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Immune effects of inhalation exposure to fragrance allergens

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J. Ezendam, Laboratory for Health Protection Research
A. de Klerk, Laboratory for Health Protection Research
J. Vermeulen, Laboratory for Health Protection Research
P.H.B. Fokkens, Centre for Environmental Health Research
H. van Loveren, Laboratory for Health Protection Research

Contact:
J. Ezendam
GBO
Janine.Ezendam@rivm.nl

This investigation has been performed by order and for the account of Food and consumer products safety authority, within the framework of V/340301: Allergene stoffen in geurproducten

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Contents

1 Introduction

There is an increasing interest in products that can be used to spread a pleasant smell in homes, cars and public buildings. In supermarkets numerous different product types can be purchased, varying from fragrant candles, scented sprays, and electrical room perfumes (see for a detailed overview (Park et al., 2006). Fragrance chemicals are not only used in scented products, but also in other consumer products, such as cosmetics. Some fragrances can cause contact dermatitis after skin exposure. This so-called perfume allergy has a prevalence of 1-3% in the general population (Nielsen & Menne, 1992; Mortz et al., 2001; Nielsen et al., 2002; Dotterud, 2007; Dotterud & Smith-Sivertsen, 2007). Currently, 26 fragrance chemicals are identified as potential contact sensitizers (SCCNFP, 1999). According to EU legislation, labelling of these 26 fragrances is mandatory when they are use in cosmetics (EU directive 2003/15/EC D (2003).

Consumers that use scented products will be exposed to the emitted ingredients predominantly via inhalation exposure, although for some products skin exposure is possible as well. At the moment, inhalation as a route of exposure is not considered in safety evaluations, because it is not regarded as a relevant route for toxic effects (Ford et al., 2000; Cadby et al., 2002). However, there is inhalation exposure, but it is unknown if inhalation of contact allergens can induce sensitization and subsequent allergic manifestations in the airways. There are only a few studies that have investigated effects of respiratory exposure to fragrance chemicals, and most of these studies do not include sensitization as an endpoint, but rather look at inhalatory toxicity or irritation (Park & Janssen, 2007). The effects of inhalation exposure to other contact allergens have been studied previously. In the respiratory local lymph node assay (LLNA) contact allergens were able to induce immune responses in the airways (Arts et al., 2008). The type of immune response that is induced by these chemicals is different from for instance proteins, that induce type I (IgE-mediated) immune responses. Contact allergens such as dinitrochlorobenzene (DNCB) (Arts et al., 1998; Vanoirbeek et al., 2006), dinitrofluorobenzene (DNFB) (Buckley and Nijkamp, 1994), and picrylchloride (Garssen et al., 1989) induced type IV (cell-mediated) responses that are also involved in contact dermatitis. It can be concluded that inhalation exposure to contact sensitizers may result in a type IV immune response in the respiratory tract and thus may pose a risk for human health. If fragrance allergens are able to do so, is unknown.

In order to assess if fragrance allergens could induce immunostimulation upon inhalation the Food and Consumer Safety Authority has initiated this project, which aims to evaluate the risks of scented consumer products. Previously, the effects of inhalation exposure to fragrances have been studied in the respiratory LLNA. This murine model is similar to the dermal LLNA, which is a validated model for hazard identification of skin sensitizers. In the respiratory LLNA exposure to substances is via inhalation instead of via topical application (Arts et al., 2008; De Jong et al., 2009). Inhalation exposure to high concentrations of cinnamal and isoeugenol induced cell proliferation in the mandibular lymph nodes. The effects of isoeugenol were more pronounced than cinnamal, suggesting that this fragrance was more potent after inhalation exposure (Ezendam et al., 2007). In contrast, after skin exposure these fragrance chemicals have a similar potency in the skin LLNA (1.2-3.3% for isoeugenol (Basketter et al., 1999; Basketter & Cadby, 2004; Takeyoshi et al., 2006) and 1.3% for cinnamal (Elahi et al., 2004)). The concentrations used in this study were quite high and induced general toxicity. Therefore, to further investigate if inhalation exposure to fragrance allergens could lead to stimulation of the immune system more substances were tested in the respiratory LLNA using concentrations that do not induce general toxicity. Besides isoeugenol, the fragrances methyl heptine carbonate, benzyl salicylate and citral were tested.

