



Assessment of sensitization potential of monoterpenes using the rat popliteal lymph node assay

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Abstract

The popliteal lymph node assay (PLNA) has been proposed as a screening test for detecting chemicals with potential of inducing allergic and auto-immune-like reactions in humans. In the present study, we used the rat PLNA to evaluate the immuno-sensitizing potential of 10 monoterpenes found in the essential oils of a variety of aromatic, edible and medicinal plants. The primary or direct PLNA was performed with the monoterpenes, and chlorpromazine (CPZ) and barbitol were used as positive and negative controls, respectively. Female, 7–8 week-old Wistar rats were injected subcutaneously (50 μ L) with the test substance (0.5, 2.5 or 5 mg) into the right hind footpad while the contralateral footpad was injected with the vehicle (DMSO) alone. Weight (WI) and cellularity (CI) indices for draining PLNs were determined 7 days after treatment. PLNA was positive (WI $\geq$ 2 and CI $\geq$ 5) for CPZ, citral, α -terpinene, β -myrcene and (–)- α -pinene, and negative for barbitol, DMSO, (–)-menthol, 1,8-cineole, ($\pm$) citronellal, (+)-limonene, ($\pm$) camphor and terpineol. A secondary PLNA, a T-cell priming test, was carried out with the four substances that had been positive in the primary assay. Six weeks after being locally primed with 5 mg/paw, rats were sc injected into the same footpad with a dose (0.5 mg/paw) of the substance that had been previously found to be insufficient to cause a positive response. WI and CI were then calculated 4 and 7 days after the second injection. CPZ was also positive in the secondary assay thereby confirming that it is a sensitizing agent. Citral, α -terpinene, β -myrcene and (–)- α -pinene, however, were negative in the secondary assay. In summary, citral, α -terpinene, β -myrcene and (–)- α -pinene induced a clear immuno-stimulatory response due to their irritant properties but no monoterpene proved to be a sensitizing agent in the PLNA.

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1. Introduction

Adverse effects of xenobiotic compounds on the immune system appear either as immuno-suppression leading to enhanced susceptibility to infections and cancer, or as immuno-stimulation and immuno-sensitization leading to auto-immune-like reactions and hypersensitivity responses in genetically predisposed individuals (Burns-Naas et al., 2001; Descotes, 1999). Potential for chemicals to induce immuno-suppression is usually revealed by routine repeated-dose toxicity assays and challenge tests. Immuno-stimulation reactions with systemic administration of drugs, however, are much more difficult to anticipate through in vitro and in vivo animal tests (Descotes, 1999). Owing to this fact, unexpected high frequencies of

Abbreviations: PLNA, popliteal lymph node assay; PLN, popliteal lymph node; WI, weight index; CI, cellularity index; IPR, individual positive response; CPZ, chlorpromazine; DMSO, dimethylsulfoxide; PBS, phosphate buffer saline; s.c., subcutaneous route.

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hypersensitivity-type adverse reactions are among the reasons why newly introduced drugs are withdrawn from the market. Immune-mediated hypersensitivity (or allergic) reactions are also among the most frequent health hazards posed by food additives, cosmetics, environmental pollutants, pesticides and industrial chemicals.

The popliteal lymph node assay (PLNA) in rats or mice has been considered as a promising screening test for the potential of low-molecular-weight chemicals to cause hypersensitivity and auto-immune-like reactions in humans (Goebel et al., 1996; Ravel and Descotes, 2005; Pieters et al., 2002). PLNA was introduced in toxicity testing by Gleichmann (1981) who had noted that subcutaneous injection of diphenylhydantoin into the mouse hind footpad induced a marked enlargement of the homolateral popliteal lymph node, a reaction very similar to that that had been previously described by Ford et al. (1970) using a rodent model of local Graft versus Host (GvH) reaction. Further studies showed that a number of drugs and chemicals known to elicit systemic auto-immune reactions in humans gave positive responses in the PLNA. Moreover, Vial et al. (1997) also showed that different laboratories testing blindly the same set of substances obtained consistent and reproducible results. Although the predictive value of PLNA was initially focused on auto-immune-like reactions, more recently it has been regarded as a screening test useful for detecting a broader class of sensitizing or immuno-stimulating chemicals (Pieters and Albers, 1999; Ravel and Descotes, 2005). There are several predictive tests for skin sensitizing potential of chemicals as the (auricular) local lymph node assay in mice, and a variety of guinea pig tests for skin sensitizers. The PLNA, nonetheless, seems to be the only reliable test

for screening chemicals causing sensitization via routes of exposure other than the skin (Goebel et al., 1996; Tuschl et al., 2002).

