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Long-term exposure to gaseous formaldehyde promotes allergen-specific IgE-mediated immune responses in a murine model

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It has long been questioned that whether exposure to formaldehyde in indoor environments may be a risk factor for developing allergen-specific IgE-mediated inflammatory responses, because there is limited clinical or experimental evidence that formaldehyde is involved in the cascade for IgE production. There is no known lower limit, below which there is no threat of serious allergic symptoms. The present study illustrates that the threshold limit of formaldehyde, 0.08 ppm (as defined by the World Health Organization), did not cause ovalbumin-specific IgE inflammatory immune responses, but higher than threshold concentrations of formaldehyde gas result in both enhanced allergen-specific IgE responses and NK (Natural Killer)-cell activity in peripheral blood cells in a murine model. Thus, formaldehyde gas may be involved in promoting allergic inflammatory

effects in subjects primed with specific allergens by NK-cell activation. These results indicate that even threshold concentrations of formaldehyde gas may play a regulatory role for 'systemic' cell-mediated immune responses. The extensive use of adhesives for building materials has resulted in higher levels of indoor air pollutants. It is conceivable that increased time indoors may enhance pre-existing allergic symptoms by concomitant exposure to volatile organic compounds and formaldehyde. The affordable limit for formaldehyde might be much lower than currently established levels in indoor environments.

Key words: formaldehyde; IgE response; indoor environment; ovalbumin

Introduction

The most common route of exposure to formaldehyde is through breathing air containing formaldehyde gas. This usually occurs in indoor environments where the gas has been emitted from formaldehyde-containing products. The most common sources of formaldehyde include pressed wood products using wood adhesives containing urea-formaldehyde resins.¹ Formaldehyde is also found in plywood, particle board, wall paneling, shelves, counter tops, hardwood plywood paneling, and medium-density fiberboard.^{2,3} The World Health Organization (WHO) defines a threshold value stating that exposure to formaldehyde should not exceed 0.08 ppm in air, based upon numerous

unpleasant clinical manifestations, including mainly sensory effects.⁴ In Japan, especially during hot and humid summers, indoor gas levels of formaldehyde in newly built dwellings can be >100 µg/m³ (0.08 ppm) over the guidelines.⁵

Breathing air containing low levels of formaldehyde can result in irritation and watering of the eyes, runny nose, and constant sneezing.^{6,7} As levels increase, burning of the nose and throat, coughing, and difficulty in breathing may occur. It is conceivable that these manifestations are the results of allergic reactions because the development of respiratory hypersensitivity and elicitation of asthmatic symptoms has been associated with exposure to chemical compounds such as formaldehyde.^{8,9} However, there is no direct evidence indicating that formaldehyde above the guideline values can directly induce allergic reactions, elicit formaldehyde-specific IgE production or worsen clinical allergic manifestations, i.e. there is no known level where there is

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threat of serious allergic symptoms. Large bodies of evidence are available addressing mechanisms of allergic responses at local sites exposed to gaseous formaldehyde. These studies discuss that the possible contribution of inflammatory cells in respiratory tract tissues have local allergic responses.^{10–12} The present study illustrates that systemic allergic responses, through NK-cell activation, rather than topical allergic responses, play a significant role in an experimental model exposed to gaseous formaldehyde. In addition, this study demonstrates for the first time the ability of formaldehyde gas to enhance allergen-specific IgE-mediated immune responses in a murine model.

Materials and methods

Experimental animals

Four-week-old female BALB/c and C₃H/He mice were purchased from Japan SLC Inc. (Hamamatsu, Japan) and housed in separate cages with outside dimensions of 300 (H) × 400 (W) × 300 (D) mm. According to the treatment protocol, all the mice were maintained on ovalbumin (OVA)-free diets. Chamber temperature was 20–22 °C and mean air humidity was 20–30%. The mice used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of Suzuka University School of Medical Science.

