

Enhanced sensitization and elicitation responses caused by mixtures of common fragrance allergens

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Summary

Background. Perfumes are complex mixtures composed of many fragrance ingredients, many of which are known to be only weak allergens when tested individually. It is therefore surprising that fragrance contact allergy is one of the most common forms of contact allergy.

Objectives. To investigate whether mixing different fragrance allergens leads to increased sensitization potency, and to examine the difference in the challenge response to one chemical in mice sensitized either with the mixture of allergens or with only the relevant allergen.

Methods. CBA mice were sensitized with three different concentrations of three fragrance allergens alone or as a mixture. The sensitization and elicitation responses were measured by ear thickness plus infiltration of B and T cells and T cell proliferation in the draining lymph nodes.

Results. We found a dose-dependent sensitization response for each of the allergens. An increased response was seen when the allergens were mixed. A stronger challenge response to cinnamal was seen in mice sensitized with the allergen mixture than in mice sensitized with cinnamal alone.

Conclusions. Our findings suggest that mixtures of allergens increase the primary response that potentiates the generation of memory T cells in response to the specific allergen. Thus, allergen mixtures enhance both induction and elicitation of contact allergy.

Key words: cocktail effect; contact allergy; perfume.

Fragrance contact allergy is seen in 1.7–4.1% of the general population, and in about 10% of patients with eczema (1). Of the contact allergies, only nickel allergy

is more prevalent than fragrance allergy (1). A perfume, also called a fragrance composition, often consists of 10–100 fragrance ingredients, or even more in the case of prestigious/luxury perfumes. When tested one by one, many fragrance ingredients are categorized as weak allergens, but moderate and strong fragrance allergens have also been identified (2). As many fragrance ingredients act as allergens with a skin-sensitizing capacity, and most perfumes contain a mixture of fragrance ingredients, the consumers are most likely exposed to mixtures, also called cocktails, of allergens when they use products

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that contain perfume (1). In three recent investigations of cosmetic products on the European market, it was found that 50–89% of these contained at least one of the 26 fragrance allergens that, according to EU regulations, should be mandatorily listed in the declaration of contents ((2) and http://ec.europa.eu/consumers/sectors/cosmetics/documents/directive/index_en.htm) (3, 4). In Denmark, 97 deodorants were analysed, and a subset of 23 of the deodorants analysed contained 5–17 of the 26 regulated fragrance allergens, with a median of eight fragrance allergens per product (5). It has been shown that exposure to two fragrance allergens together in individuals sensitized to both allergens causes a synergistic elicitation response, and that mixing a specific allergen with an irritant lowers the threshold of the elicitation response (6, 7). However, what effect allergen mixtures have on the sensitization to single allergens in the mixture is unknown. In the present study, we used mouse models to investigate the dose effect of three known fragrance allergens applied either one by one or as a mixture, and compared the sensitization capacity of a single specific fragrance allergen with that of a mixture of fragrance allergens containing identical concentrations of the specific allergen.

Materials and Methods

Mice

Female CBA mice were purchased from Harlan (Germany, crs.de@harlan.com) and housed in the specific pathogen-free animal facility at the University of Copenhagen in accordance with national animal protection guidelines (licence number 2007/501-1357). In each experiment, groups of four mice were used.

Chemicals

The following allergens were used for skin sensitization: hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (CAS 31906-04-4), isoeugenol (CAS 97-54-1) and cinnamal (CAS 104-55-2) from Sigma-Aldrich.