Monoterpenes are 10-carbon natural compounds derived from two isoprene (C_5H_8) units, the building block of all terpenes. If monoterpenes possess an oxygen atom in a functional group they are also referred to as monoterpenoids. Monoterpenes are major constituents of plant volatile oils and thus they are found in aromatic, edible and medicinal plants (Erikson, 1976; Sticher, 1977). Plant oils and their monoterpenoid constituents have been used as flavouring additives in food and beverages, as fragrance in perfumes and as scent in house-hold products (Sticher, 1977; Leung, 1980). In the industry, monoterpenes are employed as solvents and intermediates in the synthesis of several chemicals. Moreover, they are constituents of a variety of pharmaceutical products as well (Erikson, 1976; Leung, 1980). Notwithstanding the importance of human exposure to monoterpenes, studies on the immuno-sensitizing potential of these plant xenobiotics have seldom been performed.

In the present study, the rat popliteal lymph node assay (PLNA) was used to provide data on the immuno-sensitizing potential of 10 structurally distinct monoterpenes (Fig. 1) found in flavoring food additives, cosmetics, drugs as well as in a variety of other house-hold products.

2. Material and methods

2.1. Chemicals

Citral (CAS 5392-40-5), α -terpinene (CAS 99-86-5), β -myrcene (CAS 123-35-3), (–)- α -pinene (CAS 7785-26-4), (–)-menthol (CAS 2216-51-5),