Experimental protocols

Groups of mice (6–10 mice per group) were sensitized by repeated i.p. injections of 10 µg of chicken OVA (grade V; Sigma Chemical, St. Louis, Missouri, USA) emulsified in 2 mg Al(OH)₃ (Inject Alum; Pierce, Rockford, Illinois, USA) in a total volume of 100 µl, on days 0 and 7. The IgE Ab response was evaluated as described previously.¹³ Starting on week 1, OVA-sensitized mice were randomized into three groups: the first animal group was exposed to about 0.8 ± 0.1 ppm (high concentration; OVA-primed animal group, followed by exposure to 0.8 ± 0.01 ppm), second group was exposed to about 0.1 ± 0.03 ppm (low concentration; OVA-primed animal group, followed by exposure to 0.1 ± 0.03 ppm), and OVA-control group was exposed to about 0 ppm formaldehyde (OVA-primed animal group, followed by exposure to null formaldehyde), for 24 h per day, Monday through Friday, over a period of 5 weeks. The fourth non-sensitized animal group was maintained following the same protocol without exposure to formalde-

hyde (formaldehyde null control; OVA-unprimed animal group without formaldehyde exposure).

Systemic responsiveness after airway challenge with gas generating and analytical instruments

Systemic responsiveness Systemic responsiveness was assessed as a change in IgE or IgG levels after challenge with intraperitoneally injected OVA.

Gas generating and analytical instruments Formaldehyde vapour generated by means of a diffusion tube was controlled using a permeator (GASTEC, Ayase, Japan), and was pumped through a gas meter (2 l/min) into the acrylic chamber. Standard methods for monitoring formaldehyde emission levels in the chamber include using solid-phase cartridges impregnated with 2,4-dinitrophenylhydrazine (Shibata, Tokyo, Japan) to convert carbonyls to their 2,4-dinitrophenylhydrazone derivatives.¹⁴ After being sampled on the cartridges, twice a day, the sample derivatives absorbed in the cartridges were extracted and then analyzed by high-performance liquid chromatography with UV-vis detector (Hewlett Packard, Avondale, Pennsylvania, USA).

Measurement of total and OVA-specific antibody Blood samples were collected from the retro-orbital plexus by use of heparin-containing capillary tube. Plasma samples were separated by centrifugation. Samples were diluted (1:10 v/v) with PBS containing 1 U/ml heparin, and added to 96-well plates (Nalge Nunc International, Rochester, New York, USA) in triplicate. Plasma levels of total IgE or IgG and OVA-specific IgE or IgG were measured by a modification of a previously described ELISA.¹ For measurements of total IgE and IgG, the plates were coated with monoclonal rat anti-mouse IgE or anti-mouse IgG (PharMingen, San Diego, California, USA) at a concentration of 5 µg/ml in coating buffer. After addition of plasma samples, biotinylated rat anti-mouse IgE or IgG (2 µg/ml; PharMingen) was added, and amplified with streptavidin-HRP (1:2000 dilution; Zymed, San Francisco, California, USA). For OVA-specific IgE or IgG measurements, the plates were coated with rat anti-mouse IgE Ab at a concentration of 20 µg/ml or OVA in 0.1 M carbonate buffer, pH 9.6. Following an overnight incubation, the plates were washed with PBS and blocked with 10% Block Ace buffer (Dainippon Sumitomo Pharma, Tokyo, Japan). After addition of plasma samples, biotinylated anti-mouse IgE (1:1000 dilution; PharMingen) was used for detecting anti-OVA IgE, and

the reaction was amplified with streptavidin-HRP. IgG was detected using alkaline phosphatase-conjugated anti-mouse IgG (PharMingen). Total IgE or IgG levels were measured using known mouse Ab IgE standards (PharMingen). The OVA-specific IgE titers of plasma samples were calculated by comparison with known standards that were generated in the laboratory.

Cell preparation and FACS analysis

The splenic mononuclear cells were harvested from mice by passing the tissue through stainless steel mesh before FACS (Fluorescence activated cell sorter) analysis. The cells were incubated with FITC-conjugated anti-CD3, anti-CD8, anti-CD11b and PE-conjugated anti-CD4 (all from Becton-Dickinson, Bedford, UK), and PE-conjugated anti-CD19 (B220), anti-TCR $\gamma\delta$ and anti-NK1.1 (all from PharMingen) used at a concentration of 1 $\mu\text{g}/10^6$ cells. After staining, the cells were analyzed using a FACSsan (Becton Dickinson, San Jose, California, USA).