Contact sensitization and challenge experiments

For studying sensitization, mice were painted with 25 µl of either cinnamal (0.7%, 2.0%, 6.0%), isoeugenol (0.4%, 1.3%, 3.9%), HICC (2.8%, 8.3%, 25%) or a combination of the three (Table 1) in acetone/olive oil 4:1 on the dorsal side of both ears for three consecutive days (days 0–2). The concentrations of cinnamal and isoeugenol were chosen on the basis of the literature, with the expected EC3 value as the medium concentration plus a three-fold lower

Table 1. Concentrations of fragrance allergens used in the study

	Isoeugenol	Cinnamal	HICC	Mixture
Low	0.4	0.7	2.8	Combination
Medium	1.3	2.0	8.3	Combination
High	3.9	6.0	25	Combination

HICC, hydroxyisohexyl 3-cyclohexene carboxaldehyde. The numbers within the table represent the percentages (wt/vol) of the given fragrance allergen in the low-strength, medium-strength and high-strength solutions. 'Combination' denotes that the final concentration of the allergens in the mixture is as indicated for the single allergen in the corresponding row.

and a three-fold higher concentration (2). For a given allergen, the EC3 value is the concentration of the allergen that gives a three-fold increase in proliferation of lymph node cells as compared with vehicle controls as determined by local lymph node assay (8). For HICC we made an exception, as we used the approximate EC3 value as the highest concentration to avoid concentrations higher than 25% HICC (9). On day 3, mice were given 5-bromo-2-deoxyuridine (BrdU) (0.8 mg/ml) in their drinking water. BrdU is a thymidine analog that is incorporated into proliferating cells. Mice were killed on day 5. The increase in ear thickness was measured in a blinded manner with an engineer's micrometer (Mitutoyo, Tokyo, Japan), and the draining auricular lymph nodes were removed. To investigate the challenge response, mice were initially sensitized by painting with 25 µl of either 6.0% cinnamal, the high concentration of the mixture or acetone/olive oil on the dorsal side of both ears for three consecutive days. After a minimum of 21 days, mice were given BrdU (0.8 mg/ml) in the drinking water. After 24 hr, mice were challenged on both ears with 2.0% cinnamal. Mice were killed after 24 hr, the increase in ear thickness was measured, and the draining auricular lymph nodes were removed.

Flow cytometry

From the draining lymph nodes, a cell suspension was prepared manually, and the cells were counted. To determine the distribution of T and B cells, the cell suspensions were incubated with fluorochrome-conjugated anti-CD4 (RM4-5), anti-CD8a (53-6.7) or anti-CD19 (ID3) monoclonal antibodies, all from BD Biosciences (Brøndby, Denmark, bdcs_dk@europe.bd.com). Cells were incubated with the indicated monoclonal antibody diluted in ice-cold phosphate-buffered saline containing 2% foetal calf serum and 0.1% NaN₃ for 30 min on ice. After being washed, cells were analysed on a FACSCalibur (BD Biosciences) with CELLQUEST™ software.

T cell proliferation

Proliferating cells were identified by intracellular BrdU staining according to the instructions of the manufacturer (BD Biosciences). In brief, the cells were adjusted to 1×10^7 cells/ml and plated in 96-well plates (100 μ l/well). The cells were stained for surface marker expression as described above. After being washed, the cells were fixed, permeabilized, and treated with 300 μ g/ml DNase for 60 min at 37 °C. Finally, the cells were stained intracellularly with anti-BrdU and analysed by flow cytometry.

Statistical analysis

Statistically significant differences were determined by two-sample equal variance Student's *t*-test. The analyses were performed on data from two individual experiments

(*n* = 4) comparing mice exposed to the different fragrance ingredients with control mice.

Results

First, we determined the degree of local inflammation by measurements of ear swelling in mice following sensitization with three different concentrations of HICC, cinnamal, or isoeugenol, applied one by one or as a mixture (Table 1). We found that all of the allergens induced minor concentration-dependent increases in ear thickness when applied one by one (Fig. 1a). In contrast, the mixture with the highest concentrations of the three fragrance allergens induced significant ear swelling, with an ear thickness of 130% as compared with mice in the control group. In agreement with this, when studying