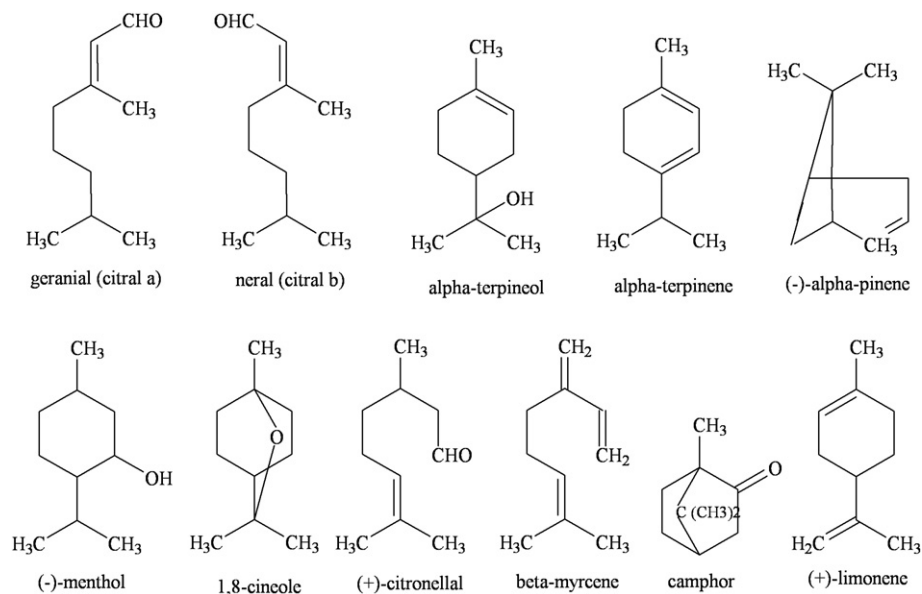


Fig. 1. Chemical structures of monoterpenes tested in the PLNA: terpineol (1-methyl-4-isopropyl-1-cyclohexen-8-ol); alpha-terpinene (1-methyl-4-propen-2-yl-cyclohexa-1,3-diene); (–) alpha-pinene ((1S,5S)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene); (–) mentol (5-methyl-2-propen-2-yl-cyclohexan-1-ol); 1,8 cineole or eucalyptol (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane); (±) citronellal (3,7-dimethyl-6-octenal); (+) limonene ((R)-4-isopropenyl-1-methyl-1-cyclohexene); beta-myrcene (7-methyl-3-methyldiene-octa-1,6-diene); camphor (1,7,7-trimethylbicyclo[2.2.1]heptan-2-one). Citral (3,7-dimethyl-2,6-octadienal) is a mixture of two geometric isomers, geranial (citral a) and neral (citral b).

1,8-cineole (CAS 470-82-6), ($\pm$) citronellal (CAS 106-23-0), (+)-limonene (CAS 5989-27-5), ($\pm$) camphor (CAS 76-22-2), terpineol (CAS 98-55-5) and dimethylsulfoxide (DMSO) were all purchased from Sigma Chemical (St. Louis MO, USA). Chlorpromazine (CAS 69-09-0) was from Farmanguinhos-FIOCRUZ (Rio de Janeiro, RJ, Brazil) and barbital (CAS 144-02-5) from Riedel Produtos Químico Farmacêuticos Ltda (Rio de Janeiro, RJ, Brazil). Except for ($\pm$) citronellal (85%), β -myrcene (90%) and α -terpinene (90%), purity of tested substances was 95% or higher.

2.2. Animals

Virgin female, 7–8 week-old Wistar rats (body weight, mean $\pm$ SD, 145 $\pm$ 23 g), from the Oswaldo Cruz Foundation (FIOCRUZ) breeding stock, were used throughout the study. All rats were individually housed in standard plastic cages with stainless steel cover lids and wood shavings as bedding and maintained at a temperature of 23 $\pm$ 1 °C, relative humidity of approximately 70%, and under a 12-h dark/light cycle. A commercial diet for laboratory rats and mice (Nuvital[®], Nuvilab, Curitiba, PR, Brazil) and tap water were provided *ad libitum*. All experiments were conducted in accordance with Brazilian animal protection and welfare laws.

2.3. Primary PLNA

Rats were subcutaneously injected (50 μ L) into the right footpad with a test substance in vehicle (DMSO for monoterpenes, and saline for barbital and CPZ). The contralateral (left) hind footpad was used as the control and thus it was injected with 50 μ L of vehicle alone. When the vehicles were tested for a primary PLNA response, the right hind footpad was injected with 50 μ L of DMSO or saline while the left hind footpad remained untreated. Seven days after footpad injection, rats were killed by CO₂ inhalation. The popliteal lymph nodes (PLN) were carefully removed, placed in phosphate buffer saline (PBS), freed from adherent fatty tissue and weighed. PLNs were then transferred to a glass tube where cells were extracted by agitation and gentle grinding using a micropestle and suspended in a known volume of PBS on ice. PLN cell number (cellularity) was counted using a Neubauer chamber. Results were expressed as weight (WI) and cellularity (CI) indices. WI and CI were calculated by dividing the value (weight or cellularity) obtained for the treated (right) side PLN by that obtained for the control (left) side PLN.

The primary PLNA was conducted in two steps. All substances were initially tested at a dose as high as 5 mg/paw and, to evaluate the existence of a dose–response relationship, those monoterpenes that gave a positive response in the first assay were subsequently tested again at the highest dose (5 mg/paw) and at two lower doses (0.5 and 2.5 mg/paw) as well.

2.4. Secondary PLNA

A secondary PLNA was undertaken to verify whether those substances, which produced a positive response in the primary assay, would also cause T-lymphocyte sensitization (Ravel and Descotes, 2005). The first step of the secondary PLNA was identical to the primary assay, except that rats were not killed on post-infection day 7. Six weeks after being primed locally with a dose of the substance that had produced a positive response (5 mg/paw), rats were challenged into the same footpad with a dose (0.5 mg/paw) that had been previously shown to be insufficient to cause a primary positive response. Rats were killed by CO₂ inhalation either 4 or 7 days after the second injection of the test substance. PLNs were removed, weighed, and cellularity was determined as described for the primary assay. In the second step of the secondary PLNA, a control group received subcutaneous injections of the vehicle into both footpads, i.e. those footpads which had been previously primed with the test substance (right) and also those which had been treated with the vehicle alone (left).