2 Materials and methods

2.1 Animals

Six to eight week old male BALB/c mice were obtained from the institute's own breeding colony. The animals were bred specific pathogen free (SPF) and kept in macrolon cages under conventional conditions. The mice were fed Hope Farms chow pellets (Woerden, the Netherlands) and water *ad libitum* during the whole experiment. The experimental setup of the study was examined and agreed upon by the institute's Ethical Committee on Experimental Animals, and all experiments were performed according to national legislation.

2.2 Fragrance chemicals

The following fragrances were used: methyl heptine carbonate (99% purity; CAS 111-12-6), benzyl salicylate (99% purity), and isoeugenol (98% purity) from Sigma (St. Louis, MO, USA) and citral (purity >95%) from Fluka (Buchs, Swizerland). These fragrances were all tested previously in the LLNA and the skin sensitizing potencies (EC3 values) of the fragrances are for methyl heptine carbonate 0.5%, for isoeugenol 1.5%, for benzyl salicylate 1.5% and for citral 5.6%.

2.3 Experimental design respiratory LLNA and skin LLNA

The respiratory LLNA was performed as described previously (Arts et al., 2008). In short, groups of male BALB/c mice (six animals per group) were exposed nose-only to one of the fragrance chemicals on three consecutive days. The animals were necropsied 3 days after the last exposure and the auricular and mandibular lymph nodes (LN) were excised, pooled for each animal, and suspended in 5 ml RPMI 1640 (Gibco, Life Technologies, Breda, the Netherlands) with 5% heat inactivated Fetal Calf Serum (FCS) (Integro, Zaandam, the Netherlands), 100 U/ml penicillin and 100 µg/ml streptomycin (standard medium). At the autopsy other lymph nodes (deep cervical, parathymic, and mediastinal lymph nodes) were macroscopically examined for lymph node enlargement to indicate possible cellular stimulation.

In all experiments, a skin LLNA was performed as a positive control. Mice were topically exposed to 10% (v/v) isoeugenol, citral, benzyl salicylate or methyl heptine carbonate in acetone: olive oil 4:1 (AOO) on the dorsum of both ears (25 µl/ear) on three consecutive days. Control mice received the same treatment with the vehicle (AOO). Mice were necropsied 3 days after the last exposure and the auricular LNs were excised and were pooled for each animal and suspended in standard medium with 5% FCS.

2.4 Inhalation exposure and atmosphere generation and analysis

Mice were exposed to one of the various test materials on three consecutive days for 45, 90, 180, or 360 min/day. Variation in exposure duration rather than in concentration was used to investigate the

dose-response relationships. During exposure all mice were placed in restraining tubes which were connected to one of the two central exposure chambers for nose-only exposure. Mice that were exposed to the vehicle control were connected to the exposure chamber for the vehicle and mice that were exposed isoeugenol or cinnamal were connected to the exposure chamber of the fragrance. All fragrances were nebulized in acetone to produce an aerosol of liquid droplets in a concentration of 75 mg/m^3. The aerosols were sampled on 47 mm Teflon filters at a flow rate of 1 litre/min for 5 minutes. The collected mass was determined gravimetrically immediately after sampling to minimize evaporations of the collected droplets and used for concentration calculations. The vapour in this mixture downstream of the filters was also sampled on activated charcoal. In addition, the test atmosphere was sampled at a flow rate of approx 1 litre/min for 5 minutes on activated charcoal and these were used for wet chemical determinations and used to calculate the average actual concentrations during the exposures.

Table 1 Aerosol, vapour and total mass concentrations of each fragrance exposure

	Mass mg/m^3	Vapour mg/m^3	Total mg/m^3
Benzyl salicylate			
Day 1	68	2	70
Day 2	75	2	77
Day 3	77	2	79
Acetone			2740
Methyl heptine carbonate			
Day 1	4	76	80
Day 2	5	76	81
Day 3	6	76	82
Acetone			2300
Isoeugenol			
Day 1		80	80
Day 2		80	80
Day 3		80	80
Acetone			1500
Citral			
Day 1		80	80
Day 2		80	80
Day 3		80	80
Acetone			2840

The actual air concentrations measured for each fragrance are presented in Table 1. These were close to the target concentration of 75 mg/m^3. The fluctuations of all test atmospheres on the three days of exposure were less than 10% as indicted by continuous mass concentration measurements using a Total Carbon Analyzer (TCA). Vaporization of benzyl salicylate resulted predominantly in aerosols, whereas vaporization of methyl heptine carbonate resulted predominantly in vapour. Vaportization of isoeugenol and citral resulted exclusively in vapour.

