Arch Dermatol Res (1992) 284: 297–302

Archives of Dermatological Research

A murine in vitro model of allergic contact dermatitis to sesquiterpene α-methylene-γ-butyrolactones*

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Received February 28, 1992

Summary: The use of a lymphocyte transformation test (LTT) to provide evidence of allergic contact dermatitis was investigated. The haptens studied were alantolactone and isoalantolactone, two moderate allergens from *Inula helenium* L., a decorative and medicinal plant. Only alantolactone showed a significant response in vivo and in vitro in mice sensitized epicutaneously, without using Freund's complete adjuvant. Isoalantolactone did not show any sensitizing capacity in the murine model studied. The comparison of in vitro lymphocyte proliferation and in vivo allergenic capacity showed a good correlation and clearly demonstrates that, of the two sesquiterpene lactones, alantolactone is the better sensitizer.

Key words: Allergic contact dermatitis – Murine model – Helenin – Alantolactone – Isoalantolactone – Cross-reaction – Lymphocyte transformation test

Helenin and two of its main constituents, two sesquiterpene lactones, alantolactone and isoalantolactone (Fig. 1), are well known contact sensitizers [3, 5, 13, 17–19]. Mitchell et al. reported allergic contact dermatitis (ACD) to alantolactone [12], but found isoalantolactone non-allergenic in humans [11]. However, further studies in guinea-pigs clearly demonstrated that both isomers were sensitizers when applied intradermally [20]. Cross-sensitization between such sesquiterpene lactones is also well established [6, 11, 19].

ACD has been widely studied in humans and guinea-pigs both in vivo and in vitro, but the majority of reports of in vitro tests concern nickel and DNCB allergy [22]. The in vitro lymphocyte transformation test (LTT) has been successfully used in this laboratory to detect contact sensitivity to alantolactone and isoalantolactone in guinea-pigs [8]. More recently, the mouse model for contact sensitization, initially developed by Asherson and Ptak [1], has been used extensively. In this model, the elicitation reaction can be quantitatively estimated by measuring the ear thickness increase after challenge in previously induced animals. The use of the mouse model, for assessment of contact sensitivity responses, has been primarily limited to the study of strong sensitizers [4, 21]. As in vitro experiments are mainly performed with mice, it seemed of interest to find out whether both in vivo and in vitro responses could be generated with a wider range of contact sensitizers [10, 16].

We recently reported successful sensitization to alantolactone in four strains of mice [7]. In view of these encouraging results, we decided to use the murine model for in vivo and in vitro studies with alantolactone and isoalantolactone (Fig. 1) using different mouse strains for both primary sensitization and cross-reactions. We now report our results.

alantolactone isoalantolactone

Inula helenium L.

Fig. 1. Alantolactone and isolantolactone

Materials and methods

Animals

Male Balb/c and DBA/2 mice were obtained from IFFA CREDO (France) and male Balb/b mice from Centre CSEAL-CNRS Orleans (France). The mice were maintained in an animal care facility at constant temperature (22 °C) and received pelleted food and water ad libitum, and were used in the experiments at 6–7 weeks of age.

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* In memory of Prof. C. Benezra and Dr. R. Fraginals accidentally deceased

Chemicals

Helenin was purchased form Sigma products, St. Louis, Mo., USA. Alantolactone was obtained as follows: helenin (10 g) was suspended in *n*-heptane (100 ml) and solubilized by heating in a water bath (60 °C) after adding methanol (10 ml). When the solution was cooled to room temperature, white crystals of pure isoalantolactone (5.0 g) were precipitated. The filtrate was evaporated under reduced pressure and alantolactone separated on a silica gel/20% silver nitrate column (500 × 25 mm, 350 g) eluted with a hexane–ethyl ether mixture (8 : 2). Alantolactone was eluted first. Its purity was ascertained by vapour phase chromatography (one peak), nuclear magnetic resonance spectra, and melting point (80.5 °C) [23]. Isoalantolactone was eluted afterwards. The criteria of purity used were the same. Its melting point was 113 °C [23].