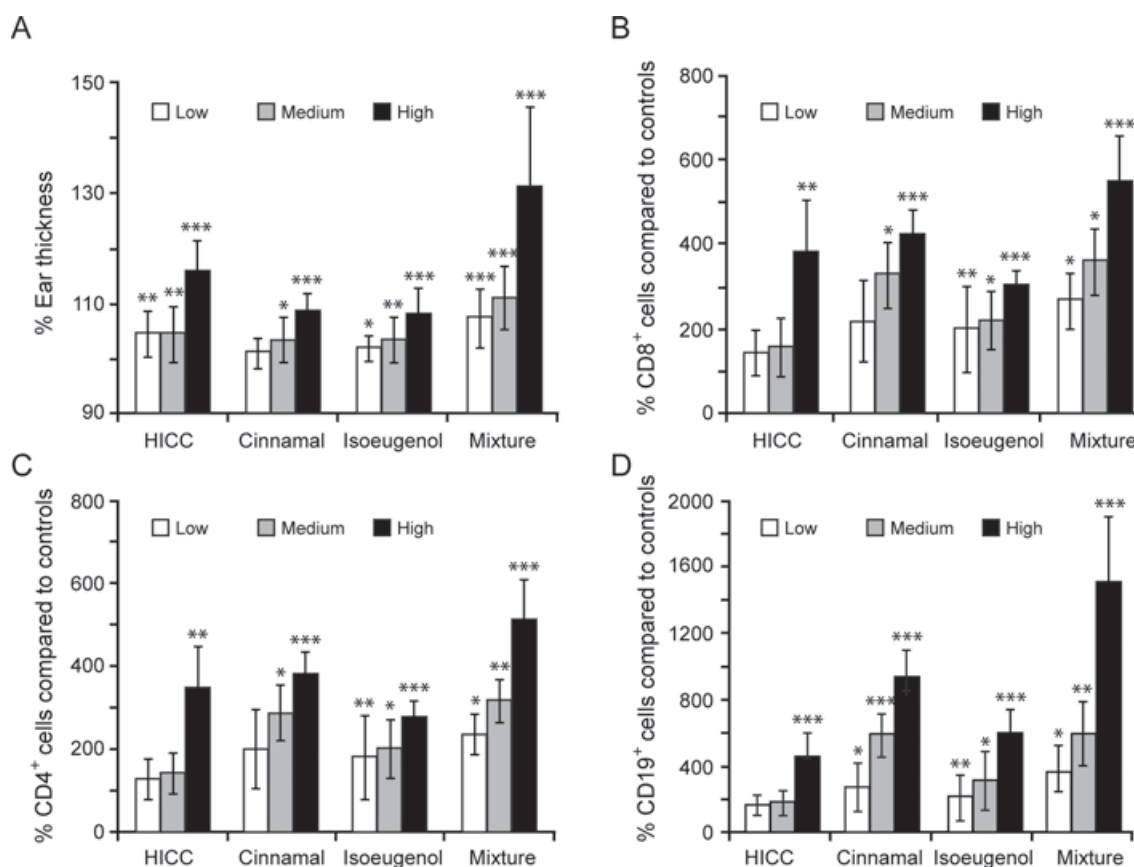


Fig. 1. Allergen dose dependency of the sensitization response. Groups of mice were exposed to three different concentrations of hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (2.8%, 8.3%, 25%), cinnamal (0.7%, 2.0%, 6.0%), and isoeugenol (0.4%, 1.3%, 3.9%), referred to as low-strength, medium-strength and high-strength solutions. Furthermore, groups of mice were exposed to allergen mixtures containing similar concentrations of each of the three allergens as given above. The vehicle was olive oil/acetone. Mice were exposed to allergen for three consecutive days (days 0–2), and were killed on day 5. (a) Change in ear thickness. (b–d) Relative numbers of CD8⁺ T cells, CD4⁺ T cells and CD19⁺ B cells in the draining lymph nodes. Results are presented as [(number of cells_{treatment})/(number of cells_{controls})] $\times$ 100% $\pm$ standard error of the mean. * *p* < 0.05, ** *p* < 0.005, *** *p* < 0.0005. The data represent two individual experiments with four mice in each group/experiment.

