25.9 ± 1.2	117.8 ± 3.4*
	+	27.5 ± 0.9	31.9 ± 1.4	168.6 ± 32.3
2000	–	27.6 ± 0.6	26.2 ± 1.1	121.0 ± 3.1*
	+	28.2 ± 0.8	27.0 ± 1.7	153.2 ± 6.7

^a Ag: antigen, *n* = 5–6.

* *P* < 0.05.

3. Results

3.1. Changes in organ weights and number of BAL cells in FA-exposed mice immunized with or without OVA

Exposure to various concentrations of FA for 12 weeks caused no significant changes in the body weight and thymus weight of female C3H/He mice (Table 1). Splenic wet weights at 400 and 2000 ppb FA were significantly reduced in nonimmunized mice compared with nonimmunized control mice. In OVA-immunized mice, FA exposure did not affect the body or organ weights.

To examine the effect of low-level FA exposure on airway inflammation, we determined the number of BAL cells immediately after exposure to FA for 12 weeks (Table 2). In nonimmunized mice, FA expo-

sure gave no significant increase in various types of inflammatory cells in BAL fluid. In OVA-immunized mice, the number of BAL cells, macrophages, and eosinophils from mice exposed to 2000 ppb FA was significantly increased relative to 0 ppb controls (*P* < 0.05). Exposure to FA induced no difference in the number of neutrophils and lymphocytes in BAL fluid of immunized mice.

3.2. Histological observation in the lungs of FA-exposed mice

In all groups of OVA-immunized and nonimmunized mice, we observed no marked histologic evidence of impairment of the epithelial cells in the lung tissues (data not shown). There was a mild infiltration of mast cells in tracheal epithelium in the groups of mice immunized with OVA, but the infiltration of mast cells was independent of FA exposure. These findings indicate that exposure of mice to 80, 400 and 2000 ppb FA did not induce any significant inflammatory features in the lungs.

3.3. Cytokine, chemokine and neuropeptides production in BAL fluid from FA-exposed mice

To identify any interaction of the increased numbers of inflammatory cells with cytokine and chemokine production in the BAL fluid, we measured the levels of proinflammatory cytokines and chemokines in BAL fluid. FA exposure decreased the production of IL-1β in immunized mice compared to 0 ppb control, immunized mice (Fig. 2, **P* < 0.05). The production of TNF-α, IL-6, and GM-CSF in BAL fluid from

Table 2
Effect of FA on the number of inflammatory cells in BAL cells^a

Group (ppb)	Ag	No. of BAL cells (×10 ⁴)	Macrophages	Neutrophils	Lymphocytes	Eosinophils
0	–	2.84 ± 0.16	2.74 ± 0.11	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.01
	+	6.73 ± 0.37	5.13 ± 0.32	0.22 ± 0.08	0.28 ± 0.05	1.07 ± 0.18
80	–	2.64 ± 0.24	2.58 ± 0.17	0.01 ± 0.01	0.03 ± 0.01	0.01 ± 0.00
	+	6.45 ± 0.28	4.85 ± 0.17	0.09 ± 0.03	0.34 ± 0.03	1.15 ± 0.14
400	–	2.90 ± 0.15	2.81 ± 0.10	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
	+	7.13 ± 0.20	5.93 ± 0.09	0.10 ± 0.03	0.27 ± 0.06	0.80 ± 0.11
2000	–	2.96 ± 0.16	2.87 ± 0.10	0.01 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
	+	9.65 ± 0.14*	7.22 ± 0.32*	0.07 ± 0.02	0.33 ± 0.05	2.00 ± 0.35*

^a Ag: antigen, *n* = 5–6.

* *P* < 0.05.

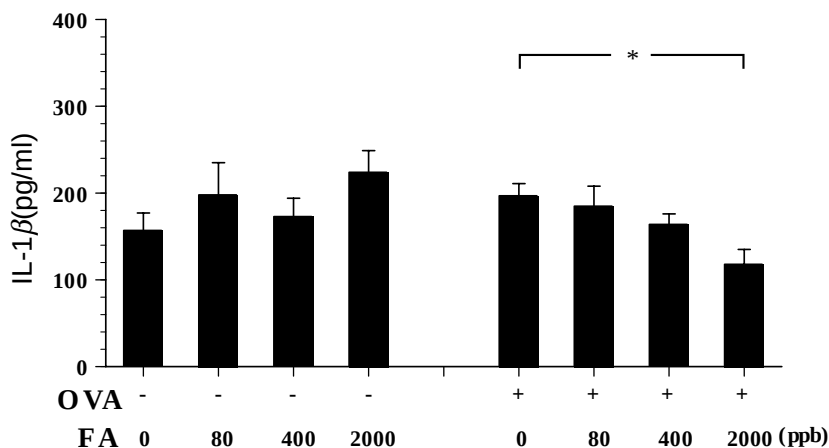


Fig. 2. IL-1 β in BAL fluid from FA-exposed mice. The day after the final FA inhalation, BAL fluid was collected from FA-exposed, nonimmunized and FA-exposed, OVA-immunized mice, and IL-1 β production was determined by ELISA. Data are presented as the mean $\pm$ S.E. ($n = 5-6$) (* $P < 0.05$).