Sensitization method

Induction was achieved in Balb/c and DBA/2 mouse strains by the epicutaneous route with helenin, alantolactone or isoalantolactone at a concentration of 10%. Groups of six animals were used for each experiment. The experimental groups received 100 µl of 10% solutions of helenin, alantolactone or isoalantolactone in acetone : olive oil (4 : 1), on the shaved abdomen.

Sensitization to Isoalantolactone

Two groups of six Balb/c mice were induced by the epicutaneous route with 10% and 20% isoalantolactone. Control groups received 100 µl of the vehicle (4 : 1 acetone : olive oil) alone. In two other groups, intradermal induction was performed in Balb/c mice, with 10% and 20% isoalantolactone. Isoalantolactone was solubilized in Feund's incomplete adjuvant (FIA) with a final induction concentration of 10% and 20% of isoalantolactone. This suspension (100 µl) was injected intradermally in the dorsal area. Control received one intradermal injection of FIA alone.

Elicitation test

Skin testing was effected 4 days after the beginning of induction. Animals were challenged on the ventral side of the right ear by depositing 25 µl of the substance (1% helenin, 1% alantolactone or 1% isoalantolactone) in acetone : olive oil (4 : 1). Ear thickness increase was measured with an engineering micrometer (Oditest, FRG). The thickness was recorded every day for 4 days after the application of the challenge dose. Intradermal-isoalantolactone induced animals were challenged in the same way 11 days after the beginning of sensitization.

For purposes of comparison, a numerical average response value was calculated for each set of readings by summing the individual rating and dividing the sum by the total number of animals in the experimental group. The results are expressed as ear thickness increase in microns or as percentage increase in ear thickness. We used the MEST (Mouse Ear Swelling Test) method for induction and challenge [9]. The thickness was measured after depositing the hapten solution or vehicle on the right ear and compared with the opposite unchallenged ear as a standard. We used as control groups non-induced mice (i.e. mice treated with the vehicle alone in the induction phase and challenged with the eliciting product). The percentage increase in ear thickness was calculated in experimental and control groups in the same way as (ear thickness in right ear minus ear thickness in left ear/ear thickness in left ear) × 100.

In vivo cross-reaction experiments

Four groups of 12 Balb/c mice were used. These groups were epicutaneously induced with 10% (groups 1 and 2) and 20% (groups 3 and 4) concentrations. Half of group 1 was used as a positive control group (induced and challenged with alantolactone) and the other half was cross-reacted with isoalantolactone (induced with alantolactone and challenged with isoalantolactone). Half of group 2 was induced and challenged with isoalantolactone as the positive control group and the other half was induced with isoalantolactone and challenged with alantolactone. Groups 3 and 4 were epicutaneously induced as above with a 20% solution of the lactones and treated in the same way.

In vitro studies

For in vitro studies, Balb/c, Balb/b and DBA/2 mice were induced by the epicutaneous route with 10% alantolactone. Naive Balb/c, Balb/b and DBA/2 mice were used as control groups. Balb/c mice were induced by the intradermal route with 10% isoalantolactone and by the epicutaneous route with 20% isoalantolactone. Naive Balb/c mice were used as a control group. Groups of four animals were used for each experiment. Epicutaneous and intradermal induction methods were performed as above.