2.5. Criterion for a positive response in the assay

If a test substance has no immuno-stimulatory effect in the PLNA, both WI and CI should be nearly 1. There has been no general agreement,

however, regarding the criterion for a positive response in the assay. As pointed out by Ravel and Descotes (2005), statistical comparisons between substance-treated (homolateral) and vehicle-treated (contralateral) PLN weights or cell numbers detect significant differences even though ratios are only slightly above the unity thereby giving rise to inconsistent conclusions on the substance immuno-stimulating potential. An alternative approach is to consider as positive any response (ratio) higher than the upper limit of the 95% confidence interval calculated for historical data of the laboratory that, as a rule, is lower than 2 for WI and lower than 5 for CI. In the present study, a twofold or higher increase in weight (WI $\geq$ 2) accompanied by a fivefold or higher increase in cellularity (CI $\geq$ 5) was fixed as the criterion for a PLNA positive response. These arbitrarily fixed WI and CI limit values have been used in most studies and are considered robust criteria for a positive response in the primary PLNA (Ravel and Descotes, 2005).

2.6. Analysis of results

Substances were considered as positive in the primary PLNA if, in the treated group, the average WI was $\geq$ 2 and the average CI $\geq$ 5, otherwise the substance was considered as negative in the assay. WI and CI were evaluated by the Kruskal–Wallis test followed by the Mann–Whitney U test, and proportions of individual positive responses (IPR) by the chi-square test or by the Fisher's exact test. In any case a difference was considered as statistically significant when $P < 0.05$.

3. Results and discussion

The PLNA was initially performed with mice but it is currently performed either in young adult rats or mice. According to Jacques Descotes (1999) there is no evidence of differences in response between rats and mice. In this study we used rats because their larger size facilitates dissection and removal of popliteal lymph nodes.

To verify to what extent the vehicles used in this study could induce PLN enlargement, we injected either saline or DMSO (50 μ L) into one hind footpad while the contralateral footpad remained untreated. As shown in Table 1, saline alone and DMSO alone did not elicit group (mean $\pm$ SD) positive responses. Although most rats did not respond to the vehicles, three out of 50 rats (6%) challenged with saline alone and five out of 47 (10.6%) challenged with DMSO alone exhibited positive (WI $\geq$ 2 and CI $\geq$ 5) responses (IPR). A possible explanation for positive responses to saline alone in the primary PLNA is that enlargement of treated side PLN was mediated by an inflammatory reaction due to tissue injury caused by sub plantar injection procedure. According to the PLNA protocol, rats bleeding or exhibiting any other overt sign of injection trauma were excluded from the study. It is possible, however, that in a few cases mild tissue damage in the injection site remained unnoticed by external examination. DMSO alone, on the other hand, produced a proportion of individual positive responses (10.6%) slightly higher than that induced by saline alone (6%) suggesting that it additionally caused local irritation in a small number of more susceptible rats. While some solvents such as ethanol and acetone are known to increase PLN weight due to primary irritancy (Choquet-Kastylevsky and Descotes, 2004), DMSO (up to 10 μ L/paw for mice and 50 μ L/paw for rats)

Table 1
Negative primary PLNA responses with monoterpenes and vehicle controls

	Monoterpenes						Negative control and vehicles		
	(–)-menthol	1,8-cineole	(±)-citronellal	(+)-limonene	(±)-camphor	Terpineol	Barbital	DMSO	Saline
N	9	10	10	10	10	10	8	47	50
WI	1.23 ± 0.41	1.46 ± 0.56	1.85 ± 1.60	1.64 ± 0.54	1.30 ± 0.47	1.32 ± 0.71	1.06 ± 0.36	1.48 ± 0.65	1.12 ± 0.90
CI	1.35 ± 0.74	2.84 ± 1.40	4.42 ± 6.42	3.38 ± 3.23	3.71 ± 6.75	2.00 ± 3.32	1.34 ± 0.92	2.95 ± 3.68	2.04 ± 2.03
IPR, no. (%)	0 (0)	0 (0)	2 (20)	2 (20)	1 (10)	2 (20)	0 (0)	5 (10.6)	3 (6)

