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INVESTIGATIVE REPORT

Inhibition of the Sensitizing Effect of Carvone by the Addition of Non-Allergenic Compounds

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We have previously reported a reduction of sensitization to carvone in guinea pigs when adding non-allergenic, structurally related compounds simultaneously at induction. This study investigates the criteria needed to obtain a reduction of sensitization in contact allergy. Linalool, a non-sensitizer structurally unrelated to carvone, significantly reduced the sensitizing capacity of carvone in guinea pigs. The effect of different concentrations of inhibitors in mixtures with carvone was investigated. No significant differences in response were obtained between the concentrations explored. A possible anti-inflammatory effect from the inhibitory chemicals was investigated *in vitro*. No suppression of the immune system was seen. This study shows that a non-allergenic compound with a structure not resembling the hapten can reduce the sensitizing effect of the hapten. It indicates that reduction of an allergenic effect might occur in consumer products that are mixtures of different chemicals. Further studies with chemically unrelated compounds with and without allergenic effect are needed. *Key words: anti-inflammatory effect; contact allergy; guinea pigs; quenching; skin.*

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Carvone (Fig. 1) is a contact allergen commonly used as a flavouring agent in toothpaste and chewing gum (1–3). In a previous study in guinea pigs, we found that the allergenic activity of *R*-carvone was significantly reduced when non-allergenic, structurally related analogues were added to carvone in the induction phase (4). The method used was a traditional guinea-pig method for predictive testing of the sensitizing capacity of fragrance compounds, the Freund's Complete Adjuvant Test (FCAT) method (5).

Carvone forms antigens after a nucleophilic attack at the β-carbon (6). This has been confirmed using two structural analogues of carvone, 2*R*,5*R*-dihydrocarvone and *R*-methylcarvone (Fig. 1) with no ability to react with nucleophiles in the β-position. Since the analogues still contain a reactive carbonyl group capable of forming Schiff's bases with primary amines, their sensitizing capacity was tested. Both analogues were shown to be non-sensitizers, indicating that the antigen formation via Schiff's base formation is of minor importance (6). The analogues have an overall structure similar to that of carvone (Fig. 1) and log P values that suggest a similar skin penetration. When 2*R*,5*R*-dihydrocarvone or *R*-methylcarvone was added to carvone in a 2:1 ratio at the induction phase a significantly reduced allergenic activity of carvone was observed. At challenge-testing in carvone-sensitized animals, no difference was obtained when using the mixture compared to when using carvone alone, showing that the inhibition is efficacious only in the induction phase (4).

Over the years, extensive studies have been carried out on the concept of antigenic competition. Strong experimental haptens have been used, such as oxazolone, dinitrochlorobenzene and picryl chloride (7, 8 and refs therein). A significant inhibition of contact sensitization to one allergen is observed in the groups of animals epidermally pretreated with another allergen

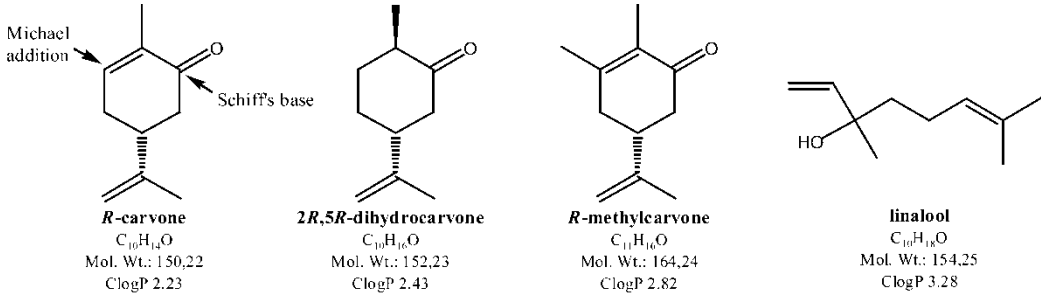

Fig. 1. Structures of compounds studied. The reactive site for antigen formation by a Michael reaction at the β-carbon or Schiff's base formation at the carbonyl carbon is indicated.