Lymphocyte proliferation assay

Balb/c, Balb/b and DBA/2 mice were sacrified 5 days after the beginning of epicutaneous induction with 10% alantolactone. Another group of Balb/c mice was sacrified 5 days after the beginning of epicutaneous induction with 20% isoalantolactone and another group was sacrificed 11 days after intradermal induction with 10% isoalantolactone. Axillary and inguinal lymph nodes were pooled from each group and cell suspensions were prepared by teasing the nodes with forceps into the culture medium. Each control group was pooled separately. Lymphocyte concentrates from homogenized lymph nodes of induced and non-induced mice, were prepared by centrifugation at 1600 rpm for 10 min. The cells were washed twice and resuspended at a standard concentration of 1×10^6 cells/ml in RPMI-1640 medium supplemented with 1 m*M* L-glutamine, 1 m*M* sodium pyruvate, 1% non-essential amino acids (L-ala, L-asn, L-glu, L-pro, L-ser), 5×10^{-5} *M* final concentration of mercaptoethanol, 100 µg/ml gentamycin (active against gram-positive bacteria, gram-negative bacteria and mycoplasma) and 10% heat-inactivated fetal calf serum. These cells were plated in quadruplicate in 96-well microtitre plate cultures. The lymphocytes were stimulated by adding 20 µl per well of a 2×10^{-5} *M*, 1×10^{-5} *M*, 2×10^{-6} *M*, 1×10^{-6} *M*, 5×10^{-7} *M* or 2.5×10^{-7} *M* solution of alantolactone or isoalantolactone in complete RPMI medium. One control culture pool received 20 µl of complete RPMI medium alone to measure intrinsic proliferative activity, and other control cell suspensions from non-sensitized mice were challenged in vitro with the same alantolactone and isoalantolactone concentrations for determining the proliferative activity of control cultures. The lymphocytes were cultivated for 2 days at 37 °C in a humidified atmosphere of 5% CO_2. The cultures were pulsed with 1 µCi/well of tritiated thymidine (^{3}H-TdR) in 20 µl complete RPMI medium and reincubated for 4 h. The cells were then harvested into glass microfibre filter strips using a semi-automatic multiple-well cell harvesting unit and dried for 24 h at room temperature in a protective paper. The filter discs were placed in scintillation vials containing a scintillation cocktail and counted in a beta counter (Kontron Beta V. Kontron Instruments, Vélizy, France).

A stimulation index (SI) was calculated for both experimental and control groups, according to the equation: SI (control group) = average counts per minute (c.p.m.) of control cells + sensitizer/average c.p.m. of control cells (without sensitizer); SI (experimental group) = average c.p.m. of sensitizer-stimulated cells + sensitizer/average c.p.m. of sensitizer-stimulated cells (without sensitizer).

In vitro cross-reaction

Three groups of four Balb/c mice were used. Cell suspensions from each group were pooled separately. One group was induced epicutaneously with 10% alantolactone. Half of this group was used as a positive control, i.e. challenged in vitro with the primary sensitizer alantolactone, and the other half was challenged in vitro

with isoalantolactone. Group 2 was induced intradermally with 10% isoalantolactone. Half of this group was challenged in vitro with isoalantolactone as the positive control group, while the other half was cross-challenged with alantolactone. Group 3 was induced epicutaneously with 20% of isoalantolactone and processed in a manner similar to that used for group 2. The lymphocyte proliferation assay was performed in the same way as above. ASI was calculated according to the formula: S.I. = average c.p.m. of primary sensitizer-stimulated cells + cross-reactant/average c.p.m. of primary sensitizer-stimulated cells (without hapten).

Results

Alantolactone has been shown to produce an ear swelling response upon ear challenge of previously induced animals [7]. Figure 2 shows the results of epicutaneous sensitization ot 10% helenin, alantolactone and isoalantolactone in Balb/c and DBA/2 strains. In Fig. 2 the results are expressed as mean percentage increases in ear thickness recorded 48 h post-challenge. The ratios of sensitized versus non-sensitized mice were analysed by Student's paired t-test using a $p < 0.05$ level of significance. The responses were significant for helenin and alantolactone in Balb/c and DBA/2 mice ($p < 0.001$). In neither strain was the response observed to isoalantolactone statistically significant as compared with controls. DBA/2 mice induced with 10% alantolactone showed signs of severe toxicity; 60% of the experimental group died after 4 days. We do not know the nature of this toxic effect. These signs were not observed in DBA/2 mice