FA-exposed mice remained at control levels (data not shown).

Chemokines, which induce the migration and attraction of inflammatory cells, may play a role in the induction of inflammation in mice exposed to FA. In this experimental condition, we detected no eotaxin, MIP-1 α and MCP-1 in BAL fluid from nonimmunized and immunized mice.

The production of neuropeptides from vagal endings may be induced as a result of irritant property of

FA. Immunization with OVA significantly increased the production of NGF in BAL fluid, but exposure to 80 and 400 ppb FA markedly decreased NGF levels in BAL fluid of immunized mice (Fig. 3). The production of NGF in BAL fluid of nonimmunized mice increased dose dependently, but not statistically different. The small amount of substance P in BAL fluid was observed in immunized mice regardless of FA exposure, but not in nonimmunized mice (data not shown). No BDNF production in BAL

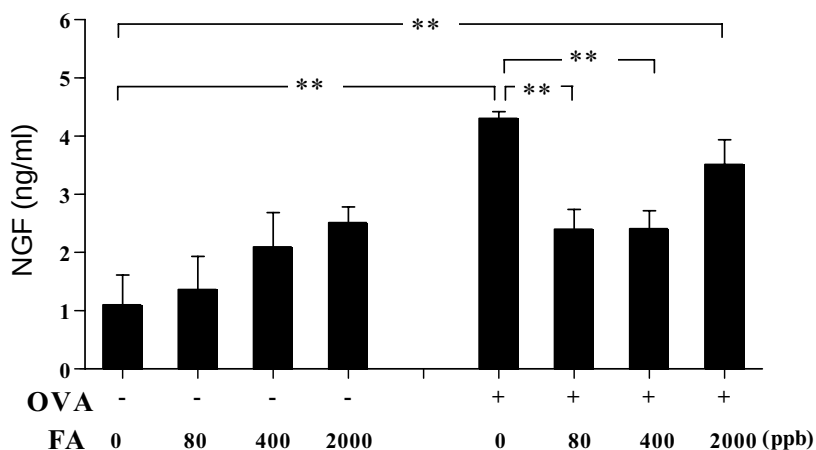


Fig. 3. NGF production in BAL fluid from FA-exposed mice. The day after the final FA inhalation, BAL fluid was collected from FA-exposed, nonimmunized and FA-exposed, OVA-immunized mice, and the production of NGF was determined by ELISA. Data are presented as the mean $\pm$ S.E. ($n = 5-6$) (** $P < 0.01$).

fluid of nonimmunized and immunized mice was detected.

3.4. Changes in the cellularity and the production of cytokine and chemokine in spleen cells from FA-exposed mice

Although the total number of spleen cells from 0, 80, 400, and 2000 ppb FA-exposed, nonimmunized mice was 2.50 ± 0.11 , 2.19 ± 0.12 , $2.01 \pm 0.07^*$, and 2.16 ± 0.10 , respectively (mean $\pm$ S.E. $\times 10^8$) (* $P < 0.05$ versus control), we observed no significant difference in the total number of spleen cells between FA-exposed and control mice immunized with OVA. The numbers of CD3-positive T cells and CD19-positive B cells, and CD4/CD8 T cell ratios in spleen cells from FA-exposed mice, both immunized and nonimmunized, were similar to those of the controls (data not shown). To investigate the effect of low-level FA exposure on systemic immunity, we investigated the cell proliferative response to mitogens or OVA in spleen cells from FA-exposed and control mice; however, we observed no significant difference between FA-exposed and control mice (data not shown).