2.5 Assessment of cell proliferation

Single cell suspensions were prepared in standard medium with 5% FCS under aseptic conditions by pressing the auricular and mandibular LN trough a 70 µm nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed in standard medium with 5% FCS (10 minutes, 300 g, 4 °C) and resuspended in 1 ml standard medium with 10% FCS. A Coulter Counter (Z2, Coulter Electronics, Mijdrecht, the Netherlands) was used to count the cells. Then the concentration of the cell suspensions was adjusted to 1×10^7 cells/ml. Of each cell suspension, 200 µl was seeded in triplicate in a U-bottom 96-well tissue culture plate (Greiner, Alphen aan den Rijn, the Netherlands). After addition of 10 µl/well (37 kBq methyl-3H-thymidine (specific activity 185 MBq/mmol, Amersham Biosciences, Buckinghamshire, UK) the cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 during 20–24 h. The cells were harvested on glass-fiber filters (LKB-Wallac, Turku, Finland) using a multiple cell culture harvester (LKB-Wallac). The [^{3}H]-thymidine activity was determined using a liquid scintillation counter (1205 Betaplate TM, LKB-Wallac). For further calculations the median of the triplicates was used. The [^{3}H]-thymidine incorporation is expressed per animal, being the measured counts per minute (cpm) times the cell number of the two LN and divided by the cell number in culture. The mean [^{3}H]-thymidine incorporation per experimental group ± SEM was calculated. Stimulation indices (SI) were calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the vehicle group. The SI after respiratory exposure was calculated by using the nose-only vehicle group and the SI after dermal exposure by using the dermal vehicle group.

2.6 Cytokine production

Cytokine production was only measured when the fragrance induced a SI ≥3. Production of cytokines was determined after culturing the isolated LN cells ($1x10^6$ cells/ml) with Concanavalin A (Con A, 5 µg/ml). After 24 h, the supernatants were harvested and levels of interferon-γ (IFN-γ), interleukin 4 (IL-4), interleukin 10 (IL-10) and interleukin 12 (IL-12 (p70)) were determined with a Bio-Plex assay (BioRad Laboratories, Hercules,California). Fluorescence was measured on a Luminex® (Biorad Life Science) and Luminex software was used to calculate the amount of cytokines (in pg/ml supernatant) using standard reference curves, included in the assay. Cytokine production was expressed in pg per lymph node, i.e. the response (in pg/10^6 cells) was multiplied by the cell number of the pooled lymph nodes. Cytokine levels below 5 pg per 10^6 cells were considered to be negative.

2.7 Statistical analysis

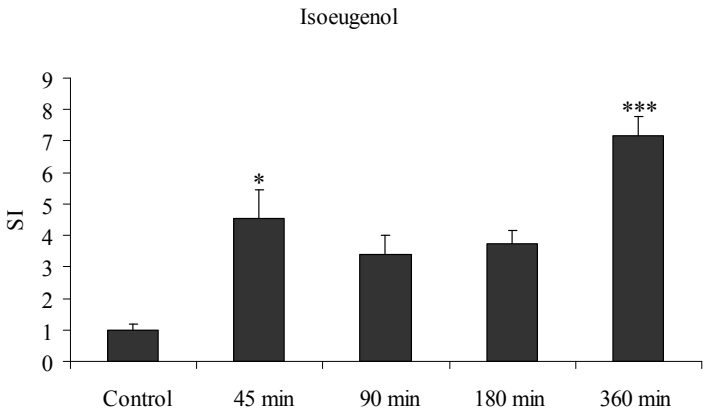
Statistical analysis was performed using one-way analysis of variance (ANOVA). Significant differences of the control group were determined with the Bonferroni post hoc test, using a significance level of $p=0.05$.

