

Lack of evidence for allergenic properties of coumarin in a fragrance allergy mouse model

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Background: There is controversy as to whether coumarin, an ingredient in cosmetics and fragrances, is a contact allergen involved in fragrance allergy. We recently showed that the purity of coumarin is a critical parameter for its allergenicity because coumarin preparations containing trace amounts of contaminants induced cell proliferation in the local lymph node (LN) assay whereas pure coumarin did not.

Objective/Method: In the present study, we analyzed the sensitizing properties of coumarin (purity > 99.9) and of dihydrocoumarin (DHC), in a recently developed model of fragrance allergy in mice.

Results: DHC was able to prime T cells in LNs draining the sensitization skin site and to induce a typical allergic contact dermatitis (ACD) reaction upon challenge, confirming that DHC is endowed with moderate sensitizing properties. In contrast, no T-cell activation and no ACD responses were obtained following sensitization and challenge with coumarin.

Conclusion: These results confirm that pure coumarin is endowed with very weak sensitizing capacities, if any, and suggest that the presence of contaminants in coumarin preparations may account for the previously reported allergenic properties of coumarin.

Key words: allergic contact dermatitis; coumarin; fragrance allergy; mouse ear-swelling test; murine model; T cell priming. © Blackwell Munksgaard, 2007.

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Allergic contact dermatitis (ACD) is a public health concern in industrialized countries and one of the commonest occupational diseases (1, 2). There is controversy as to whether coumarin, an ingredient in cosmetics and fragrances, is a contact allergen involved in fragrance allergy. Indeed, coumarin allergy, defined by positive skin patch tests, has been observed in up to 6.8% of patients with ACD (3–6). Moreover, it has been shown that coumarin is endowed with irritant properties at the concentration of 8% (7). However, in a recent study on the skin reactivity to 14 frequently used chemicals, Frosch et al. (8) observed positive patch tests to coumarin in less than 0.3% of the 1855 ACD patients tested. The main pitfall of the studies showing the allergenicity of coumarin was the lack of information concerning the purity of the coumarin used for patch testing. In

most of the studies, coumarin was used at the concentration of 5% in petrolatum and was said to be 95–99% pure. Indeed, coumarins comprise a large variety of natural or synthetic compounds, in which some chemicals are known as moderate (5,7-dihydroxycoumarin, 5,7-dihydroxy-4-methylcoumarin) or weak (scoparone, isoscopoletin and 4-hydroxycoumarin) contact allergens (9). These chemicals are different from coumarin but are often confused with it. Because the chemical process of coumarin is critical for the purity of the molecule and because coumarin derivatives and contaminants are endowed with irritant and allergenic properties, we hypothesized that the reported allergenic properties of coumarin could in fact be attributed to contaminants. In a recent study combining murine experiments and human clinical studies, we showed that the coumarin

chemical (pure coumarin preparation) was extremely well tolerated (10). In contrast, derivatives contaminating some coumarin preparations were endowed with sensitizing properties detected in the local lymph node assay (LLNA) suggesting that contaminants but not the pure chemical are responsible for both the irritant and sensitizing properties previously attributed to coumarin (10).

Experimental studies from the last 15 years have shown that CD8+ type 1 T cells are effector cells of ACD through cytotoxicity and IFN- γ (interferon gamma) production while CD4+ T cells are endowed with down-regulatory functions (11). We have recently reported on a mouse model of fragrance allergy in which mice deficient in CD4+ T cells develop a robust ACD to hexylcinnamaldehyde, eugenol, and hydroxycitronellal. The ACD reaction is mediated by specific IFN- γ -producing CD8+ T cells, which are primed in lymph nodes (LNs) draining the sensitization site (12). In contrast, no ACD could be obtained in normal mice suggesting that CD4+ T cells contain a subset of T-regulatory cells (Treg), which control the priming and activation of fragrance-specific CD8+ T cells. This observation opened new opportunities to develop maximalized murine models of ACD for the detection of weak contact sensitizers.

In the present study, we investigated the sensitizing properties of pure coumarin and of 3,4-dihydrocoumarin (DHC) in the newly developed model of ACD to fragrances in CD4+ T cell-deficient mice. We show that DHC, but not coumarin, behaves as a contact sensitizer and is able to prime T cells and to induce ACD responses.

Materials and Methods

Mice

Female BALB/c or C57BL/6 mice (Charles River Laboratories (L'Arbresle, France), were bred at the institute's animal facility (AniRA.PBES – ENS-Lyon, France) under pathogen free conditions. They were left to acclimate before entering the study and provided for food and water *ad libitum*. All the mice were used between 6 weeks and 10 weeks of age. Experimental procedures have been approved by the institutional review board on animal experiments (Comité Régional d'Ethique sur l'Expérimentation Animale).