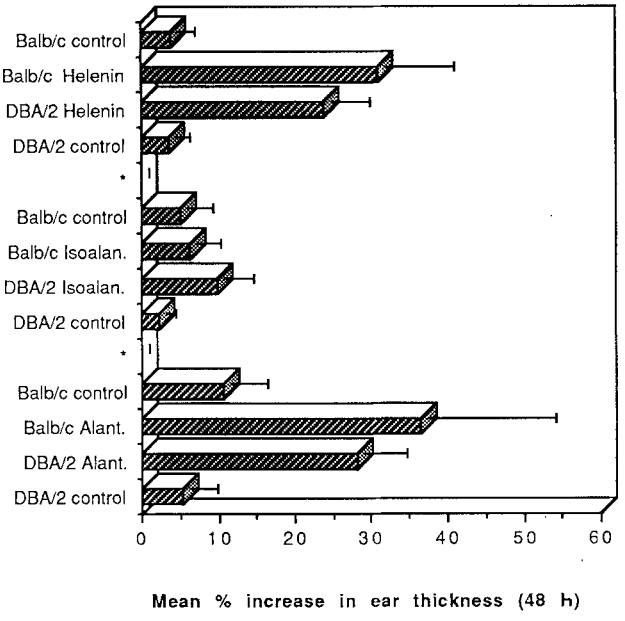

Fig. 2. Percentage of ear thickness increase in epicutaneously sensitized Balb/c and DBA/2 mice with 10% helenin, isoalantolactone and alantolactone induction dose in acetone/olive oil (4:1)

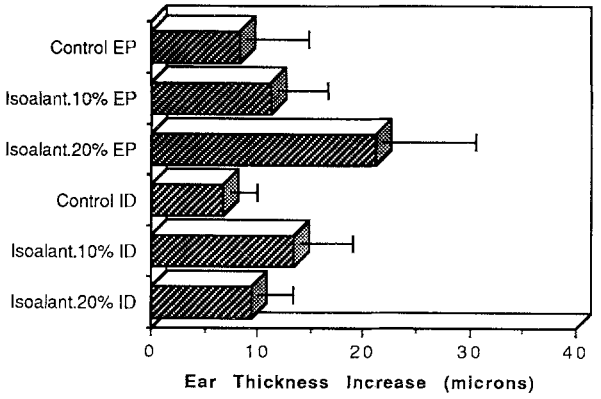

Fig. 3. Ear thickness increase (microns) 48 h after challenge with 1% isoalantolactone in Balb/c mice sensitized to isoalantolactone by two different methods (epicutaneous and intradermal route) and at two different concentrations (10% and 20%). ID, intradermal; EP, epicutaneous