Cytotoxicity assay

In BALB/c mice, only 1% or less of splenic lymphoid cell, are NK1.1 positive. In contrast, in C₃H/He mice, >10% NK1.1⁺ cells can be isolated from splenic compartments thus providing sufficient cell numbers to more accurately measure differences in NK-cell activity between formaldehyde-exposed and non-exposed mice than those in BALB/c mice. Therefore, splenic cell suspensions isolated from both BALB/c, and C₃H/He mice were evaluated for NK-cell activity. Because, NK-cell activity was measured using a 4-h ⁵¹chromium-release assay as described previously.¹⁵ Splenic effector cells were plated onto microtiter plates and assayed against a fixed number of YAC-1 target cells (10⁴/well) to achieve effector to target ratios (E/T ratio) of 200:1, 100:1, 50:1, 25:1, and 12.5:1. The plates were incubated in 5%CO₂/95% air for 4 h at 37°C, after which 100 μl of the supernatants were harvested from each well and counted on a gamma counter. Specific cytotoxicity was calculated according to the standard formula: % specific lysis = 100 $\times$ (mean experimental release – mean spontaneous release)/(mean maximum release – mean spontaneous release).

The spontaneous release for each target used in these assays was <10%. Lytic units were calculated from the curve of the percentage lysis. Lytic units per 10⁷ effector cells were calculated, where one lytic unit was defined as the number of effector cells required to lyse 20% of a population of 10⁴ tar-

get cells in a 4-h assay.¹⁶ Lytic unit values are expressed as mean $\pm$ SE.

Statistics

Mean and SE of the data were determined and are shown in the text and figures. Analyses of phenotypic differences and analyses of differences in lytic units between groups were performed using Student's *t*-test for paired comparisons. Differences in levels of antibody production over time were analyzed with Dunnett's test for significance where appropriate. A *P*-value <0.05 was considered statistically significant.

Results

IgE antibody production

To begin, we evaluated total IgG and IgE levels in female BALB/c mice housed in strictly controlled gaseous formaldehyde over a period of 5 weeks. Figure 1 shows levels of total IgE in mice exposed to high, low, or null (control) concentrations of formaldehyde over time. No significant change in total IgE levels was seen in mice exposed to a high concentration (0.7971 $\pm$ 0.0890 ppm), low concentration (0.1364 $\pm$ 0.0284 ppm), or null concentration (0.0025 $\pm$ 0.0018 ppm) of formaldehyde gas (Figure 1). Similar results were seen with total IgG levels (data not shown). These findings suggest that the exposure of gaseous formaldehyde itself resulted

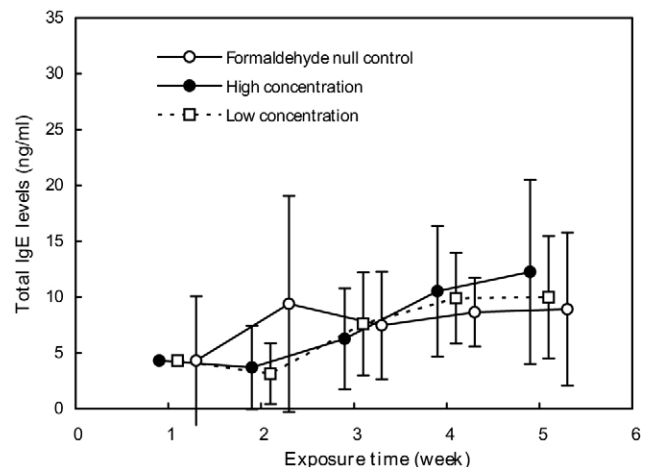


Figure 1 Effect of gaseous formaldehyde on the production of total IgE antibody. Blood was collected once weekly via retro-orbital plexus. Total plasma IgE levels in the samples from BALB/c mice exposed to approximately 0.0, 0.1 or 0.8 ppm of formaldehyde gas were measured by an ELISA. The longitudinal bar represents the mean $\pm$ SE of five mice.

in neither IgE nor IgG production in mice during an exposure period of 5 weeks.