3 Results

3.1 Effects of inhalation exposure to fragrances on cell proliferation

Inhalation exposure to the fragrance chemicals did not induce any macroscopically visible toxic effects, neither did it affect body weight gain (not shown). The effects on cell number and cell proliferation in the mandibular lymph nodes are summarized in Table 2 and the SI values are depicted in Figure 1. The fragrance chemicals did not affect cell proliferation in the auricular lymph nodes (not shown). The only fragrance that significantly increased cell number and proliferation in the mandibular lymph nodes was isoeugenol. After 45 min/day exposure, cell proliferation was already significantly increased >4-fold compared to the control group. At the time points 90 min/day and 180 min/day SI values of 3.5 were observed, and in the group that was exposed for 360 min/day cell proliferation increased further to a SI value of 7.2.

A



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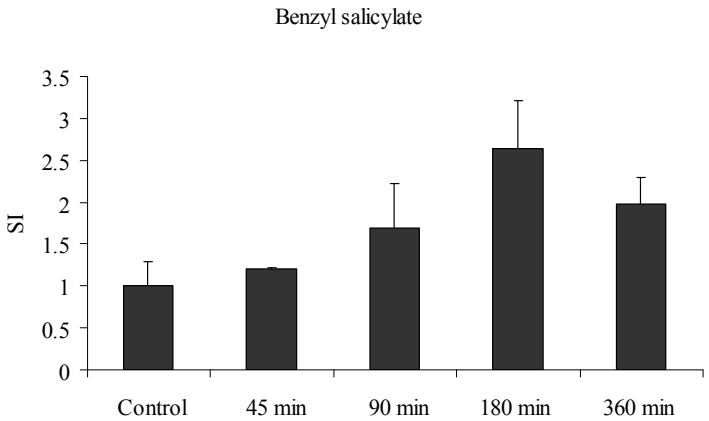

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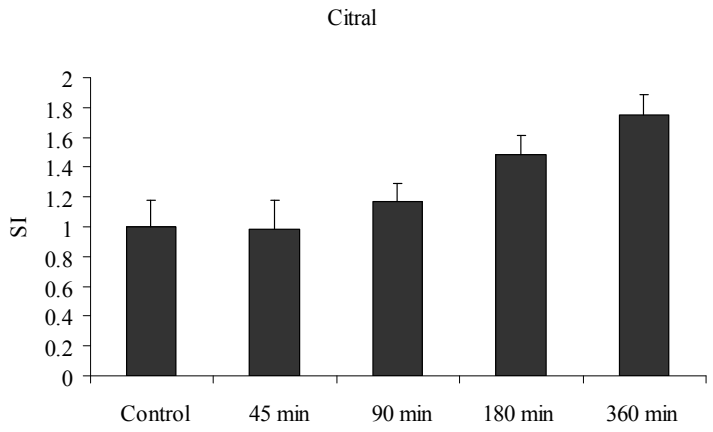

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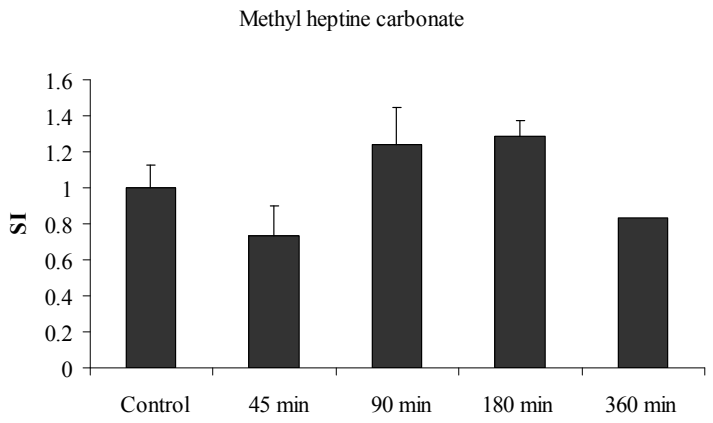

Figure 1: SI values in the mandibular LNs upon inhalation exposure to isoeugenol (A), benzyl salicylate (B), citral (C) and methyl heptine carbonate (D). SI values were calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group. Stimulation indices are shown as mean ± SEM (n=6 mice per group). Statistically significant differences were assessed with a one-way ANOVA with a Bonferonni's post hoc test. Asterisks depict significant differences from the control group: * $p<0.05$, *** $p<0.001$.