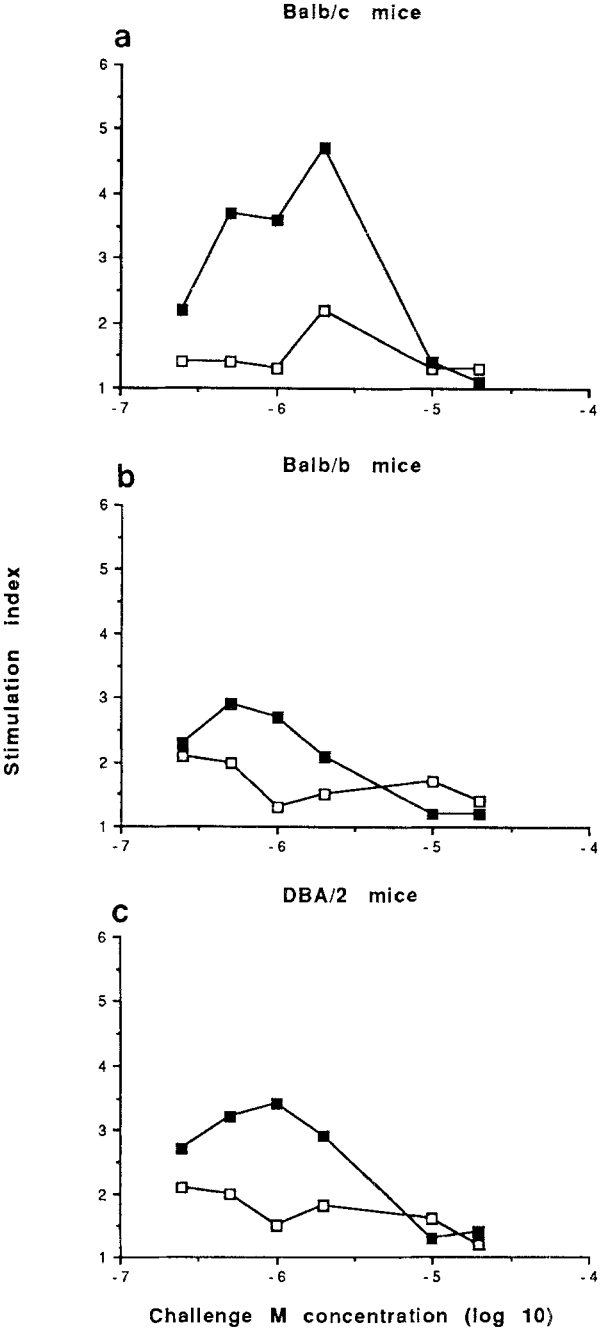

Fig. 4 a–c. ^{3}H-Thymidine incorporation. Blastogenesis response in three mouse strains sensitized to alantolactone: Balb/c **(a)**, Balb/b **(b)**, and DBA/2 **(c)**. Challenge concentration are expressed as log values; —□— control; —■— sensitized

sensitized to 10% helenin and isoalantolactone. In view, of the toxicity observed in the DBA/2 strain with high doses of alantolactone, we decided to continue with the Balb/c mouse strain. The results of intradermal and epicutaneous induction with 10% and 20% isoalantolactone are shown in Fig. 3 which shows an ear thickness increase in animals epicutaneously induced with 20% isoalantolactone, but the increase was non-significant ($p < 0.3$) compared with the control group which was not induced but challenged with isoalantolactone. Animals intradermally induced with 10% isoalantolactone had responses stronger than animals epicutaneously induced with 10% isoalantolactone. This is probably due to better penetration of isoalantolactone when injected by the intradermal route.

In view of our results, we chose to use 20% isoalantolactone epicutaneous induction in order to observe possible cross-reaction. Mice induced with alantolactone did not respond when challenged with isoalantolactone. However, we found a significant ear thickness increase in mice induced with isoalantolactone and challenged with alantolactone.

Lymph node cell proliferation is shown in Fig. 4 and Tables 1 and 2. A stimulation index $\geqq 2.5$ was considered as a significant measurement of contact hypersensitivity. The statistical significance of the SI was assessed by Student's t-test at the $p \leqq 0.05$ level of significance (SI $= 2.5$, $p < 0.05$). Table 1 shows the SI in cell cultures from alantolactone-sensitized mice. Non-induced or vehicle-induced control mice showed no blastogenesis. The maximum response was observed for 2×10^{-6} M challenge concentration in sensitized Balb/c mice (Fig. 4). Table 1 shows the results of blastogenesis assay cultures in Balb/c mice sensitized to isoalantolactone. Both groups of 20% epicutaneous isoalantolactone-induced Balb/c mice, or the group of 10% intradermally isoalantolactone-sensitized Balb/c mice, did not show any significant response to any challenge concentrations between

Table 1. Isoalantolactone lymphocyte proliferation test in Balb/c mice

Sensitization concentration	Sensitization route	Group	Stimulation index (M)					
			2×10^{-5}	1×10^{-5}	2×10^{-6}	1×10^{-6}	5×10^{-7}	2.5×10^{-7}
20%	Epicutaneous	Control	1.5	1.4	1	1.2	1.5	1.1
		Sensitized	1.4	1.2	0.9	1.1	1.6	1.3
10%	Intradermal	Control	1	0.9	1	1	0.9	1
		Sensitized	0.9	1	0.8	0.9	0.8	1.1

$$\text{Stimulation index (control group)} = \frac{\text{c.p.m. of control cells + sensitizer}}{\text{c.p.m. of control cells ((without sensitizer)}}$$

$$\text{Stimulation index (sensitized group)} = \frac{\text{c.p.m. of sensitizer-stimulated cells + sensitizer}}{\text{c.p.m. of sensitizer-stimulated cells (without sensitizer)}}$$