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before induction, compared to animals pretreated with vehicle alone (8, 9). In contrast to these studies, our previous study (4) demonstrates an inhibitory effect of a non-allergenic compound on a moderate allergen commonly used. Furthermore, the animals are exposed to the inhibiting compound and the allergen simultaneously in a mixture at induction (4).

Abrogation of the sensitizing effect from specific chemicals by the addition of other specific chemicals is stated in the guidelines from the International Fragrance Industries Association (IFRA) (10). Based on investigations presented in the middle of the 1970s (11), these guidelines state that the sensitizing capacity of certain fragrance aldehydes (cinnamal, citral and phenylacetaldehyde) is "quenched" by the addition of certain other fragrance chemicals (eugenol, limonene and dipropyleneglycol). However, in studies performed by other investigators using the sensitizers and their quenchers in stipulated ratios it was not possible to confirm the results (12).

The mechanism of the inhibitory effect of the carvone analogues on carvone sensitization cannot readily be explained. It could be speculated that the analogues hinder the antigen formation from occurring as a result of structure similarity to carvone. Since both analogues used were non-sensitizers, a competitive interaction via a covalent bond formation must be considered unlikely; instead, the analogues might interact non-covalently. Furthermore, we have to consider that the analogues were added in excess and thereby might temporarily have blocked the binding site of carvone by a dilution effect. Another possibility is that the analogues induced an antigen non-specific anti-inflammatory response, thereby suppressing the immune reaction against carvone.

The aim of the present study was to investigate the inhibitory effect on carvone sensitization using a chemically unrelated compound. The non-sensitizing monoterpene linalool (Fig. 1) (13) with a branched flexible carbon chain, an alcohol function and a log P value similar to that of carvone was chosen. In addition, the effect of the concentrations of the inhibitors was investigated using linalool and *R*-methylcarvone. The investigations were performed in guinea pigs using the same method as in our first study (4, 5). Furthermore, a potential down-regulatory effect of carvone analogues and linalool on immune reactivity was analyzed *in vitro* using murine lymphocytes.

MATERIAL AND METHODS

Chemicals

(*R*)-(-)-Carvone (98%) [6485-40-1] was purchased from Aldrich Chemical (Stockholm, Sweden). Linalool (97%) [78-70-6] was purchased from Lancaster Synthesis Ltd (Eastgate, Lancashire, UK). Linalool was distilled under vacuum prior to animal experiments. No contaminants were detected with gas chromatographic (GC) analysis. *R*-Methylcarvone [85710-65-2] and 2*R*,5*R*-dihydrocarvone [5524-05-0] were synthesized as previously described (6, 14, 15). Freund's complete (FCA) and incomplete (FIA) adjuvants were obtained from Difco (Detroit, MI, USA). White non-stabilized petrolatum was obtained from VWR International (Stockholm, Sweden). Fetal calf serum (FCS) was purchased from Biological Ind. (Beit Haemek, Israel), concanavalin A (ConA) from ICN Biomedicals, Inc. (Aurora, OH, USA), [^{3}H]-thymidine from Amersham (Buckinghamshire, UK), IFN-γ monoclonal antibodies and recombinant IFN-γ from PharMingen (San Diego, CA, USA) and streptavidine alkaline phosphatase from DAKO (Glostrup, Denmark). L-Glutamine, lipopolysaccharide (LPS), gentamicin, bovine serum albumine (BSA), iscove's modified Dulbecco's medium and 4-nitrophenylphosphate tablets were purchased from Sigma (Stockholm, Sweden). Cytokine ELISA kits for TNF-α (MTA00) and IL-13 (M1300C) were purchased from R&D systems (Abingdon, UK). Other chemicals used were of pharmaceutical or analytical grade.