To investigate the effect of low-level FA exposure on the secretory properties of spleen cells, we examined cytokine and chemokine production in spleen

cells stimulated with mitogens or antigen. The production of IFN- γ by LPS-stimulated spleen cells from FA-exposed, nonimmunized mice was significantly increased at 2000 ppb FA relative to the control (Fig. 4b), but that from PHA-stimulated spleen cells was not significantly changed (Fig. 4a). The production of IL-4 by PHA-stimulated spleen cells from mice exposed to 0, 80, 400, and 2000 ppb FA was 28.3 ± 3.7 , 27.8 ± 4.2 , 23.4 ± 2.2 , and 22.3 ± 3.8 , respectively (mean $\pm$ S.E., pg/ml). Levels of IL-4 in the culture supernatants from LPS-stimulated spleen cells of all FA-exposed mice were below the limit of detection (>5 pg).

Forty-eight hours after in vitro OVA stimulation, the production of IL-5 (Fig. 5a) and IFN- γ (Fig. 5b) by cultured spleen cells from FA-exposed, OVA-immunized mice was not significantly different from that in control mice. Levels of IL-4 in the culture supernatants from OVA-stimulated spleen cells of all FA-exposed mice were below the limit of detection. On the other hand, exposure of immunized mice to 400 and 2000 ppb FA resulted in a significant increase in the production of MCP-1 from spleen cells stimulated with OVA for 24 h (Fig. 6b), but no significant difference in the production of MIP-1 α (Fig. 6a). In nonimmunized mice, FA exposure gave no significant changes in both MCP-1 and MIP-1 α production from spleen cells.

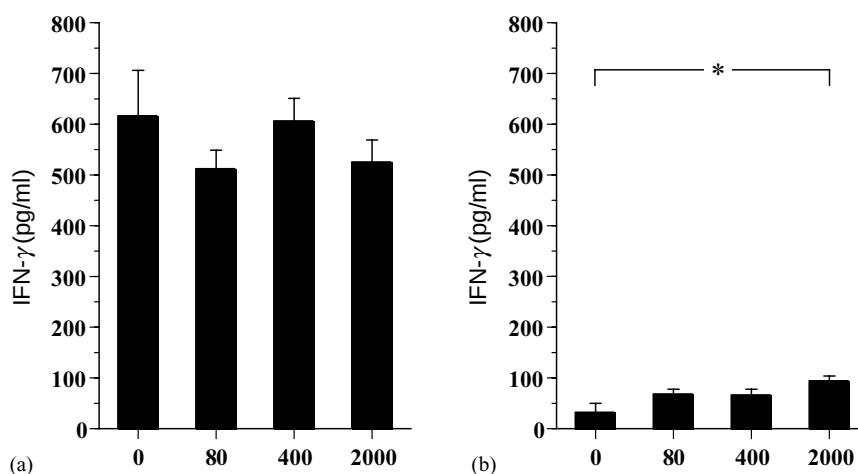


Fig. 4. IFN- γ production by spleen cells from FA-exposed, nonimmunized mice. The day after the final FA inhalation, spleen was collected from the mice. Spleen cells were cultured for 48 h in the presence of PHA (a) and LPS (b), then the IFN- γ levels in the supernatants were determined by ELISA. Data are presented as the mean $\pm$ S.E. ($n = 5$) (* $P < 0.05$).

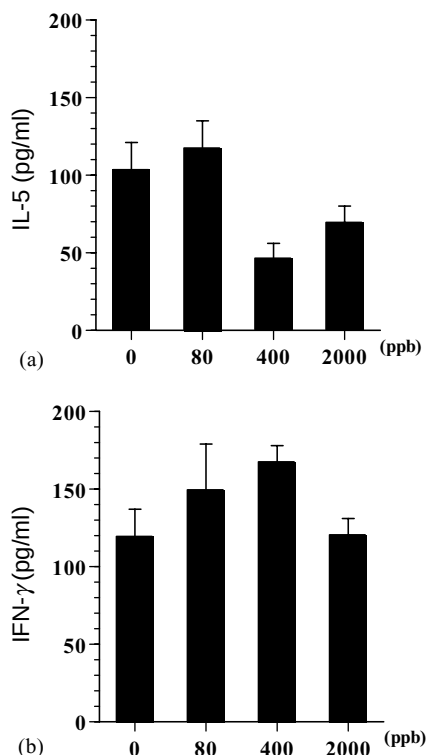


Fig. 5. IL-5 and IFN- γ production by spleen cells from FA-exposed, OVA-immunized mice. The day after the final FA inhalation, spleen was collected from the mice. Spleen cells were cultured for 48 h in the presence or absence of OVA, then the IL-5 (a) and IFN- γ (b) levels in the supernatants were determined by ELISA. Levels of both in the absence of OVA were below the limit of detection (not shown). Data are presented as the mean $\pm$ S.E. ($n = 6$).