Injected doses were either 5 mg/paw (monoterpenes and barbital) or 50 µL /paw (vehicles). Values are means ± SD; Weight (WI) and Cellularity (CI) indices: values for the draining popliteal lymph node of the treated (right paw) side divided by that of the control (left paw) side. IPR, number (%) of rats within the group with WI ≥ 2 and CI ≥ 5. Substances were classified as negative because group mean values for WI and CI were lower than 2 and 5, respectively.

has been generally regarded as a suitable vehicle for PLNA (Descotes et al., 1997). The effects of DMSO alone, nevertheless, had seldom, if ever, been previously examined in studies with the PLNA. In addition to testing the vehicles, negative (barbital) and positive (CPZ) control substances were concurrently tested in the primary PLNA. As expected, response to barbital (5 mg/paw) was negative (Table 1) while CPZ (2.5 and 5 mg/paw) was clearly positive in the primary assay (Table 2). These results were consistent with those that had been obtained for similar doses of these control substances in an inter-laboratory validation study (Vial et al., 1997).

All monoterpenes were initially tested at an arbitrarily fixed high dose (5 mg/paw). It is of note that, as a rule, 5 mg/paw has been the highest dose of a test compound assayed in the rat PLNA (Vial et al., 1997; Descotes et al., 1997). In a pilot study (data not shown) we had previously noted that, at higher doses, some monoterpenes caused pain and local edema. Signs of local pain or inflammatory reaction, however, were not observed with any of the tested monoterpenes after injection of a dose as high as 5 mg/paw. As shown in Table 1, (–)-menthol, 1,8-cineole and (±) camphor produced clearly negative responses in the primary PLNA. Although average WI and CI indices for (±)-citronellal, (+)-limonene and terpineol-treated animals were lower than those required for classifying a substance as positive in the assay, two out of 10 rats (20%) treated with any of the three monoterpenes exhibited individual positive responses (IPR) (Table 1).

As far as we are aware, monoterpenes had not been tested in the PLNA, therefore, the inter-animal variability of PLNA response to these substances cannot be compared among studies. For barbital (negative control) and CPZ (positive control), however, inter-animal variability (coefficient of variation) of weight (WI) and cellularity (CI) indices in our study tended to be higher than that observed in the preliminary inter-laboratory validation study reported by Vial et al. (1997). Although variations in the magnitude of WI and CI were also noted between laboratories involved in the inter-laboratory validation study, all three laboratories identified correctly the test compounds as positive or negative in the primary PLNA. Our study also identified correctly barbital and CPZ as negative and positive compounds, respectively. The source of inter-animal

Table 2
Positive primary PLNA responses with monoterpenes and chlorpromazine

Monoterpene	Dose (mg/paw)		
	0.5	2.5	5.0
<i>Citral</i>			
N	7	8	10
WI	1.22 ± 0.89	1.52 ± 0.63	2.46 ± 1.48*
CI	2.71 ± 3.04	3.31 ± 3.12	13.16 ± 15.68*
IPR, no. (%)	14.3	0.0	40.0
<i>α-Terpinene</i>			
N	7	7	10
WI	1.04 ± 0.70	1.09 ± 0.44	3.42 ± 3.13*
CI	1.31 ± 1.60	1.23 ± 1.11	9.57 ± 12.32*
IPR, no. (%)	0.0	0.0	60.0* ⁺
<i>β-Myrcene</i>			
N	7	8	11
WI	1.32 ± 0.47	1.65 ± 1.20	2.73 ± 1.10
CI	2.01 ± 1.56	4.65 ± 4.64	10.94 ± 8.53*
IPR, no.(%)	0.0	25.0	63.6* ⁺
<i>(–)-α-pinene</i>			
N	8	8	10
WI	1.41 ± 0.91	1.91 ± 0.96	3.17 ± 1.29*
CI	2.88 ± 5.23	2.53 ± 1.64	11.55 ± 9.16*
IPR, no (%)	12.5	0.0	80.0*
<i>Chlorpromazine</i>			
N	4	6	11
WI	1.30 ± 0.35	2.06 ± 0.81*	3.22 ± 1.13*
CI	1.26 ± 0.81	8.49 ± 8.91*	8.28 ± 8.19*
IPR, no. (%)	0.0	50.0	63.6*