Calculations

Log P values (Fig. 1) were calculated using the computer program ACD Log P DB from Advanced Chemistry Development. Conformational analysis of carvone and linalool were performed using a 1000 (carvone) and 5000 (linalool) step Monte Carlo conformational search with the BatchMin program and the MM3 force field, as implemented in the MacroModel program version 7.0. All conformers with a relative steric energy <13 kJ/mol were saved and analyzed (Fig. 2).

Experimental animals

Female, outbred, albino Dunkin Hartley guinea pigs weighing 250–300 g were purchased from Bio Jet service (Uppsala, Sweden). The guinea pigs were housed in Macrolon cages, kept on a guinea pig standard diet and water *ad lib*. Female C57BL/6 mice weighing 20–30 g were purchased from B-K Scanbur (Sollentuna, Sweden). The mice were housed in individually ventilated cages (BioZone, Margate, UK) and kept on standard laboratory diet and water *ad lib*. The local ethics committees in Stockholm and Gothenburg approved the studies.

Effect of linalool on R-carvone sensitization

The FCAT (5) on guinea pigs was used. On days 0, 6 and 9 the animals received one intradermal injection (0.1 ml) on the upper back for induction. At the first injection the animals

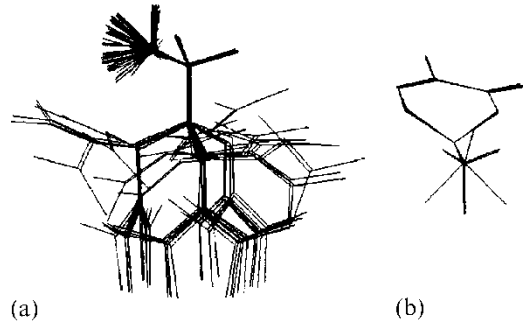

Fig. 2. Conformational analysis of linalool (a) and carvone (b). A total of 88 low-energy conformers were found for linalool and six for carvone. The lowest energy conformations (<2 kcal/mol) are shown. These correspond to 45 conformers for linalool (a) and 3 for carvone (b). The hydrogen atoms have been removed for clarity.

received the test material in an FCA/H_2O emulsion, while an FIA/H_2O emulsion was used at the second and third inductions, which was in accordance with recommendations from the ethics committee in Stockholm. The compounds in the concentrations used for induction and challenge were shown to be non-irritating in pretests on FCA-treated and non FCA-treated guinea pigs.

Three groups with 14 animals in each were used. The first group was injected with *R*-carvone 3.3×10^{-4} mol/g (5% w/w) in FCA(FIA)/H_2O emulsion (1:1). The second group was injected with a mixture of *R*-carvone and linalool in concentrations 1:2 (3.3×10^{-4} mol/g: 6.7×10^{-4} mol/g) in FCA(FIA)/H_2O emulsion (1:1). The third group was a control group sham-treated with an FCA(FIA)/H_2O emulsion. Closed challenge testing was performed on day 19 (16). The test material (0.015 g) was applied on the shaved flanks of the animals for 24 h using alumina test chambers (Finn Chambers®, i.d. 8 mm; Epitest Tuusla, Finland). The reactions were assessed at 48 h and 72 h after application. The minimum criterion for a positive reaction was a confluent erythema. The test concentrations of *R*-carvone were chosen according to earlier experiences (1, 4). All animals were challenged with *R*-carvone 3.3×10^{-5}, 1.3×10^{-5}, 0.67×10^{-5}, 0.47×10^{-5}, 0.33×10^{-5} and 0.13×10^{-5} mol/g (0.5, 0.2, 0.1, 0.07, 0.05, 0.02%), with linalool 3.3×10^{-4} mol/g (5.13%) and with a vehicle control. Petrolatum was used as vehicle for all test materials.

Rechallenge. On day 48, all animals were retested with *R*-carvone 6.7×10^{-5}, 3.3×10^{-5}, 1.3×10^{-5}, 0.67×10^{-5}, 0.47×10^{-5}, 0.33×10^{-5} and 0.13×10^{-5} mol/g (1.0, 0.5, 0.2, 0.1, 0.07, 0.05, 0.02%) and with petrolatum.