3.5. Antigen-specific antibody titers in plasma from FA-exposed and control mice

To determine the systemic effect of low-level FA exposure, IgE antibody production in plasma was determined by using ELISA. FA exposure induced no significant difference in anti-OVA IgE production in plasma compared with controls (Fig. 7a). Total IgE production in plasma of FA-exposed mice showed no significant difference from that of control mice (data not shown). Anti-OVA IgG1 production was significantly reduced after exposure to 400 ppb FA (Fig. 7b, $*P < 0.05$). It failed to induce a significant difference in anti-OVA IgG2a production between FA-exposed and control mice (Fig. 7c). However, exposure to 80

and 400 ppb FA significantly reduced anti-OVA IgG3 production in plasma (Fig. 7d).

3.6. Neuropeptides production in plasma in FA-exposed mice

Since the production of NGF in BAL fluid of immunized mice was significantly suppressed by 80 and 400 ppb FA inhalation, the levels of NGF and substance P were measured in plasma. Although there are no changes in the production of NGF in plasma of FA-exposed, nonimmunized mice, immunization with OVA markedly increased the production of NGF (Fig. 8, $**P < 0.01$). Exposure to 80 and 400 ppb FA significantly decreased the production of NGF in plasma of OVA-immunized mice compared to that of 0 ppb control, immunized mice. Similar pattern of FA-induced suppression was shown in NGF levels between BAL fluid and plasma in immunized mice. The amount of substance P in plasma in nonimmunized mice was increased in a dose-dependent manner (Fig. 9). However, no significant increase in the levels of substance P in plasma in OVA-immunized mice was seen.

4. Discussion

In this study, we investigated the long-term effects of low-level FA exposure on the magnitude of immunogenic and neurogenic inflammatory response in allergic model mice. The results indicated that exposure of immunized mice to 2000 ppb FA increased the number of inflammatory cells in BAL fluid and enhanced the production of MCP-1 in culture supernatants from spleen cells. In OVA-immunized mice, however, the production of IL-1 β in BAL fluid was significantly decreased by 2000 ppb FA. In nonimmunized mice, the production of IFN- γ in culture supernatants from LPS-stimulated spleen cells was affected by 2000 ppb FA. Our results are in agreement with recent studies showing that FA exposure does not induce serious respiratory allergic inflammation (Gannon et al., 1995; Smedley, 1996) or acts through non-IgE-mediated immune mechanisms (Arts et al., 1997).

Compared with the effect of short-term FA exposure, the most striking findings is that the enhanced

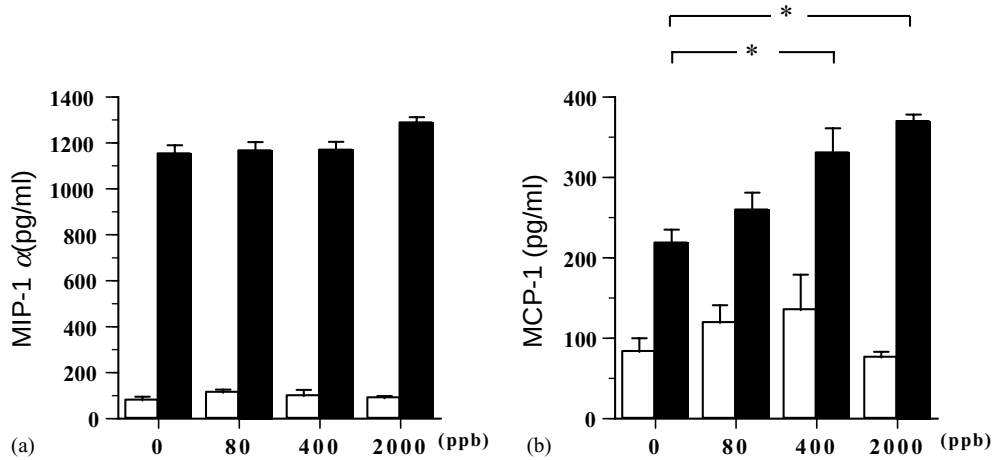


Fig. 6. MIP-1 α and MCP-1 production by spleen cells from FA-exposed, OVA-immunized mice. The day after the final FA inhalation, spleen was collected from the mice. Spleen cells were cultured for 24 h in the presence (solid column) or absence (open column) of OVA, then the MIP-1 α (a) and MCP-1 (b) levels in the supernatants were determined by ELISA. Data are presented as the mean $\pm$ S.E. ($n = 6$) (* $P < 0.05$).