Table 2. Lymphocyte proliferation test in Balb/c mive: cross-reactions

Sensitization in vivo	Challenge in vitro	Stimulation index (M)					
		2×10^{-5}	1×10^{-5}	2×10^{-6}	1×10^{-6}	5×10^{-7}	2.5×10^{-7}
10% Alantol. (EP)	Alantol.	1.1	1.4	4.7**	3.6*	3.7*	2.2
10% Alantol. (EP)	Isoalantol.	2	1.9	1.8	1.2	1.7	1.4
20% Isoalan. (EP)	Isoalantol.	1.4	1.2	0.9	1.1	1.6	1.3
20% Isoalan. (EP)	Alantol.	0.9	1	1.2	1.2	1.5	1.1
10% Isoalan. (ID)	Isoalantol.	0.9	1	0.8	0.9	0.8	1.1
10% Isoalan. (ID)	Alantol.	1.1	1	1	1	1	1

EP, epicutaneous; ID, intradermal

$$\text{Stimulation index (primary)} = \frac{\text{c.p.m. of primary stimulated cells + primary sensitizer}}{\text{c.p.m. of primary sensitizer stimulated cells (without sensitizer)}}$$

$$\text{Stimulation index (cross-reaction)} = \frac{\text{c.p.m. of primary sensitizer stimulated cells + cross-reactant}}{\text{c.p.m. of primary sensitizer stimulated cells (without hapten)}}$$

* $p < 0.02$, ** $p < 0.001$

2×10^{-5} *M* and 2.5×10^{-7} *M* (maximum SI = 1.6). Table 2 shows the results of in vitro cross-reaction. Table 2, indicates a significant response only in Balb/c mice induced and challenged with alantolactone. Thus, in vitro cross-reaction results are in good agreement with in vivo cross-reaction responses.

Discussion

During the last 20 years, many investigators have demonstrated that peripheral T lymphocytes from contact-sensitized animals can show a proliferative response when cultured with the relevant hapten. The majority of studies have examined the capacity of strong haptens to induce a significant proliferative response, but weak allergens have not been studied. This particular problem has been addressed in the present work in which a comparison of the allergenic potential of helenin and two of its main constituents, alantolactone and isoalantolactone, has shown that alantolactone is the better sensitizer in the mouse model.

In a recent study, we reported differences in sensitization rate with alantolactone in relation to concentration of sensitizer and strain of mouse used [7]. We found that the mice most sensitive to alantolactone were Balb/b, Balb/c and DBA/2 strains. For purposes of comparison of the allergenic potential of helenin, alantolactone and isoalantolactone, we used Balb/c and DBA/2 strains with an equal induction concentration (10%). Figure 2 clearly shows that helenin is a sensitizer and that alantolactone is the better of the two main sensitizers present in helenin. Similar conclusions have been reported in humans [12] and guinea-pigs [19]. The Balb/c strain is more sensitive to helenin and alantolactone than DBA/2. We did not observe any significant response to isoalantolactone in either Balb/c or DBA/2 strains. Stampf et al. [20] found differences in sensitization rate with alantolactone and isoalantolactone in relation to concentration and strain of guinea-pig used, when these sesquiterpenic lactones were applied epicutaneously. They observed that both isomers were equally good sensitizers when applied intradermally, but suggest that skin penetration of the two isomers might be very different. However, the responses were considered positive, according to the criteria of 'all-or-none reaction': even if 11 out of 12 animals were sensitized, the skin reaction to isoalantolactone was very weak.