Effect of different inhibitor concentrations on R-carvone sensitization

R-Methylcarvone and linalool (Fig. 1) were used as inhibitors in this experiment, which was performed as described above. Five groups of animals were used. For induction: group 1 ($n = 10$) was injected with *R*-carvone 3.3×10^{-4} mol/g, group 2 ($n = 11$) was injected with a mixture of *R*-carvone and *R*-methylcarvone in concentrations 1:1 (3.3×10^{-4} mol/g: 3.3×10^{-4} mol/g), group 3 ($n = 11$) was injected with *R*-carvone and *R*-methylcarvone in concentrations 1:2 (3.3×10^{-4} mol/g: 6.7×10^{-4} mol/g), group 4 ($n = 11$) was injected with *R*-carvone and linalool in concentrations 1:1 (3.3×10^{-4} mol/g: 3.3×10^{-4} mol/g) and group 5 ($n = 11$) with *R*-carvone and linalool in concentrations 1:2 (3.3×10^{-4} mol/g: 6.7×10^{-4} mol/g). All animals were challenged with *R*-carvone 6.7×10^{-5}, 3.3×10^{-5}, 1.3×10^{-5}, 0.67×10^{-5}, 0.47×10^{-5}, 0.33×10^{-5}, 0.13×10^{-5} mol/g and with petrolatum as vehicle control.

In vitro exposure of splenocytes with R-carvone or the inhibitors

A single cell preparation of murine splenocytes was performed as previously described (17). Spleen cells (1×10^6/ml) were incubated in 96-well (for proliferation experiments) or 24-well (for cell supernatants) flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) in volumes of 0.2 or 1 ml, respectively. Iscove's medium (supplemented with 10% FCS, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and 50 μg/ml gentamicin) was used. Solutions of *R*-carvone, 2*R*,5*R*-dihydrocarvone, *R*-methylcarvone or linalool (33 mM) in dimethylsulphoxide (DMSO) were filtered through a 0.22 μm sterile filter (Acrodisc® syringe filters, Pall Corporation, Ann Arbor, MI, USA). The filtrates were diluted with medium to give final concentrations of 5, 50 and 500 μM. Control cultures were exposed to DMSO in corresponding concentrations. ConA or LPS was included at a final concentration of 0.6 μg/ml or 5 μg/ml, respectively. Cultures incubated without mitogens were also investigated. After 48 h the cell supernatants were collected and centrifuged to discard cell debris. The supernatants were stored at −20°C until use.

Lymphocyte proliferation

Spleen cells (1×10^6/ml) were incubated for 72 h as described above. For the final 18 h, 1 μCi [^{3}H]-thymidine was added to each well. The cultures were harvested into glass-fibre filters and counted in a β-scintillation counter (Matrix™ 96 Packard). The different treatments were set up in triplicate and the results were expressed as the mean of counts per minute (cpm).

Cytokine production

Enzyme-linked immunosorbent assay (ELISA) was used to measure cytokines in the supernatants. For detection of IFN-γ in 48 h supernatants, 96-well flat-bottomed plates (Nunc Maxisorp) were coated with a capture rat anti-mouse IFN-γ monoclonal antibody (2 μg/ml in 0.05 M carbonate buffer, pH 9.6) overnight at 4°C in a moist chamber. After being washed with a solution of 0.05 M tris(hydroxymethyl)-aminomethane (Tris): 0.05% Tween 20 (Tween), the plates were blocked for 1 h with BSA (1%) in Tris (0.05 M, pH 7.4) at 37°C. After the blocking solution was discarded, solutions with recombinant mouse IFN-γ diluted in twofold steps in Tris (0.05 M):NaCl (0.015 M) (pH 7.4) were added to the unwashed wells to obtain a standard curve. Supernatants were added undiluted. Plates were incubated for 2 h at 37°C, washed with Tris:Tween, and 2 μg/ml of biotinylated rat anti-mouse IFN-γ monoclonal detection antibody diluted in Tris:NaCl was added and left overnight at 4°C in a moist chamber. After being washed, streptavidine alkaline phosphatase diluted in Tris:NaCl to a final concentration of 0.2 μg/ml was added and the mixture was left for 1 – 1.5 h at room temperature. Alkaline phosphatase substrate (4-nitrophenyl phosphate) dissolved in diethanolamine buffer (1 mg/ml, pH 9.8) was added after washing. The plates were read within 1 h at 405 nm in a spectrophotometer (SPEKTRAmax PLUS, Molecular Devices Corporation, Sunnyvale, CA, USA).