IgE antibody production in allergen-primed hosts

In order to determine whether formaldehyde gas can be involved in specific antigen-mediated IgE production, mice were immunized with OVA plus aluminum hydroxide on days 1 and 7. Figure 2 illustrates the time-course of OVA-specific IgE levels in mice receiving OVA allergens, following an exposure to formaldehyde gas. Five weeks after the last immunization, plasma levels of OVA-specific IgE were significantly increased in mice exposed to high concentrations of formaldehyde (OVA-primed, and then exposed to ~0.8 ppm) compared with those exposed to low concentrations (OVA-primed, and then exposed to ~0.1 ppm), OVA-control (OVA-primed but no exposure to formaldehyde), or formaldehyde null control (OVA-unprimed, and exposed to ~0.0 ppm) ($P < 0.01$; Figure 2). In contrast, mice exposed to any concentration of formaldehyde did not demonstrate any enhanced OVA-specific IgG production over the observation period of 5 weeks (data not shown). These results indicate that levels of OVA-specific IgE antibody in mice exposed to formaldehyde levels of >0.8 ppm were significantly increased when compared with those in control mice ($P < 0.05$). In contrast, levels of OVA-specific IgE production in mice exposed to 0.1 ppm or less were comparable to those in control circumstances

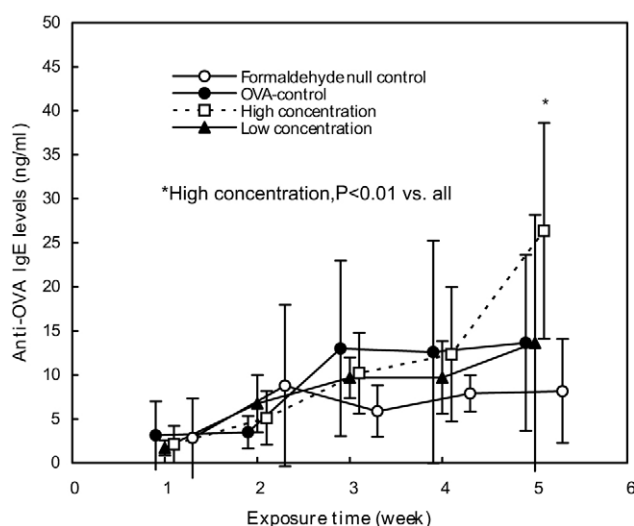


Figure 2 Effect of gaseous formaldehyde on the production of anti-OVA-specific IgE antibody. Following immunization with OVA-aluminum hydroxide twice during a week, total OVA-specific IgE levels were measured weekly from BALB/c mice exposed to formaldehyde gas. Bar represents the mean $\pm$ SE of five mice.

(low, $P > 0.05$ vs. control). This concentration of formaldehyde, which is close to the threshold value of 0.08 ppm recommended by WHO, did not facilitate accelerated responses in specific antigen-mediated IgE production.

NK-cell activation in BALB/c and C3H/He splenic compartments

Associated with the development of allergic asthma, mice with normal NK1.1⁺CD3⁻ cells develop pulmonary eosinophilic inflammation and produce allergen-specific IgE as well as cytokines.¹⁷ In order to further ascertain whether gaseous formaldehyde itself would contribute to any non-beneficial cellular immune responses, we assessed levels of CD3, CD4, CD8, CD19, CD11b, TCR- $\gamma\delta$ and NK1.1-expressing leukocytes in splenic cells in allergen-unprimed BALB/c mice exposed to low (threshold; 0.08 ± 0.01 ppm) concentrations of formaldehyde when compared with those in control mice. In order to focus on whether formaldehyde is a critical regulator of NK 1.1 expression and NK activity, we performed FACS analysis and ⁵¹Cr-release cytotoxicity assays. A greater expression of NK cells, defined by NK1.1, was seen in BALB/c mice exposed to threshold concentrations (Table 1; $P = 0.0195$ vs. control), or greater concentrations than threshold concentrations (data not shown) of formaldehyde when compared to control mice. T-cell subsets and B cells, defined by CD3, CD4, CD8 and CD19 monoclonal antibodies, did not show significant changes in expression. Similar results were obtained in experiments using C₃H/He mice (data not shown). As shown in Figure 3, enhanced NK-cell activity in splenic cells from either BALB/c or C3H/He mice exposed to high, or low concentrations of formaldehyde was seen when compared with that in cells after exposed to null concentrations (control) (Control, $P < 0.05$ vs. low and high). These observations suggest that even threshold concentrations of formaldehyde gas may play a regulatory role for cell-mediated immune responses, i.e. enhanced NK-cell activity.