We used concentrations of 10% and 20% of isoalantolactone both for epicutaneous and intradermal induction. Figure 3 shows that no significant response was observed, with respect to the controls for any of the concentrations and induction methods used. However the maximum (but not significant) responses, occurred with 20% isoalantolactone by epicutaneous induction. The above results suggest that responses to isoalantolactone and helenin depend on concentration, mouse strain and support the same 'strain and dose-dependence' observed with alantolactone [7]. The isoalantolactone response in shown Fig. 3 suggests a possible overdose effect to 20% isoalantolactone intradermal induction. This phenomenon was described by Roberts and Williams [15] and was also found in mice epicutaneously sensitized to alantolactone [7].

Cross-reactions to structurally related lactones have been reported [6, 8, 11, 19]. In this work, we have observed differences in the responses of mice sensitized to alantolactone and isoalantolactone and challenged with the primary sensitizer or its isomer. Mice induced with alantolactone did not respond when challenged with isoalantolactone, while mice induced with isoalantolactone and challenged with alantolactone showed a response slightly stronger than mice induced and challenged with isoalantolactone. These results suggest that there are differences in the sensitizing and eliciting capacity of both isomers in the mouse model. As in the in vivo responses, the results of the lymphocyte proliferation in assay primary sensitization to isoalantolactone were negative.

The reactions involved have generally exhibited high specificity in binding to receptors. In recent studies from this laboratory, we showed that response to enantiomers (i.e. mirror-image compounds) was specific [2, 14]. The results described here for alantolactone and isoalantolactone, compounds with minor structural differences (isomers), seem to show that these sesquiterpene-α-methylene-γ-butyrolactone also have isomeric specificity.

In the generally accepted mechanism of ACD, the hapten pentrates the skin, becomes bound to a protein carrier which is taken up by antigen-presenting cells (APC), and is presented to T lymphocytes, triggering a number of reactions that eventually lead to the contact dermatitis observed. If high specificity in binding to receptors occurs with both lactone isomers, it is possible that different properties and particularly recognition by T-lymphocyte receptors would be different. It is probable, in this way, that alantolactone is active in the in vivo mice model and the LTT, while the other isomer is not. Alternatively, it is also possible that skin penetration of the two isomers is very different due to different physical and chemical properties of the two isomers.

Gabriel-Robez et al. [8] reported a lymphocyte transformation test study using skin protein extracts–alantolactone conjugates in guinea-pigs sensitized to alantolactone. Cross-sensitivity in alantolactone-induced guinea-pigs and in vitro when challenged with skin protein extracts–isoalantolactone conjugates was also observed. However, when the hapten alone was used, in primary reaction to alantolactone or in cross-reactivity with isoalantolactone, isoalantolactone was toxic or slightly stimulating in a few cases. Primary sensitization to isoalantolactone was not studied. In this in vitro study, we used induction and challenge to alantolactone and isoalantolactone and cross-reaction with both isomers. The results of in vitro experiments using the LTT are compatible with our results from in vivo studies. Fig. 4, clearly shows a significant SI after primary induction with alantolactone in Balb/c mice with in vitro challenge concentrations between 2×10^{-6} *M* ($p < 0.001$) and 5×10^{-7} *M* ($p < 0.02$). Similarly, the DBA/2 strain shows a significant SI maximun after primary induction with

alantolactone to challenge concentrations between 1×10^{-6} and 5×10^{-7} M ($p < 0.02$) and the Balb/b strain shows a significant SI after primary sensitization to alantolactone with challenge concentrations between 1×10^{-6} and 5×10^{-7} M ($p < 0.05$). It is important to note that the Balb/b strain showed an overdose effect in the in vivo study at the same induction dose [7]. As in the in vivo study, the results of the in vitro experiments an primary sensitization ot isoalantolactone were negative. There was no significant SI in any of the animals sensitized and challenged with isoalantolactone (see Table 1).

Conclusion

This study suggests differential immunological activity between the two isomers. Our experience with the LTT suggests that this assay may be of value in the diagnosis of alantolactone and isoalantolactone sensitizing capacity and, more generally, in ACD to sesquiterpene lactones.

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