Cytokine ELISA kits were used for detecting TNF-α and IL-13 in supernatants. The analyses were performed following the instructions of the producer.

Statistics

A difference in response between exposed guinea pigs and controls was evaluated using the Fisher exact test. The two-factor analysis of variance was used for evaluation of an inhibitory effect.

RESULTS

Effect of linalool on R-carvone sensitization

The group of animals induced with a 1:2 mixture of *R*-carvone and linalool showed a significantly lower response than the group induced with *R*-carvone alone, when challenge-tested with *R*-carvone 3.3×10^{-5}, 1.3×10^{-5} and 0.47×10^{-5} mol/g (Table I).

Compared to the response in control animals, a significant response was observed for all concentrations

of carvone except 0.33×10^{-5} and 0.13×10^{-5} mol/g in the group induced with carvone alone (72 h reading, Table I), while a significant response was seen only for the highest concentration (3.3×10^{-5} mol/g, 72 h reading, Table I) in the group induced with the mixture of carvone and linalool. One positive reaction was observed for linalool (72 h) in the group exposed to the mixture. No positive reactions were seen in the control animals.

Rechallenge. A significant difference ($p < 0.0001$) was observed between the response rates to carvone of the group induced with the mixture (carvone + linalool 1:2) compared to the group induced with carvone alone (Fig. 3). Compared to the response in the controls, a significant difference ($p < 0.05$) was seen for all concentrations of carvone except the lowest (0.13×10^{-5} mol/g) in the group induced with carvone alone, while the group induced with the 1:2 mixture showed a significant response ($p < 0.05$) only for the highest concentrations (6.7×10^{-5} and 3.3×10^{-5} mol/g). No positive reactions were seen in the control animals.

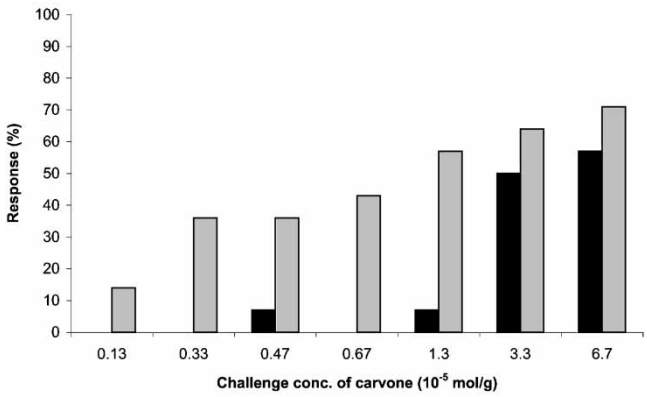

Fig. 3. Inhibition with linalool, results at rechallenge (72 h reading). Response rates (%) to *R*-carvone in guinea pigs induced with *R*-carvone alone or *R*-carvone in mixture with linalool 1:2. A significant difference in the response rates ($p < 0.0001$) was seen between the animals induced with the mixture (carvone:linalool 1:2) (■) compared to those induced with *R*-carvone alone (▒).