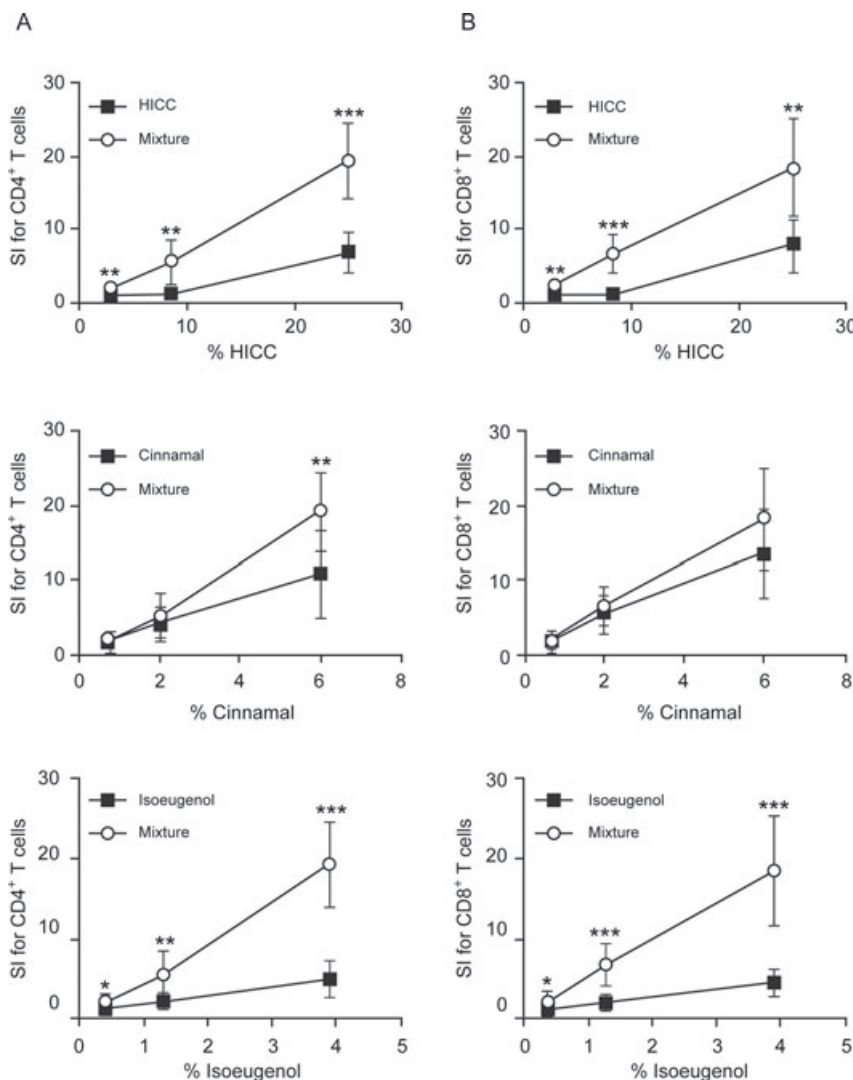


Fig. 2. T cell proliferation following exposure to isolated or mixed fragrance allergens. Mice were exposed to low-strength, medium-strength or high-strength solutions of fragrance allergens for three consecutive days (day 0–2). On day 5, mice were killed, and T cell proliferation in draining lymph nodes was analysed by flow cytometry following 5-bromo-2-deoxyuridine (BrdU) staining. (a) Stimulation index (SI) of the CD4⁺ T cell response calculated as $(CD4^+ BrdU^+_{treatment}) / (CD4^+ BrdU^+_{control})$. (b) SI of the CD8⁺ T cell response calculated as $(CD8^+ BrdU^+_{treatment}) / (CD8^+ BrdU^+_{control})$. Results are presented as mean $\pm$ standard error of the mean. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. The data represent two individual experiments with four mice in each group/experiment. HICC, hydroxyisohexyl 3-cyclohexene carboxaldehyde.

the draining lymph nodes, we found a concentration-dependent increase in the infiltration of CD8⁺ and CD4⁺ T cells and CD19⁺ B cells for all three allergens (Fig. 1b–d). The mixture containing the highest concentrations of the three allergens potentiated the infiltration of all three cell types (Fig. 1b–d).