Values are Means ± SD; Weight (WI) and Cellularity (CI) indices: value for draining popliteal lymph node of the treated (right paw) side divided by that of the control (left paw) side. IPR, number (%) of rats within the group with WI ≥ 2 and CI ≥ 5. WI and CI were analyzed by the Kruskal–Wallis test followed by the Mann–Whitney U test. % of IPRs were compared by the chi-square test or by the Fisher's exact test. An asterisk (*) indicates that the value differs from that of the lowest dose group (0.5 mg/paw) and a plus (+) indicates that it differs from the intermediate dose (2.5 mg/paw).

variability of (WI and CI) response to a given substance in the primary PLNA is not entirely clear.

Pieters and Albers (1999) remarked that some chemicals could cause systemic effects after subcutaneous injections into the footpad thereby affecting the contralateral (vehicle-treated control) PLN as well. If a response is also induced in the contralateral (control) PLN, it could

attenuate eventual differences of weight and cellularity between treated and control side PLNs thereby reducing the assay sensitivity. To examine the possibility that control PLNs had also been affected through a systemic effect, we compared weights and cellularities of PLNs homolateral to paws treated with DMSO alone (from DMSO-vehicle control group rats the contralateral paws of which remained untreated) with those of control side PLNs (treated with DMSO alone) of each monoterpene-treated group. Weights and cell numbers of control PLNs from monoterpene-treated groups did not differ from those of a separate DMSO-treated control group (data not shown). These results indicated that, in the PLNA, there was no noticeable systemic effect of these 10 monoterpenes (5 mg/paw) on control side PLNs.

Contrasting to the six monoterpenes listed in Table 1, citral, α -terpinene, β -myrcene and (–)- α -pinene, tested at a dose as high as 5.0 mg/paw, produced clearly positive responses in the primary PLNA (Table 2). Additionally to having group mean values for WI and CI higher than 2 and 5, respectively, rats treated (5 mg/paw) with any of these monoterpenes also exhibited at least 40% of IPR (Table 2). To investigate whether the substance-induced PLN increase was dose dependent, monoterpenes that had been positive in the initial test were further tested at lower dose levels (2.5 and 0.5 mg/paw) as well. At doses lower than 5 mg/paw, however, no monoterpene was positive in the primary PLNA (Table 2).

Although being a rather useful tool to screen low-molecular-weight chemicals with immuno-stimulating potential, the primary PLNA does not distinguish between chemicals that induce hapten-specific T-cell memory responses from those that elicit non-specific adjuvating inflammatory responses (i.e., irritant chemicals) (Pieters and Albers, 1999; Ravel and Descotes, 2005). To evaluate whether positive responses in the primary PLNA were mediated by T-lymphocyte sensitization or not, all four positive monoterpenes and CPZ were subsequently tested in the secondary assay. In the secondary PLNA, a locally – or systemically – sensitized animal is challenged with a dose of the chemical that is insufficient to elicit a primary response (sub-optimal dose). The secondary response of previously sensitized animals to a sub-optimal dose is then compared with the response of non-sensitized controls challenged with the same dose of the chemical. Since anamnestic immune responses, in addition to being triggered by previously insufficient doses, also present shorter latencies, secondary assay responses were evaluated both at 4 and 7 days after the challenge with a sub-optimal dose. As shown in Table 3, secondary assays for α -terpinene, β -myrcene and (–)- α -pinene were clearly negative. The secondary responses of previously sensitized rats challenged with a sub-optimal dose of the monoterpene were not different from those of non-challenged controls at either 4 or 7 days after challenge. Moreover, 7 days after treatment, secondary responses of primed rats were not greater than those of non-sensitized animals challenged with the same sub-opti-