Effect of different inhibitor concentrations on R-carvone sensitization

All exposed animal groups were sensitized to carvone (Fig. 4). However, the groups induced with mixtures of carvone and linalool or methylcarvone all showed a lower response rate compared to the group induced with carvone alone. The significance was calculated giving: group 2 (carvone:methylcarvone 1:1; $p < 0.01$), group 3 (carvone:methylcarvone 1:2; $p < 0.0001$), group 4 (carvone: linalool 1:1; $p < 0.01$) and group 5 (carvone:linalool 1:2; $p < 0.001$) (Fig. 4). No significant differences were obtained between the response rates when the inhibitors were added in excess compared to when added in concentrations equimolar to carvone (group 3 vs. group 2 and group 5 vs. group 4, respectively; Fig. 4).

Table I. *The inhibitory effect of linalool on R-carvone sensitization*

Test material in challenge (mol/g × 10^{-5})		Animals induced with	
		R-carvone	*R*-carvone + linalool (1:2)
R-carvone (3.3)	48 h	9[a, b]	3[a, e]
	72 h	9[b]	7[c]
R-carvone (1.3)	48 h	6[c]	1[f]
	72 h	7[c]	3
R-carvone (0.67)	48 h	3	0
	72 h	5[d]	1
R-carvone (0.47)	48 h	2	0
	72 h	4[d]	0[g]
R-carvone (0.33)	48 h	0	0
	72 h	2	0
R-carvone (0.13)	48 h	0	0
	72 h	0	0
Linalool (33)	48 h	0	0
	72 h	0	1
Petrolatum	48 h	0	0
	72 h	0	0

[a]The results are given as number of animals responding in the exposed groups ($n = 14$, respectively).
[b]p(exposed/controls) < 0.001.
[c]p(exposed/controls) < 0.01.
[d]p(exposed/controls) < 0.05.
[e]$p = 0.024$, [f]$p = 0.036$ and [g]$p = 0.049$ compared with animals induced with carvone.

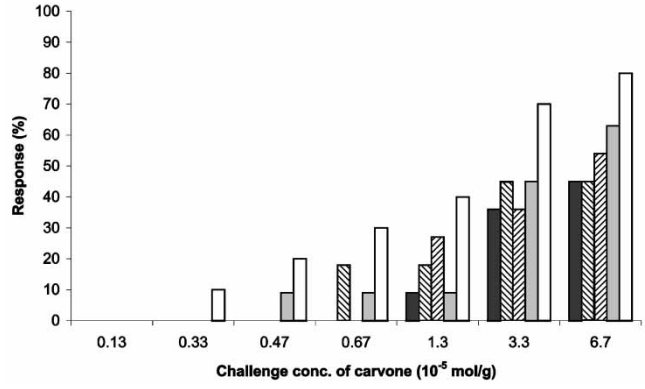

Fig. 4. Effect of different inhibitor concentrations on *R*-carvone sensitization, results at challenge (72 h reading). Response rates (%) to *R*-carvone in animals induced with carvone (group 1; □), carvone + linalool 1:1 (group 4; ▒) ($p < 0.01$), carvone + linalool 1:2 (group 5; ▨) ($p < 0.001$), carvone + methylcarvone 1:1 (group 2; ▧) ($p < 0.01$) and carvone + methylcarvone 1:2 (group 3; ■) ($p < 0.0001$). A significant decrease (p values given in parentheses) was seen in the response rates for all groups induced with the mixtures compared to that for the group induced with carvone alone. No significant difference was obtained with the various concentrations of the inhibitors.

Lymphocyte proliferation

The proliferative response of spleen cells was not affected by the addition of *R*-carvone nor of the inhibitors. The addition of mitogens increased the proliferative response of the cells, but an addition of carvone, the analogues or linalool did not affect the overall proliferative response (data not shown). At the highest dose (500 μM), a decrease in the proliferative response was seen, probably as a consequence of the high DMSO concentration.