Chemicals

DHC, 98% pure and α -hexylcinnaldehyde (HCA, 85% pure) were purchased from Sigma-Aldrich (Saint Quentin en Fallavier, France).

Pure coumarin (Rhodiascent extra pure[®], >99.9% pure) was produced in Rhodia Organics (Lyon, France) (10). All the chemicals were prepared freshly before application, and dissolved in *N,N*-dimethylformamide (DMF; Sigma-Aldrich, Saint Quentin en Fallavier, France) or in acetone/olive Oil (AOO, Carlos Erba and Lessieur) (4:1, v/v). They were dispensed with gloves for dermal protection.

Antibody depletion of CD4+ T cells in vivo

The rat anti-mouse CD4 monoclonal antibody (mAb) GK 1.5 was obtained from ATCC (Rockville, MD, USA). Hybridomas were grown as ascites in pristane-primed Swiss-nude mice. BALB/c or C57BL/6 mice were given i.p. injections of 20 μ g of anti-CD4 mAbs in 200 μ l on D-3, D-2, D-1 and D4. Depletions > 98% were observed in all the experiments.

Mouse ear swelling test

The mouse ear-swelling test (MEST) was used for the study of ACD reactions. C57BL/6 mice were sensitized on 3 consecutive days (D-2, D-1 and D0) by epicutaneous application of 50 μ l of DHC (50%) or pure coumarin (50%) onto abdominal skin, and challenged 5 days after the last sensitization dose with 12.5 μ l of a non-irritant concentration of DHC (40%) or pure coumarin (40%) onto the left ear.

Analysis of chemical-induced T cell priming in draining LNs

Sensitization procedure. The sensitization procedure was conducted according to the standard LLNA protocol (13). Briefly, groups of BALB/c mice ($n = 3-4$) were painted by topical application of 25 μ l/ear of the appropriate dilution of the test substance, or of vehicle alone, on the dorsum of both ears, daily for 3 consecutive days (D-2, D-1 and D0). 5 days after the first application of chemicals (D3), draining auricular LNs were collected into complete RPMI medium and a single cell suspension of LN cells was prepared by mechanical disaggregation through a 100 μ m nylon filter (Becton Dickinson, Le Pont de Claix, France). Viable cell counts were carried out by exclusion of 0.5% trypan blue.

IFN- γ ELISA. Production of IFN- γ by LN cells of mice was determined by enzyme-linked immunosorbent assay (ELISA). A total of 2 millions LN cells/ml was cultured in 96-well plates in RPMI complete medium, in presence of a submitogenic concentration (5 μ g/ml) of

phytohemmagglutinin (PHA; Sigma), at 37° cel-sius, 5% CO₂. Preliminary experiments have shown that this dose is not mitogenic for resting T cells, but can potently stimulate proliferation of and IFN- γ production by T cells previously activated *in vivo*, as previously described (14). Of note, the same batch of PHA was used for all the experiments. Cell-free supernatants were harvested after 20 hr, and IFN- γ production was determined by ELISA using the R&D System (Lille, France) DuoSet kit following the manufactural protocol.

Interexperiment standardization of the IFN- γ results. We observed that the magnitude of IFN- γ secretion by LN cells of vehicle- and fragrance allergen-painted mice varied greatly, but in similar proportions, from 1 experiment to another. Therefore, in order to compare results obtained from different experiments, HCA, the recommended LLNA positive control (13), was introduced in each experiment and the IFN- γ results (ng/ml) recorded for DHC and coumarin haptens were expressed as percent of the HCA response (IFN- γ by LN cells of DHC- and coumarin-sensitized mice/IFN- γ by LN cells of HCA-sensitized mice).

Results and Discussion

DHC, but not coumarin, primes IFN- γ -producing T cells in CD4+ T cell-deficient mice

In vivo priming of hapten-induced T cells was assessed by IFN- γ -ELISA assays performed on LN cells recovered from immunized mice at D3 and restimulated *in vitro* for 20 h with sub-mitogenic doses of PHA, as previously reported (12). IFN- γ secretion induced by DHC and coumarin in several experiments are reported in Table 1. Results are expressed as: (i) raw IFN- γ concentrations in the culture supernatant (ng/ml)

and (ii) percent of the IFN- γ production by LN cells of HCA-sensitized mice.

DHC induced significant IFN- γ production by LN cells in 3 out of 4 experiments, representing 22, 36, and 75% of that induced by HCA. In contrast, only very low amounts of IFN- γ were produced by LN cells of coumarin-sensitized mice. The values obtained in the 4 experiments (3, 6, 7, and 3% of the HCA response) were in the range of the background values obtained for dimethylformamide and acetone/olive oil, the 2 vehicles used to dissolve coumarin and DHC.