mal dose (0.5 mg/paw) of the monoterpene. Citral was also negative in the secondary PLNA thereby suggesting that it is likely to be rather an irritant than a sensitizing substance. The possibility that citral is a weak sensitizer, however, cannot be entirely ruled out by our data. Citral was near positive in the secondary assay with a challenge dose of 0.5 mg/paw, and thus the possibility exists that a positive response might have been obtained if we had tested a somewhat higher challenge dose.

Contrasting to these four monoterpenes, CPZ gave a clear positive response in the secondary assay. WI and CI indices of primed rats challenged with a sub-optimal dose of CPZ were higher than those of non-primed rats challenged with the same dose and also higher than those of non-challenged (primed) controls. In addition, there was 50% of individual positive responses among primed rats challenged with the sub-optimal dose of CPZ against no IPR (0%) among non-sensitized and non-challenged controls (Table 3). The positive response to CPZ in the secondary PLNA was consistent with previous results from the human maximization test and the mouse local (auricular) lymph node assay that had indicated that this drug is a contact allergen (Basketter et al., 1994). CPZ has also been reported to induce or exacerbate auto-immune reactions in humans (Bigazzi, 1997).

As far as we are aware, so far no monoterpene has been associated to the induction or worsening of auto-immune reactions in humans. Although exposure to some plant essential oils has been associated to contact dermatitis in humans, there are only a few studies on the irritant and sensitizing properties of monoterpenes. Regarding this topic, citral has been the most studied monoterpene, but it remains to some extent controversial whether it is to be classified as a contact allergen, or as a skin irritant agent (Heydorn et al., 2003). While some human patch test studies have classified citral as a skin irritant (Allenby et al., 1993; Rothenborg et al., 1977) other studies in volunteers have described it as a contact allergen (Frosch et al., 2002; Steltenkamp et al., 1980). Animal predictive tests such as the guinea pig maximization test and the murine local (auricular) lymph node assay have also identified citral as a skin sensitizer (Basketter and Scholes, 1992; Basketter et al., 1994). It is of note that, in the presence of substantial irritant activity, it is difficult to demonstrate weak sensitizing properties using either the guinea pig maximization test (Basketter et al., 1994) or human patch tests (Heydorn et al., 2003). Results from the present study (PLNA) are consistent with the interpretation that citral is an irritant substance but they did not support the view that – by an exposure route other than the skin – it is also a sensitizer.

The other monoterpenes (α -terpinene, β -myrcene and (–)- α -pinene) that were positive in the primary PLNA, however, have been far less studied. We previously reported that β -myrcene caused a chemical (aseptic) peritonitis when injected into the peritoneal cavity of rats and mice, a finding that is consistent with an irritant effect

Table 3
Secondary PLNA responses with monoterpenes and chlorpromazine (positive control)