Cytokine production

Neither the inhibitors nor *R*-carvone affected the IFN-γ, IL-13 or TNF-α levels in supernatants of naive splenocytes or splenocytes triggered with mitogens. The highest levels of IFN-γ were detected in ConA stimulated cultures, whereas the TNF-α levels were highest in cultures stimulated with LPS. IL-13 was readily detected only in ConA stimulated cultures. Also for the cytokine production, a toxic effect was seen for the highest dose of DMSO (data not shown).

DISCUSSION

Our findings indicate that compounds with no allergenic activity can reduce the sensitizing effect of a contact allergen. The structure similarity between the inhibitor and the allergen appears to be of minor importance. By adding non-allergenic structural analogues to carvone we have shown in a previous study in guinea pigs (4) that it is possible to reduce the sensitizing capacity of carvone. The present study demonstrates that linalool with a structure unrelated to carvone can reduce the sensitizing capacity of carvone and that the inhibitors do not need to be added in excess. By determining lymphocyte proliferation and cytokine production we have also demonstrated that the reduction of carvone sensitization cannot be explained by a general immunosuppressive effect exerted by the inhibitory chemicals.

In our first study (4), we found that 2*R*,5*R*-dihydrocarvone and *R*-methylcarvone (Fig. 1) could significantly reduce the sensitizing effect of carvone. Since both the carvone analogues and linalool are non-sensitizers (6, 13), a competitive inhibition for antigen formation is unlikely. We have speculated that antigen formation might be hindered by the analogues dihydrocarvone and methylcarvone due to the structure similarities with carvone. However, this seems unlikely, since also linalool can significantly reduce the sensitizing effect of carvone. If a compound hinders an interaction between carvone and the reactive site of a macromolecule, the structure of carvone and the interfering compound should be related. Carvone and linalool contain too many structural and geometrical differences and therefore cannot be expected to compete for the same binding site. Linalool is a monoterpene with the same number of carbon atoms and a molecular weight similar to that of carvone. However, linalool consists of a flexible branched carbon chain with an alcohol function in contrast to carvone, which contains a six-membered ring and a carbonyl group. The 3D structure and chemical properties of the molecules are thereby different (Fig. 2). Under the conditions investigated, the inhibitors will not react with carvone and thereby change the allergenicity of this hapten.

In our previous study (4) the ratio between carvone and the analogues was 1:2 in the mixture used for induction. To rule out that our findings were a result of a dilution effect, that is, analogues temporarily blocking the binding site of carvone, we tested the sensitizing capacity of carvone when methylcarvone or linalool was added to carvone in the ratios 1:1 and 1:2. All four animal groups induced with mixtures of carvone and inhibitor (groups 2, 3, 4 and 5; Fig. 4) showed a significantly lower response rate compared to the group induced with carvone alone (group 1). However, there was no significant difference in response between the groups induced with the 1:2 mixture compared to those induced with the 1:1 mixture (group 2 vs. 3 and group 4 vs. 5). This indicates that an excess of the inhibitor does not cause the inhibitory effect.

An important factor for sensitization is the skin penetration capacity of a compound. It is considered that lipophilic compounds penetrate the skin more easily than hydrophilic compounds (18). The partition coefficient between octanol and water (log P) was calculated in order to estimate the skin penetration. The log P values for carvone and its analogues are about 2.5, while the value for linalool is slightly higher, 3.3. In FCAT (5), compounds to be investigated are injected intradermally at induction, which diminishes the importance of the difference in lipophilicity. However, some investigations (19) indicate that a difference in the uptake route of the compound and in the formation and presentation of the antigen is dependent on the lipophilicity of the compound.

Corticosteroids are traditionally used for treatment of allergic contact dermatitis owing to their anti-inflammatory effect. The corticosteroids can also act as contact allergens, which is difficult to detect since the anti-inflammatory effect can mask the elicitation (20). Also non-steroid compounds with an anti-inflammatory effect are described in the literature (21–23). Since the inhibition observed in the present study might be due to an anti-inflammatory effect, a possible decrease in proliferation and cytokine levels in inhibitor-exposed cell culture supernatants was investigated. The cells were stimulated either with LPS (stimulating macrophages and B cells) or ConA (mainly a T cell mitogen). The different test compounds affected neither the

proliferative response nor the cytokine production of the cells.