Discussion

Formaldehyde is frequently used in wooden housing materials as resin or adhesive. In Japan, especially during summer months, warm temperatures and high humidity in indoor environments result in gas levels, ranging from 0.08 to 0.20 ppm higher than during any other season.⁵ The most common symptoms after frequent or prolonged exposure to formaldehyde include wheezing, watering eyes, runny

Table 1 Phenotypic expression of splenic cells in BALB/c mice exposed to low threshold concentrations of formaldehyde gas

Subset	n	Low concentration (0.08 ± 0.01 ppm)	Control (0.0 ppm)	P-value ^b
CD19 ⁺	10	39.3 ± 1.3 ^a	39.3 ± 1.7	0.993
CD3 ⁺	10	54.3 ± 1.2	51.5 ± 1.2	0.106
CD8 ⁺	10	30.8 ± 0.4	30.0 ± 0.4	0.196
CD4 ⁺	10	61.0 ± 0.5	61.3 ± 0.4	0.583
CD4 ⁺ CD8 ⁺	10	0.39 ± 0.03	0.35 ± 0.04	0.421
CD4 ⁺ CD8 ⁻	10	7.81 ± 0.40	8.29 ± 0.36	0.389
CD3 ⁺ TCR-γδ ⁺	10	2.02 ± 0.14	2.24 ± 0.07	0.185
CD11b ⁺	10	6.87 ± 0.37	7.21 ± 0.27	0.457
NK1.1 ⁺	10	0.32 ± 0.01	0.27 ± 0.02	0.020

^aMean (%) ± SE; ^bTested by Student's *t*-test for comparisons between control and exposure groups.

nose, and constant sneezing and may occur as a result of not only topical but also systemic allergic reactions. In spite of this, there has been limited experimental or clinical evidence that formaldehyde gas, at concentrations above or below the threshold level of 0.08 ppm, has an effect on allergen-specific IgE-mediated antibody responses.^{18,19} The present study evaluated the association of formaldehyde gas levels with total or OVA allergen-specific IgE or IgG responses in an animal model using strictly controlled formaldehyde gas exposures. The experiments were performed by supplying a constant level of gas in air using reliable instruments as well as accurate monitoring techniques, thereby demonstrating the involvement of formaldehyde gas in facilitating IgE antibody-mediated responses and

ascertaining whether formaldehyde gas at threshold concentrations in air affects IgE-mediated allergic responses. Formaldehyde itself induced neither formaldehyde-specific IgE nor IgG antibody production in mice exposed to either high or low concentrations of formaldehyde gas in the air for 24 h 5 days a week over 5 weeks (Figure 1). However, when animals were sensitized with OVA allergen prior to gas exposure, greater IgE antibody production specific for the allergen was observed in mice exposed to high concentrations of gaseous formaldehyde when compared with low concentrations, OVA-control, or formaldehyde null control (high concentration, $P < 0.05$ vs. all; Figure 2). These results suggest that formaldehyde at concentrations above the threshold (0.08 ppm) may promote specific allergen-directed IgE-mediated immune responses, resulting in increased risk of developing exacerbations of allergic symptoms. A panel of expert concluded that eye irritation does not occur for healthy volunteers until at least 1.0 ppm following a short period of exposure and may not be attributable to allergic responses.²⁰ Although a review has argued that the data presented are not convincing evidence that low levels of indoor formaldehyde increase the risk of allergies, this epidemiological survey can be further extended by evaluating the effects of formaldehyde on subjects with active allergic symptoms or those with history of allergic symptoms.