We then analysed T cell proliferation in the draining lymph nodes in mice following sensitization with the fragrance ingredients, applied either one by one or as a mixture (10). A concentration-dependent increase in the stimulation index (SI) of both CD4⁺ and CD8⁺ T cells as determined by BrdU⁺ cells was seen for all three allergens, both when applied one by one and when applied as a mixture (Fig. 2). As compared with the isolated fragrance allergens, the mixture induced a significant two-fold to five-fold increase in the SI of CD4⁺ T cells (Fig. 2a). Likewise, as compared with the isolated

fragrance allergens, the fragrance mixture induced a significant increase in the SI of CD8⁺ T cells, except for cinnamal (Fig. 2b).

The results described above indicate that mixtures of fragrance allergens induce a stronger inflammatory response, both locally and in the draining lymph nodes, than isolated fragrance allergens. In turn, a stronger primary inflammatory response could lead to increased T cell activation and generation of memory T cells, and thereby to an enhanced challenge response. Consequently, we next studied the challenge response to isolated cinnamal in mice that had been sensitized either with isolated cinnamal or with the allergen mixture. We sensitized mice with either 6% cinnamal or the allergen mixture that contained 6% cinnamal (high). After a minimum of 21 days, the mice were challenged with 2% cinnamal, and after 24 hr the immune response was determined. A significant

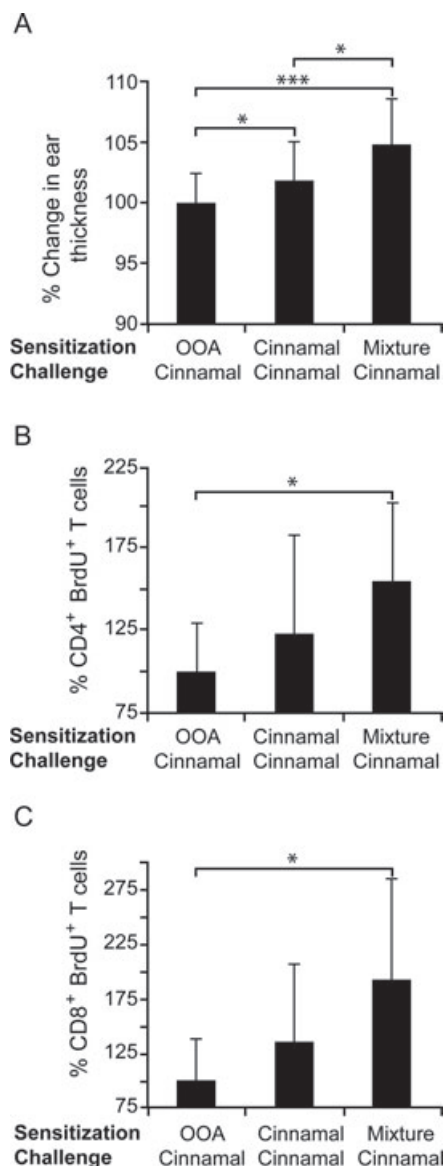


Fig. 3. Sensitization with the allergen mixture increases the challenge response to cinnamal. Mice were sensitized with either 6% cinnamal or the high-strength allergen mixture solution by exposure to the allergen for three consecutive days. After a minimum of 21 days, mice were challenged with 2% cinnamal. (a) Change in ear thickness. (b) Proliferating CD4⁺ T cells calculated as $[(CD4^{+} BrdU^{+}_{treatment}) / (CD4^{+} BrdU^{+}_{control})] \times 100\%$. (c) Proliferating CD8⁺ T cells calculated as $[(CD8^{+} BrdU^{+}_{treatment}) / (CD8^{+} BrdU^{+}_{control})] \times 100\%$. Results are presented as mean $\pm$ standard error of the mean. * $p < 0.05$, *** $p < 0.0005$. The data represent two individual experiments with four mice in each group/experiment. OOA: olive oil/acetone; mixture: 3.9% isoeugenol, 6.0% cinnamal, 25% HCC.