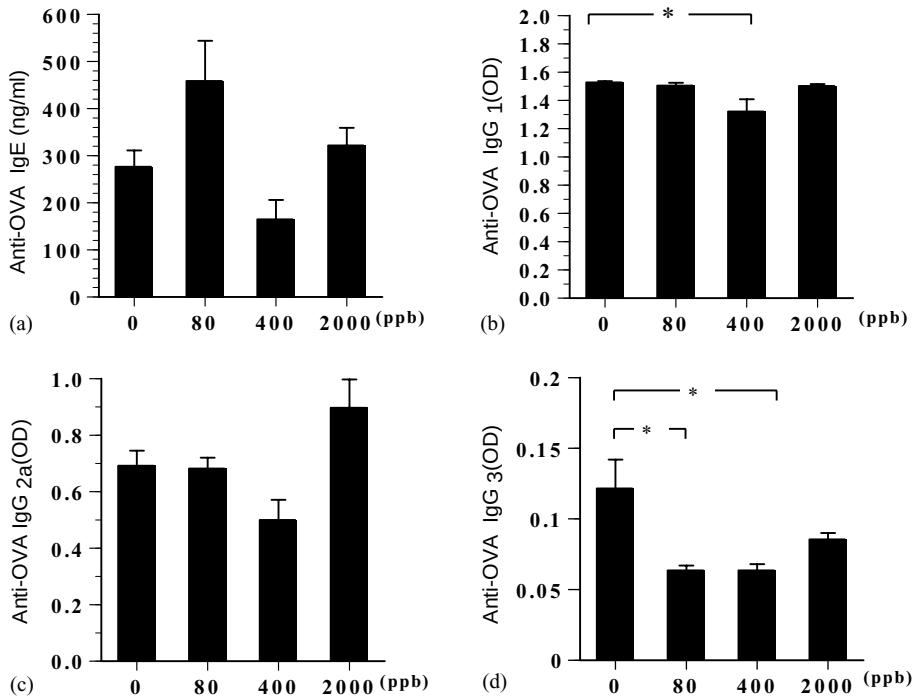


Fig. 7. Effects of low levels of FA inhalation on OVA-specific antibody production in plasma of mice. The day after the final FA inhalation, plasma samples were collected from the mice in each treatment group, and the levels of anti-OVA IgE (a), anti-OVA IgG1 (b), anti-OVA IgG2a (c), and anti-OVA IgG3 (d), in diluted plasma were measured by ELISA. Data are presented as the mean $\pm$ S.E. ($n = 6$) (* $P < 0.05$).

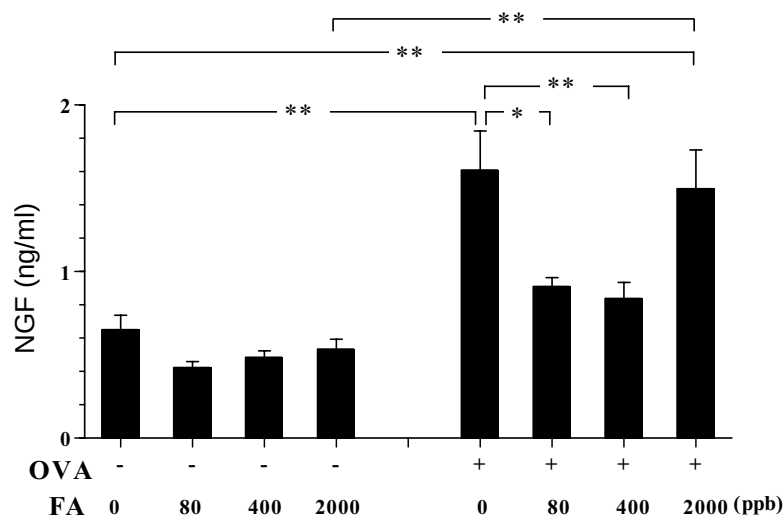


Fig. 8. NGF production in plasma of FA-exposed mice. The day after the final FA inhalation, plasma samples were collected from FA-exposed, nonimmunized and FA-exposed, OVA-immunized mice, and the production of NGF was determined by ELISA. Data are presented as the mean $\pm$ S.E. ($n = 5-6$) (* $P < 0.05$, ** $P < 0.01$).