Benzyl salicylate and citral increased cell proliferation in the mandibular LNs slightly. These effects were not statistically significant. Exposure to benzyl salicylate induced a dose (concentration x time)-dependent increase of cell proliferation which peaked at the exposure time of 180 min/day, reaching a SI value of 2.6. After inhalation exposure to citral the maximum SI value that was reached after 360 min/day exposure was 1.8. Finally, methyl heptine carbonate did not increase cell proliferation in the mandibular LNs.

Table 2A: Effects of inhalation exposure to isoeugenol on the mandibular LNs

Group	Cell number ($x10^6$ cells)	Proliferation (CPM)	Stimulation index (SI)
Inhalatory exposure			
Control	4.8 ± 0.39	2316 ± 398	1.0 ± 0.17
45 min/day	8.2 ± 0.81*	10545 ± 2098*	4.6 ± 0.91*
90 min/day	8.3 ± 0.41**	7853 ± 1409	3.4 ± 0.61
180 min/day	9.1 ± 0.88***	8660 ± 998	3.7 ± 0.43
360 min/day	9.6 ± 0.64***	16596 ± 1419***	7.2 ± 0.61 ***

Table 2B: Effects of inhalation exposure to benzyl salicylate on the mandibular LNs

Group	Cell number ($x10^6$ cells)	Proliferation (CPM)	Stimulation index (SI)
Inhalatory exposure			
Control	2.7 ± 0.28	1574 ± 461	1.0 ± 0.29
45 min/day	3.1 ± 0.31	1898 ± 28	1.2 ± 0.02
90 min/day	3.2 ± 0.47	2654 ± 838	1.7 ± 0.53
180 min/day	4.7 ± 0.83	4157 ± 895	2.6 ± 0.57
360 min/day	4.4 ± 1.9	2894 ± 527	2.0 ± 0.32

Table 2C: Effects of inhalation exposure to citral on the mandibular LNs

Group	Cell number ($x10^6$ cells)	Proliferation (CPM)	Stimulation index (SI)
Inhalatory exposure			
Control	3.4 ± 0.31	2095 ± 259	1.0 ± 0.12
45 min/day	3.6 ± 0.70	2058 ± 332	0.98 ± 0.16
90 min/day	3.2 ± 0.39	2457 ± 171	1.2 ± 0.08
180 min/day	4.4 ± 0.22	3104 ± 221	1.5 ± 0.11
360 min/day	3.4 ± 0.23	3667 ± 198	1.8 ± 0.09

Table 2D: Effects of inhalation exposure to methyl heptine carbonate on the mandibular LNs

Group	Cell number ($x10^6$ cells)	Proliferation (CPM)	Stimulation index (SI)
Inhalatory exposure			
Control	2.8 ± 0.08	2011 ± 256	1.0 ± 0.13
45 min/day	2.7 ± 0.3	1472 ± 176	0.73 ± 0.09
90 min/day	3.7 ± 0.54	2492 ± 342	1.2 ± 0.17
180 min/day	3.8 ± 0.51	2581 ± 412	1.3 ± 0.21
360 min/day	3.1 ± 0.28	1673 ± 177	0.83 ± 0.09

Results are shown as mean ± SEM (n=6 per group). SI values were calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group. Statistically significant differences were assessed with a one-way ANOVA with a Bonferonni's post hoc test. Asterisks depict significant differences from the control group: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2 Effects of isoeugenol on cytokine production

In the experiments reported previously (Ezendam et al., 2007), supernatants were collected from the mice exposed to the high concentrations of isoeugenol and cinnamal (300 mg/m^3). High exposure to cinnamal induced only a slight increase in proliferation after 180 min/day exposure, inducing a 2-fold increase in cell proliferation. Furthermore, there was no effect on the production of IL-4 and IFN-γ (data not shown). Inhalation exposure to isoeugenol increased the production of all cytokines measured, the most pronounced increase was observed for IFN-γ. These effects were not significantly different from the control group, probably due to high variance in the experimental groups. Inhalation exposure to lower concentrations of isoeugenol (75 mg/m^3) induced both IL-4 and IFN-γ production, which was for both cytokines significantly higher than the control groups when mice were exposed for 360 min/day. Again, isoeugenol induced higher amounts of IFN-γ than IL-4.