increase in ear swelling was seen in mice sensitized with the allergen mixture as compared with mice sensitized

with cinnamal alone (Fig. 3a). As for the local immune response, sensitization with the allergen mixture seemed to potentiate the challenge response to cinnamal as measured by T cell proliferation in the draining lymph nodes (Fig. 3b and c). Whereas sensitization with cinnamal alone led to an approximately 20% increase in the number of proliferating CD4⁺ T cells, sensitization with the allergen mixture led to an approximately 35% increase (Fig. 3b). Likewise, a 50% increase in the number of proliferating CD8⁺ T cells was seen in mice sensitized with cinnamal alone, whereas mice sensitized with the allergen mixture had a 90% increase in the number of proliferating CD8⁺ T cells (Fig. 3c).

Discussion

In this study, we found that mixtures of fragrance allergens have an increased potency in sensitization and elicitation of contact allergy reactions as compared with the isolated fragrance allergens. These results may explain why fragrance allergy is a prevalent phenomenon in spite of the fact that many fragrance allergens are categorized as weak sensitizers. In agreement with Nicolas and co-workers, the changes in ear swelling in our experiments, during both sensitization and challenge, were minor in comparison to what is seen for 2,4-dinitrofluorobenzene and *p*-phenylenediamine (PPD) (10–12). Even though Nicolas and co-workers used the fragrance ingredients α -hexylcinnamaldehyde (hexylcinnamal), eugenol and hydroxycitronellal at concentrations three-fold to six-fold higher than the EC3 value, they did not see any change in ear swelling in immunocompetent animals (12). In contrast, we found significant ear swelling in mice sensitized with a mixture of three fragrance allergens, comparable to the ear swelling observed after exposure to 1% PPD (11).

It has previously been shown that application of an irrelevant allergen during challenge lowers the reaction threshold (13). In the present study, we found that sensitization with a mixture of fragrance allergens containing cinnamal induced a stronger challenge response to cinnamal than sensitization with cinnamal alone. As contact allergy is a T cell-mediated reaction, the potency of an allergen depends on its ability to induce activation of T cells. A naïve T cell requires two signals to be activated: (i) signal 1, mediated by the T cell receptor when it recognizes specific major histocompatibility complex–peptide complexes presented on the cell surface of an antigen-presenting cell (APC); and (ii) signal 2, mediated by CD28 when it binds the co-stimulatory molecules CD80 and CD86 expressed at the cell surface of activated APCs. Furthermore, recent studies have shown that cytokines also play a role in T cell activation and the generation of