The concept that one chemical in a mixture might reduce the allergenic activity of another has been debated and investigated since the first publication on quenching 27 years ago (11). That study reports quenching, i.e. total abrogation, of the allergenic effect in a human maximization test (24, 25) of three fragrance aldehydes (cinnamal, citral and phenylacetaldehyde) by the presence of certain other fragrance chemicals (eugenol, limonene and dipropyleneglycol). During the years, this observation has been reinvestigated using the stipulated quenchers in different experiments (12). No quenching, not even a reduction of the sensitizing potential, could be observed in these experiments. However, no considerations regarding the structure similarity and sensitizing capacity of the quenching chemicals were made. Basketter & Allenby (26) presented an extensive work where they investigated the allergic response in guinea pigs of two of the three quenching pairs, cinnamic aldehyde and citral in combinations with eugenol or limonene. They studied different dose levels, dosing routes and the use of FCA in relation to quenching. However, these experiments did not include dose-response evaluations, neither in induction nor in challenge. When using only one concentration of either test chemical, a possible decrease in the allergic response is difficult to detect. In our studies, the guinea pigs are challenged with a serial dilution of carvone and the dose-response rates between the different groups are compared.

The effect of limonene on citral sensitization has been investigated on both a macroscopic and a microscopic level (27), with groups of guinea pigs induced with citral alone or with citral in a mixture along with limonene, according to the FCAT method (5). After an open epicutaneous test, no difference was observed in the response of the group induced with the citral:limonene mixture compared to the group induced with citral alone, when challenge-tested with citral or the mixture (27). At the microscopic level, stronger reactions towards citral in animals induced with citral alone compared to reactions in animals induced with the mixture were observed. However, these investigations were performed in few animals and the reports are not conclusive (27).

In a recent study presented by the Research Institute for Fragrance Materials (28), the effects of limonene on citral sensitization and of phenylethyl alcohol on phenylacetaldehyde sensitization were investigated using the human maximization test (17, 18) and a human repeated insult patch test (29). No sensitization was obtained from either mixture. However, data when applying either allergen alone were not presented.

In studies on antigenic competition, Kimber et al. (9) demonstrated a hapten non-specific decrease in the allergenic response in mice pretreated with oxazolone before induction and challenge with picryl chloride and vice versa. The effect measured as ear swelling was transient, lasting no longer than 10 days after the pretreatment. Our investigations demonstrated a longer-lived inhibition since a significant decrease of the response to carvone was still obtained at rechallenge on days 49 (4) and 48 (present study) after induction for all studied inhibitor-carvone mixtures.

The mechanism behind the reduction of carvone sensitization remains unclear. Further *in vivo* and *in vitro* studies using other chemically unrelated compounds with and without allergenic effect are needed. In future studies, experiments using the murine local lymph node assay (LLNA) (30) should be of interest. The method seems suitable for this kind of experiment, since inhibition according to prior experiments (4) works only at the induction phase. Previous experiments regarding inhibition of sensitization have been performed using the LLNA (31). The quenching pairs cinnamic aldehyde:eugenol and citral:limonene were investigated. Different mixtures for induction were used and compared to groups induced with cinnamic aldehyde or citral alone, but no details about the experiments are presented (24). No reduction of the sensitizing capacity of the allergens was observed.

The mechanism of induction in contact allergy is complicated and the impact of the chemical properties of a hapten on the antigen formation and the immunogenic response is still unclear (19). Our study shows that the sensitizing effect of a hapten can be reduced by a structurally unrelated compound when the hapten and the inhibitor are administered in a mixture at induction. Since consumers are exposed to allergens in mixtures, a provocative thought is that not only do we find a synergetic effect of allergens but also a reduction due to the influence of other chemicals present in the mixture.

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