An statistically significant increase in the percentage of splenic NK1.1⁺ cells was seen in mice exposed to threshold concentration of formaldehyde gas (0.08 ± 0.01 ppm) for 5 weeks, however, the percentage of other cell types remained unchanged after exposure to formaldehyde (Table 1). Additionally, it was found that exposure to high or low concentrations of formaldehyde gas resulted in significant increases above control in NK-cell cytotoxicity. This suggests that exposure to either low or high concentrations of formaldehyde gas itself could result in significant increases in the percentage of NK1.1⁺ cells as well as increases in NK-cell cytotoxicity. Depletion of NK1.1⁺CD3⁻ cells before priming with allergens inhibits pulmonary eosinophil and T-cell infiltration as well as inflammatory cytokine production in a murine model of allergic asthma.^{17,21} This effect of NK cells is demonstrated upon the stimulation of IgE specific for allergens.^{14,19} FACS analysis of lymphocytes in mice after allergen provocation has shown the pronounced increase in the number of both NK1.1⁺CD3⁺ (NKT), and NK1.1⁺CD3⁻ in peritoneal lavages that could produce IL-5.²¹ NK cells can contribute to IgE-mediated allergic responses by

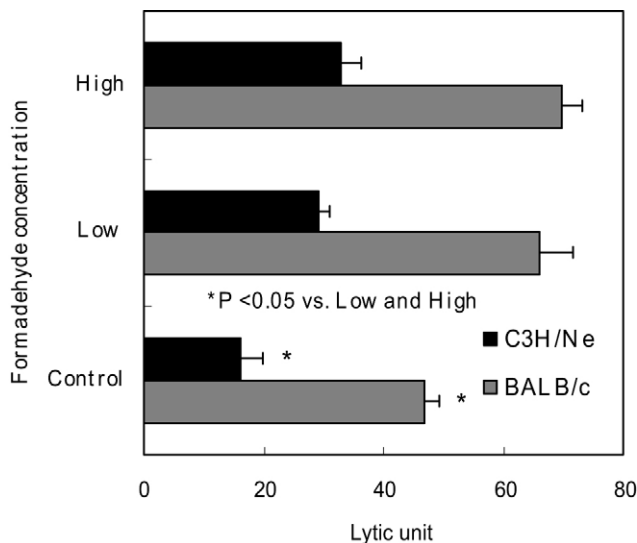


Figure 3 Splenic NK-cell activity in mice after exposure to formaldehyde. NK activity of splenocytes in BALB/c or C3H/He mice after exposure to low, high, or null concentrations of formaldehyde was evaluated against YAC-1. NK activity was expressed in lytic units. Results are the mean ± SEM of three independent experiments.

producing a variety of cytokines upon IgE stimulation.²² Along with the fact that NKT cells are a major source of IL-4 in response to other cytokines,^{23,24} it seems likely that NKT cells are involved in allergy-induced inflammatory responses. Although, the present study does not provide any data on the mechanism leading to enhanced antigen-specific IgE-mediated responses by formaldehyde, it is likely that either low or high concentrations of formaldehyde gas can selectively regulate NK-cell function, which could promote allergen-specific IgE production possibly in allergen-primed hosts. These results also strongly suggest that in addition to local immunity, systemic immunity plays a regulatory role in exacerbating allergic symptoms, however, additional studies may be needed to demystify detailed mechanisms.

Although, formaldehyde gas, at threshold levels of 0.08 ppm, is not directly responsible for exacerbating IgE-mediated immune responses (Figure 2), it is suggested that formaldehyde gas itself induce enhanced IgE-mediated allergic responses via a cascade of NK-cell activation. Although, less is known

about the health effects of other harmful gases, such as volatile organic compounds (VOC) emitted from housing products, it is possible that concomitant exposure to formaldehyde and VOC may induce IgE-mediated allergic inflammation especially in indoor environments in which the adhesives responsible for remitting formaldehyde are extensively used. Exposure to low concentrations of formaldehyde gas or VOC may put patients with pre-existing airway inflammation, such as asthma, or patients with heightened sensitivity to chemicals, at risk for developing exacerbations of their illness.

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