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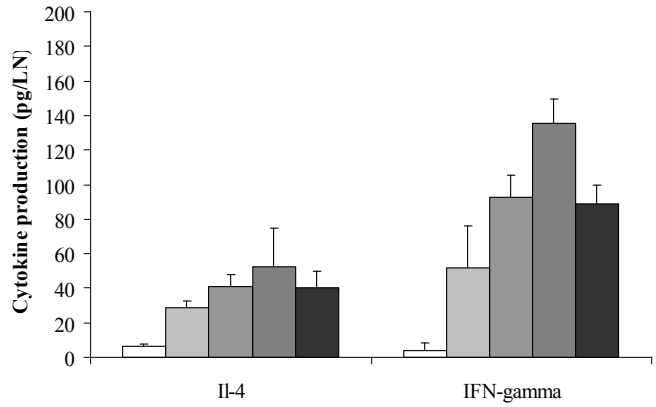

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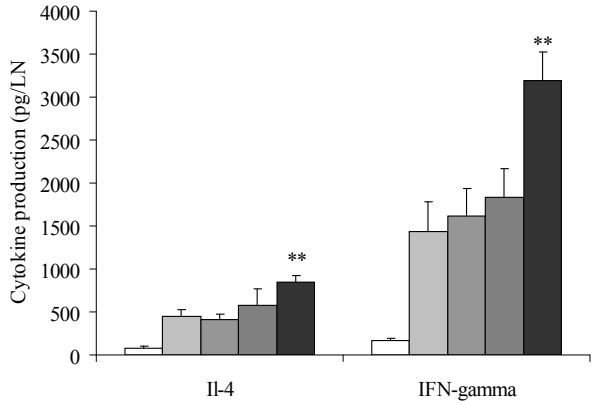

Figure 2: Cytokine production after inhalation exposure to 300 mg/m^3 (A) and 75 mg/m^3 (B) isoeugenol. Mice were exposed to acetone (white bars) or to one of the fragrances for 45 (light grey bars), 90 (darker grey bars), 180 (dark grey bars) or 360 (black bars) min/day for three consecutive days. Cytokine production was determined in supernatants of mandibular lymph node cells cultured with ConA. Data are expressed as pg per lymph node. A one-way ANOVA with a Bonferonni post hoc test was used to calculated statistical significant differences from the control group (**: $p<0.01$).

Benzyl salicylate, methyl heptine carbonate and citral had only minor effects on cell proliferation. From our studies with cinnamal and other sensitizers it is known that chemicals that induce moderate effects on cell proliferation, do not enhance cytokine production. Therefore, in these experiments cytokine production was not assessed.

3.3 Effects of fragrance chemicals in the dermal LLNA

Table 3 summarizes the effects of topical application of fragrance allergens in the LLNA. All chemicals were applied in a concentration of 10% (v/v). Isoeugenol, benzyl salicylate and methyl heptine carbonate induced SI values ≥ 3. The highest SI values were induced by benzyl salicylate, followed by isoeugenol and methyl heptine carbonate. Citral did not induce cell proliferation in the auricular lymph nodes.

Table 3: Effects of dermal exposure to the fragrances

Group	Cell number ($x10^6$ cells)	Proliferation (CPM)	Stimulation Index (SI)
Control	4.4± 0.32	2291 ± 151	1.0 ± 0.07
10% methyl heptine carbonate	9.9 ± 1.0	10030 ± 1298	4.4 ± 0.77
Control	4.3 ± 0.29	1934 ± 168	1.0 ± 0.09
10% benzyl salicylate	10.9 ± 1.5	17985 ± 2498	9.3 ± 1.4
Control	5.6 ± 0.35	4984 ± 869	1.0 ± 0.17
10% isoeugenol	15.6 ± 2.1	33449 ± 7817	6.7 ± 1.6
Control	3.8 ± 0.22	2406 ± 167	1.0 ± 0.07
10% citral	6.1 ± 1.1	3450 ± 434	1.4 ± 0.18

Results are shown as mean ± SEM (n=4 per group). Mice were treated topically with AOO (control) or the different fragrance chemicals in a concentration of 10% in AOO. Stimulation indices are calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group.