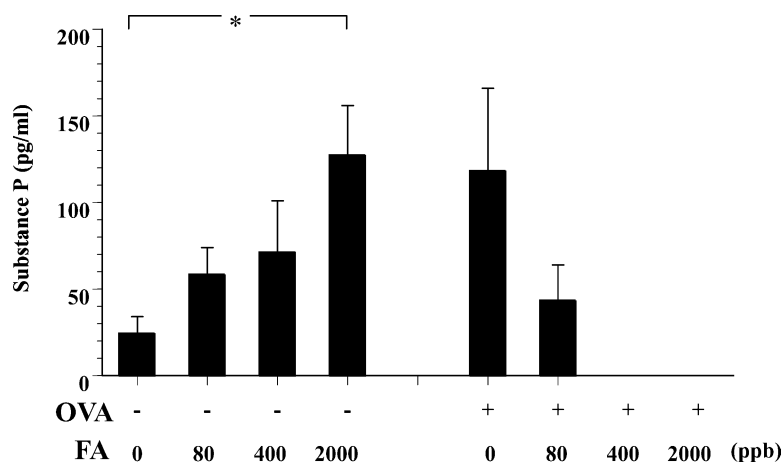


Fig. 9. Substance P levels in plasma of FA-exposed mice. The day after the final FA inhalation, plasma samples were collected from FA-exposed, nonimmunized and FA-exposed, OVA-immunized mice, and the levels of substance P were determined by ELISA. Data are presented as the mean $\pm$ S.E. ($n = 5-6$) (* $P < 0.05$).

production of NGF in BAL fluid and plasma from control immunized mice was markedly suppressed by 80 and 400 ppb FA exposure. Proinflammatory cytokines regulate the production of NGF in immune systems. IL-1 mediates and regulates NGF production in lymphoid tissues and peripheral nervous system (Kannan

et al., 1996; Herx et al., 2000). TNF- α influences the synthesis and release of NGF (Aloe et al., 1993; Fiore et al., 1996). Our results showed that exposure to 80 and 400 ppb FA did not affect the levels of IL-1 β and TNF- α in BAL fluid. These things suggest that the decrease of NGF production in OVA-immunized mice

by FA exposure may be independent of the levels of proinflammatory cytokines. It is possible to speculate that the production of proinflammatory cytokines in BAL fluid may be modulated by early FA exposure. NGF induces B cells to Ig producing cells (Otten et al., 1989; Kimata et al., 1991). NGF as well as CD40 have been shown to modulate B cell proliferation and immunoglobulin secretion (Brodie and Gelfand, 1994). Our results indicate that exposure to 400 ppb FA decreased the production of antigen-specific IgG1 and IgG3 in plasma correlated with the decreased NGF production. It is likely that exposure to 80 and 400 ppb FA may act to inhibit the immunologically mediated augmentation of NGF and IgG3 production.

FA is an irritant and stimulates C-fiber endings (Nielsen, 1991). FA can stimulate the release of substance P, which may induce a neurogenic inflammatory response, in the eyes and nose (Nielsen, 1991). An increase in vascular permeability induced by FA exposure in the rat airway was mediated predominantly by stimulation of the tachykinin NK1 receptor (Ito et al., 1996). Our study indicated that exposure to 2000 ppb FA significantly increased the level of substance P in plasma. These findings suggest that the stimulation of pulmonary C-fiber endings by long-term FA inhalation may be related to the modulation of neurogenic inflammation in peripheral or central nervous system, resulting in dysregulation of neuro-immune interactions. Further studies on the effect of FA exposure on neurogenic inflammatory response in brain or central nervous system will be needed.

In addition to augmentation of inflammatory properties of NGF, NGF has anti-inflammatory properties. For example, NGF levels in brain of systemic lupus erythematosus mice decreased during the development of the disease, but increased in the levels of circulating NGF (Bracci-Laudiero et al., 1996). NGF regulates calcitonin gene-related peptide expression in activated B cells, and interferes with T cell mediated autoimmune disease in the central nervous system (Flugel et al., 2001; Bracci-Laudiero et al., 2002). Further studies on the effect of low-level FA exposure on the role of anti-inflammatory properties in NGF will be needed.

Chronic exposure to low levels of FA is suspected to impair neuro-immune interactions, which may be in-

involved in the initiation or triggering of multiple chemical sensitivity, chemical intolerance, and sick building syndrome (Meggs, 1995). The WHO has established 80 ppb as a guideline for the FA (WHO, 1999). Epidemiologically, as an indoor pollutant, 100–800 ppb FA in homes and offices was estimated (Samet et al., 1987; Matsumura, 1991; Institute of Medicine, 2000). If the decreased NGF following exposure to low-level FA is related to unfavorable modulation of sensory neurons (which can yield a variety of peripheral and central symptoms) and to immune abnormalities, these association may provide a new explanation for the multi-organ symptoms of patients with chemical sensitivities. Understanding the role of NGF in FA exposure may provide an insight into the modulation of neuro-immune interaction and provide opportunities for clarifying unexplained symptoms in chemically sensitive subjects related to nervous system. Although the mechanism by which exposure to low levels of FA markedly decreased the production of NGF in BAL fluid and in plasma of OVA-immunized mice remains unclear, NGF may be established as a sensitive marker for evaluating a novel finding caused by low-level FA exposure.