We therefore conclude that pure coumarin is devoided of sensitizing potency as it is unable to prime IFN- γ -producing T cells. These results are in line with the LLNA studies we have recently obtained showing that pure coumarin is unable to stimulate LN cells (10).

DHC, but not coumarin, induces a potent ACD in CD4+ T cell-deficient mice

We next investigated the ability of pure coumarin and DHC to induce an ACD in CD4+ T cell-deficient mice using the standard MEST. C57BL/6 mice were sensitized (50% concentration) for 3 consecutive days (D-2, D-1 and D0) by skin painting on the abdomen, and challenged on the ear 5 days later by a non-irritant concentration of chemical (40%).

DHC induced a classical ACD response, which peaked at 24 hr post-challenge (Fig. 1). In contrast, no ACD reaction could be obtained with pure coumarin even when administered at high concentrations over 3 consecutive days. These *in vivo* results are in agreement with the IFN- γ -production by LN cells and show that coumarin cannot prime T cells and sensitize animals even in a maximization test using mice deficient in

Table 1. *In vivo* priming of chemical-specific T cells

		Chemical									
		HCA		Dihydrocoumarin		Pure coumarin		Dimethylformamide		Acetone/olive oil	
		IFN- γ secretion									
		ng/ml	% HCA response	ng/ml	% HCA response	ng/ml	% HCA response	ng/ml	% HCA response	ng/ml	% HCA response
Experiment	1	25.4	100	3.55	14	0.8	3	0.3	1		
	2	72.9	100	16.1 ^a	22 ^a	4.1	6	2.9	4		
	3	81.1	100	29.4	36	5.3	7	1.3	2		
	4	28.9	100	21.7 ^a	75 ^a					0.1	0.3
	5	10.9	100			0.3 ^a	3 ^a			0.3	3

HCA, hexylcinnaldehyde; PHA, phytohemmagglutinin.

CD4+ T cell-depleted BALB/c mice were sensitized on both ears for 3 consecutive days (D-2, D-1 and D0) by different chemicals. HCA was tested at 25% concentration. Pure coumarin and dihydrocoumarin were tested at 25%^a or 50%. At D3, draining lymph node cells were collected and cultured *ex vivo* in presence of 5 μ g/ml PHA for 20 hr. IFN- γ (interferon gamma) secretion induced by the different chemicals was compared with that obtained with HCA, and the results were expressed as percent of the HCA response.

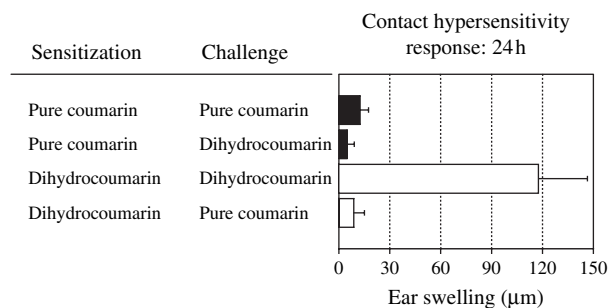


Fig. 1. Allergic contact dermatitis to pure coumarin and dihydrocoumarin (DHC). CD4⁺ T cell-depleted C57BL/6 mice were sensitized by 3 applications of pure coumarin (black bars) or DHC (white bars) at D-2, D-1 and D0 and challenged at D5. Results are expressed as the mean ear swelling $\pm$ SD at 24 hr post-challenge. They are representative of 3 different experiments with 4 mice per group.

Treg cells which are appropriate for the detection of weak fragrance allergens. It is noteworthy that no cross-reactivity could be detected between DHC and coumarin inasmuch as: (i) DHC-sensitized animals failed to develop ACD response when coumarin was used for challenge; (ii) no ACD reaction was observed after DHC challenge of coumarin-sensitized mice. It would be interesting to determine whether this lack of cross-reactivity between these 2 compounds is correlated to the lack of pro-inflammatory properties of pure coumarin, which are essential for the recruitment of effector CD8⁺T cells at the site of challenge, or is because of genuine differences in the antigenic structures responsible for the activation of effector T cells.

Conclusions

The present study shows that pure coumarin cannot prime T cells and is unable to induce an ACD reaction in an optimized mouse model of fragrance allergy. This model was reported to be sensitive enough to detect the sensitizing properties of hexylcinnamaldehyde, eugenol, and hydroxycitronellal. That coumarin is unable to induce sensitization in this model strongly supports the hypothesis that coumarin is endowed with very weak sensitizing capacities if any. This report further emphasizes that the purity of a chemical is a major parameter for its sensitizing and allergenic properties. Indeed, some coumarin derivatives are known contact allergens and we provide here evidence that DHC is able to induce ACD in mice. Therefore, manufacturers should precisely check the purity of the chemical they

propose to introduce in preparations used for either patch testing or for cosmetics in order to prevent risks of skin sensitization and allergy.

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