4 Discussion

There is limited human data available on the effects of inhalation exposure to fragrances in humans. In epidemiological studies it has been shown that inhalation of fragrances can aggravate respiratory symptoms in patients with asthma or chronic obstructive pulmonary disease. However, it is unknown if these symptoms are caused by non-specific irritation of the airways or by a specific allergic reaction (Elberling et al., 2005). There are two case reports that have shown that occupational exposure to fragrances could lead to asthma and rhinitis. In a saleswoman working in a perfumery suffering from respiratory distress it was shown that the respiratory symptoms could be reproduced by inhalation of different perfume brands (Baur et al., 1999). In addition, in a hair dresser it was shown that inhalation exposure to eugenol caused occupational asthma (Quirce et al., 2008). Often, adverse effects of chemicals are first detected in an occupational setting, in which exposure is often more chronic and to higher concentrations. Schnuch et al. (2009) have studied the effects of inhalation of isoeugenol or Lyral in subjects with a contact allergy for these fragrances. They have shown that inhalation did induce subjective respiratory symptoms in some patients. Furthermore, in some patients the contact dermatitis worsened after inhalation exposure (Schnuch et al., 2009). These studies indicate that inhalation of fragrances could lead to respiratory allergies. However, if this will occur in the general population that use scented products is unknown.

In this study we demonstrate that isoeugenol can induce immune effects in the respiratory LLNA, whereas the other tested fragrance chemicals induce slight or no immune effects. The respiratory LLNA is an animal model that is based on the protocol of the LLNA but exposure is via inhalation (Arts et al., 2008). Unlike the skin LLNA, which is extensively validated and is the method of choice for the hazard identification of skin sensitizers, the respiratory LLNA has not been validated for hazard identification. In the skin LLNA, a chemical is designated as a skin sensitizer when it induces a SI value of 3 or greater. For the respiratory LLNA it is unknown if the same cut-off value can be used. The penetration of compounds through the skin barrier is different from penetration in the airways. Furthermore, stimulation of the immune system via the skin will involve other cell types than in the lungs, which could lead to a quantitatively and qualitatively different immune response. After inhalation, the cytokine profile induced by isoeugenol shows that a Th1-mediated immune reaction was induced. This is typical for a delayed-type hypersensitivity reaction induced by contact sensitizers. This reaction is characterized by high IFN-γ and low IL-4 production, both after dermal (Vandebriel, et al. 2000; Dearman et al., 2002) and inhalation exposure (De Jong et al, 2009).

The fragrances that were tested in the respiratory LLNA are all, with the exception of citral, stronger skin sensitizers (EC3 ≤ 2%) (OECD, 2008). In humans, the most potent contact allergen is methyl heptine carbonate, followed by isoeugenol, cinnamal, citral and benzyl salicylate (Api & Vey, 2008; Basketter et al., 2008). In the respiratory LLNA, however, the ranking of chemicals is different than in the skin LLNA. The most potent contact allergen tested, i.e. methyl heptine carbonate, was negative in the respiratory LLNA. Furthermore, the only fragrance that induced a significant increase in cell proliferation was isoeugenol, while cinnamal and benzyl salicylate, that have similar skin sensitizing potencies as isoeugenol, were less potent after inhalation exposure. These data suggest that the skin sensitizing potency does not predict the potency in the respiratory LLNA. This has been shown previously for DNCB and oxazolone, which are have a comparable potency in the skin LLNA, but in the respiratory LLNA oxazolone was more potent than DNCB (Arts et al, 2008).

It can be concluded that isoeugenol can stimulate the immune system upon inhalation inducing a Th1 type of reaction. The other tested fragrance allergens, induced only insignificant or no effects. To further investigate the relevance of these findings, additional experiments that investigate effects in the elicitation phase are needed. Experiments in which the effects of an inhalation challenge with fragrance

allergens are measured in sensitized animals will provide more data that can be used for the hazard identification of this fragrance. Especially functional lung function parameters, such as breathing patterns, should be assessed in order to obtain insight in clinical relevant parameters.

In conclusion, some but not all contact sensitizers are able to induce sensitization after inhalation, but the clinical consequences need to be further investigated.

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National Institute
for Public Health
and the Environment

P.O. Box 1
3720 BA Bilthoven
The Netherlands
www.rivm.com