In conclusion, low-level FA inhalation can induce differential immunogenic and neurogenic responses in allergic mice.

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References

- Aloe, L., Probert, L., Kollias, G., Bracci-Laudiero, L., Spillantini, M.G., Levi-Montalcini, R., 1993. The synovium of transgenic arthritic mice expressing human tumor necrosis factor contains a high level of nerve growth factor. *Growth Factors* 9, 149–155.
- 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.
- Arts, J.H.E., Dröge, S.C.M., Spanhaak, S., Bloksma, N., Penninks, A.H., Kuper, C.F., 1997. Local lymph node activation and IgE responses in Brown Norway and Wistar rats after

- dermal application of sensitizing and non-sensitizing chemicals. *Toxicology* 117, 229–237.
- Bracci-Laudiero, L., Lundeberg, T., Stenfors, C., Theodorsson, E., Tirassa, P., Aloe, L., 1996. Modification of lymphoid and brain nerve growth factor levels in systemic lupus erythematosus mice. *Neurosci. Lett.* 204, 13–16.
- Bracci-Laudiero, L., Aloe, L., Buanne, P., Finn, A., Stenfors, C., Vigneti, E., Theodorsson, E., Lundeberg, T., 2002. NGF modulates CGRP synthesis in human B-lymphocytes: a possible anti-inflammatory action of NGF? *J. Neuroimmunol.* 123, 58–65.
- Braun, A., Lommatzsch, M., Renz, H., 2000. The role of neurotrophins in allergic bronchial asthma. *Clin. Exp. Allergy* 30, 178–186.
- Brodie, C., Gelfand, E.W., 1994. Regulation of immunoglobulin production by nerve growth factor: comparison with anti-CD40. *J. Neuroimmunol.* 52, 87–96.
- Burge, P.S., Harries, M.G., Lam, W.K., O'Brien, I.M., Patchett, P.A., 1985. Occupational asthma due to formaldehyde. *Thorax* 40, 225–260.
- Dearman, R.J., Basketter, D.A., Evans, P., Kimber, I., 1999. Comparison of cytokine secretion profiles provoked in mice by glutaraldehyde and formaldehyde. *Clin. Exp. Allergy* 29, 124–132.
- Donnerer, J., Schuligoi, R., Stein, C., 1992. Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. *Neuroscience* 49, 693–698.
- Fiore, M., Probert, L., Kollias, G., Akassoglou, K., Alleva, E., Aloe, L., 1996. Neurobehavioral alterations in developing transgenic mice expressing TNF- α in the brain. *Brain Behav. Immunity* 10, 126–138.
- Flugel, A., Matsumuro, K., Neumann, H., Klinkert, W.E., Birnbacher, R., Lassmann, H., Otten, U., Wekerle, H., 2001. Anti-inflammatory activity of nerve growth factor in experimental autoimmune encephalomyelitis: inhibition of monocyte transendothelial migration. *Eur. J. Immunol.* 31, 11–22.
- Gannon, P.F., Bright, P., Campbell, M., O'Hickey, S.P., Burge, P.S., 1995. Occupational asthma due to glutaraldehyde and formaldehyde in endoscopy and X-ray departments. *Thorax* 50, 156–159.
- Grammer, L.C., Harris, K.E., Shaughnessy, M.A., Sparks, P., Ayars, G.H., Altman, L.C., Patterson, R., 1990. Clinical and immunologic evaluation of 37 workers exposed to gaseous formaldehyde. *J. Allergy Clin. Immunol.* 86, 177–181.
- Hendrick, D.J., Rando, R.J., Lane, D.J., Morris, M.J., 1982. Formaldehyde asthma: challenge exposure levels and fate after five years. *J. Occup. Med.* 24, 893–897.
- Herx, L.M., Rivest, S., Wee Yong, V., 2000. Central nervous system-initiated inflammation and neurotrophism in trauma: IL-1 β is required for the production of ciliary neurotrophic factor. *J. Immunol.* 165, 2232–2239.
- Hori, H., Arashidani, K., 1997. Basic characteristics of a formaldehyde gas generator using solid paraformaldehyde. *J. UOEH* 19, 123–131.
- Imai, T., Shimizu, F., Fujimaki, H., Watanabe, N., 1983. Enhancement of IgE antibody production by ovalbumin aerosol in mice. *Int. Arch. Allergy Immunol.* 70, 368–372.