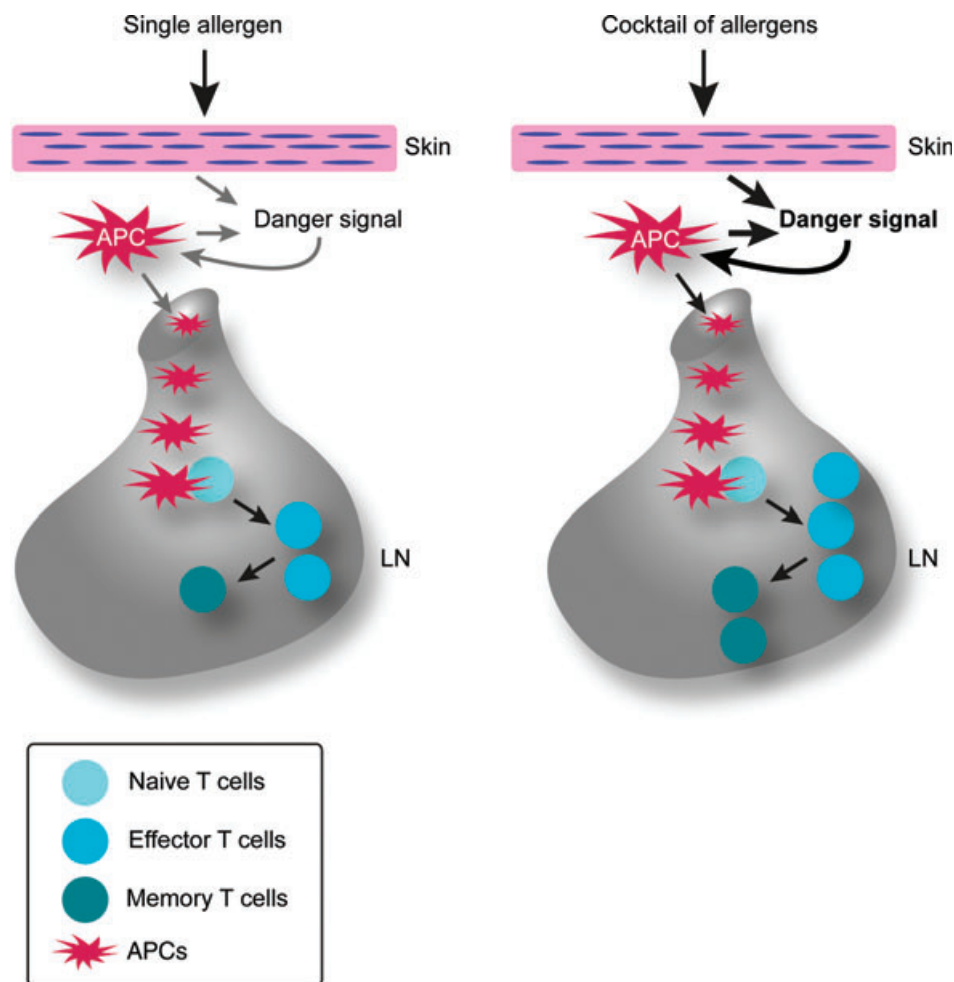


Fig. 4. Model for the cocktail effect of fragrance allergens. Exposure to a single allergen induces only weak danger signals, and thereby weak activation of antigen-presenting cells (APCs), T cell activation, and memory T cell generation. However, exposure to a cocktail of allergens leads to enhanced danger signals, and thereby to a higher degree of APC and T cell activation and memory T cell generation. LN, lymph.

T cell effector functions, survival, and memory T cell formation (14, 15). We therefore propose the following model to explain the enhanced sensitization and elicitation responses caused by allergen mixtures/cocktails. Exposure to a cocktail of fragrance allergens induces greater production of pro-inflammatory cytokines, for example interleukin (IL)-1 and IL-12, than exposure to a single fragrance allergen. This leads to enhanced activation of the APCs and to the generation of the optimal cytokine milieu. Subsequently, this leads to more efficient T cell activation and generation of memory T cells. Because of the increased generation of memory T cells, subsequent challenge with a single allergen will induce a stronger response in individuals initially sensitized with a cocktail of allergens than in individuals sensitized with the single allergen (Fig. 4).

The routine diagnosis of fragrance allergy is made by patch testing with two mixtures of fragrance allergens: fragrance mix I, a mixture of eight ingredients, two of which are natural extracts, and fragrance mix II, a mixture of six ingredients (16, 17). It seems that these mixtures represent not only a good reflection of normal exposure to perfumes, but also provide the optimal stimulus to the immune system, and thereby have a high diagnostic ability. There is long-term experience that patients who react to one of the fragrance mixtures may be patch test-negative to all of the individual ingredients, even if tested at higher concentrations. This has often been interpreted as a false-positive reaction to the mixture, but, in light of the current study, it may also be attributable to false-negative reactions to the ingredients.

Fragrances and hair dyes are two groups of consumer products composed of cocktails of allergens. The current study indicates that mixtures of allergens not only increase the risk of allergy being induced, but also increase the severity of subsequent symptoms upon re-exposure. It indicates that risk assessment and management should

be based on knowledge of the effects of allergen mixtures and not only those of isolated allergens.

Acknowledgement

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