Pre-treatment Treatment	Time after treatment				
	4 days		7 days		
	Primed Control	Primed Challenged	Primed Control	Non-primed ^a Challenged ^a	Primed Challenged
<i>Citral</i>					
N	5	5	10	7	10
WI	1.03 ± 0.64	1.49 ± 1.00	1.32 ± 0.54	1.22 ± 0.89	1.71 ± 0.76
CI	0.71 ± 0.62	5.94 ± 7.59	1.65 ± 0.89	2.71 ± 3.04	4.59 ± 3.78
IPR, no. (%)	0 (0.0)	1 (20.0)	0 (0.0)	1 (14.3)	2 (20.0)
<i>α-Terpinene</i>					
N	8	8	10	7	10
WI	0.84 ± 0.35	1.58 ± 0.42	1.46 ± 0.96	1.04 ± 0.70	1.45 ± 0.84
CI	1.20 ± 1.50	1.69 ± 1.13	1.89 ± 1.19	1.31 ± 1.60	1.76 ± 0.89
IPR, no. (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>β-Myrcene</i>					
N	8	8	10	7	10
WI	1.74 ± 0.53	1.51 ± 0.89	1.77 ± 0.88	1.32 ± 0.47	0.96 ± 0.88
CI	3.98 ± 4.41	1.30 ± 1.29	1.98 ± 3.64	2.01 ± 1.56	1.76 ± 1.35
IPR, no. (%)	1 (12.5)	0 (0.0)	1 (10.0)	0 (0.0)	0 (0.0)
<i>(-)-α-pinene</i>					
N	8	7	10	8	10
WI	1.43 ± 0.56	1.53 ± 1.17	1.37 ± 0.57	1.41 ± 0.91	1.46 ± 0.46
CI	1.71 ± 0.75	1.41 ± 1.24	2.16 ± 3.14	2.88 ± 5.23	1.55 ± 0.84
IPR, no. (%)	0 (0.0)	0 (0.0)	1 (10.0)	1 (12.5)	0 (0.0)
<i>Chlorpromazine</i>					
N	–	–	10	4	10
WI	–	–	1.26 ± 0.63	1.30 ± 0.35	2.05 ± 1.22*
CI	–	–	2.02 ± 1.53	1.26 ± 0.81	5.96 ± 4.49*
IPR, no. (%)	–	–	0 (0.0)	0 (0.0)	5 (50.0)*

Rats were locally primed with the test substance (5 mg/paw) and 6 weeks later challenged into the same paw with a dose (0.5 mg/paw) previously demonstrated to be insufficient to cause a positive response in the primary assay. Values are means ± SD; weight (WI) and cellularity (CI) indices: value for draining popliteal lymph node of the treated (right paw) side divided by that of the control (left paw) side. IPR, number (%) of rats within the group with WI ≥ 2 and CI ≥ 5. WI and CI were compared by the Mann–Whitney U test and the Fisher's exact test. An asterisk (*) indicates that response of primed rats challenged with a sub-optimal dose of CPZ differs ($P < 0.05$) from that of primed non-challenged controls and also from that of not primed rats challenged with a sub-optimal dose.

^a The non-primed group challenged with 0.5 mg/paw was the same shown in Table 2.

(Paumgartten et al., 1990). Acute inhalation of α -pinene was reported to cause pulmonary and respiratory tract irritation in BalbC mice (Nielsen et al., 2005). Except for these two evidences of peritoneal and pulmonary irritation, we have found no report on the irritant properties of β -myrcene and α -pinene. We have found no study on the irritant properties of α -terpinene either.

As pointed out by several authors, more than 130 compounds have already been tested in the primary PLNA and, so far, a rather good correlation has been found between responses in the assay and the reported capacity of the chemical to induce auto-immune reactions or contact dermatitis in humans (Pieters and Albers, 1999; Pieters et al., 2002; Ravel and Descotes, 2005). A few cases of drugs that are associated to auto-immune-like reactions in humans and gave negative results in the assay (e.g. procainamide, isoniazid) are known. These false negative cases were apparently due to a limited drug metabolism at the injection site because positive responses were obtained when some of their metabolites were tested in

the PLNA. Along this line, we cannot rule out the possibility of false negative results due to an eventual immunogenicity of monoterpene metabolites that are not produced at the injection site. At any rate, although a limited local metabolic capacity is likely to be a problem for the prediction of potential for chemicals to induce auto-immune (systemic) reactions in humans, it seems to be a less important limitation for the prediction of potential to cause local hypersensitivity reactions – such as contact dermatitis.

In conclusion, while (–)-menthol, 1,8-cineole, (±) camphor, (±)-citronellal, (+)-limonene and terpineol were negative, citral, α -terpinene, β -myrcene and (–)- α -pinene gave positive responses in the rat primary PLNA. These four monoterpenes, however, gave negative results in the secondary PLNA. Taken together, these findings suggest that the ten monoterpenes evaluated in the present study are not immuno-sensitizing substances, and that primary PLNA positive responses to citral, α -terpinene, β -myrcene and (–)- α -pinene were due to their irritant properties.

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