- Institute of Medicine, 2000. Committee on the assessment of asthma and indoor air. Indoor chemical exposures. In: *Clearing the Air: Asthma and Indoor Air Exposures*. National Academy Press, Washington, pp. 223–262.
- Ito, K., Sakamoto, T., Hayashi, Y., Morishita, M., Shibata, E., Sakai, K., Takeuchi, Y., Torii, S., 1996. Role of tachykinin and bradykinin receptors and mast cells in gaseous formaldehyde-induced airway microvascular leakage in rats. *Eur. J. Pharmacol.* 307, 291–298.
- Kannan, Y., Bienenstock, J., Ohta, M., Stanisiz, A.M., Stead, R.H., 1996. Nerve growth factor and dytocrine mediated lymphoid tissue-induced neurite outgrowth from mouse superior cervical ganglia in vitro. *J. Immunol.* 156, 313–320.
- Kimata, H., Yoshida, A., Ishioka, C., Mikawa, H., 1991. Stimulation of Ig production and growth of human lymphoblastoid B-cell lines by nerve growth factor. *Immunology* 72, 451–452.
- Matsumura, T., 1991. Present indoor air pollution. *J. Jpn. Soc. Atmos. Environ.* 26, A99–A109.
- Meggs, W.J., 1995. Neurogenic switching: a hypothesis for a mechanism for shifting the site of inflammation in allergy and chemical sensitivity. *Environ. Health Perspect.* 103, 54–56.
- Nielsen, G.D., 1991. Mechanisms of activation of the sensory irritant receptor by airborne chemicals. *Crit. Rev. Toxicol.* 21, 183–208.
- Otten, U., Ehrhard, P., Peck, R., 1989. Nerve growth factor induces growth and differentiation of human B lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 86, 10059–10063.
- Riedel, F., Hasenauer, E., Barth, P.J., Kozirowski, A., Rieger, C.H.L., 1996. Formaldehyde exposure enhances inhalative allergic sensitization in the guinea pig. *Allergy* 51, 94–99.
- Rowat, S.C., 1998. Integrated defense system overlaps as a disease model: with examples for multiple chemical sensitivity. *Environ. Health Perspect.* 106 (Suppl. 1), 85–109.
- Samet, J.M., Marbury, M.C., Spengler, J.D., 1987. Health effects and sources of indoor air pollution. Part 1. *Am. Rev. Respir. Dis.* 136, 1486–1508.
- Samet, J.M., 1990. Environmental controls and lung disease. *Am. Rev. Respir. Dis.* 142, 915–939.
- Smedley, J., 1996. Is formaldehyde an important cause of allergic respiratory disease? *Clin. Exp. Allergy* 26, 247–249.
- Sorg, B.A., Tschirgi, M.L., Swindell, S., Chen, L., Fang, J., 2001. Repeated formaldehyde effects in an animal model for multiple chemical sensitivity. *Ann. NY Acad. Sci.* 933, 57–67.
- Swiecichowski, A.L., Long, K.J., Miller, M.L., Leikauf, G.D., 1993. Formaldehyde-induced airway hyperreactivity in vivo and ex vivo in guinea pigs. *Environ. Res.* 61, 185–199.
- Tarkowski, M., Gorski, P., 1995. Increased IgE antiovalbumin level in mice exposed to formaldehyde. *Int. Arch. Allergy Immunol.* 106, 422–424.
- Thrasher, J.D., Broughton, A., Madison, R., 1990. Immune activation and autoantibodies in humans with long-term inhalation exposure to formaldehyde. *Arch. Environ. Health* 45, 217–223.

- Vedder, H., Affolter, H.U., Otten, U., 1993. Nerve growth factor (NGF) regulates tachykinin gene expression and biosynthesis in rat sensory neurons during early postnatal development. *Neuropeptides* 24, 351–357.
- World Health Organization (WHO), 1999. Guidelines for Air Quality. WHO, Geneva, pp. 42–51.
- Wood, R., Coleman, J.B., 1995. Behavioral evaluation of the irritant properties of formaldehyde. *Toxicol. Appl. Pharmacol.* 130, 67–72.
- Ziem, G., McTamney, J., 1997. Profile of patients with chemical injury and sensitivity. *Environ. Health Perspect.* 105 (Suppl.), 417–431.