A Successful Murine Model for Contact Sensitization to a Sesquiterpene-α-Methylene-γ-Butyrolactone: Sensitization to Alantolactone in Four Strains of Mice

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Induction of allergic contact hypersensitivity to a sesquiterpene lactone, alantolactone, was studied in four strains of mice: C3H/He, DBA/2, Balb/b, and Balb/c. The last three were successfully sensitized. A significant dose/response was demonstrated in these species, as well as an experimental "overload effect" in Balb/c and Balb/b strains. Histologic studies confirmed the allergic nature of the reaction. From the overall results, alantolactone can be considered a moderate sensitizer in mouse as well as in guinea pig. This study shows that the murine model can be used for experimental contact sensitization with moderate allergens, without the use of Freund's adjuvant for induction. *J Invest Dermatol 97:473–477, 1991*

Alantolactone, isoalantolactone, and dihydroalantolactone are sesquiterpene lactones isolated from the roots of a Compositae plant *Inula helenium* L [1–5]. Compositae (Asteraceae) plants are one of the major sources of allergic contact dermatitis (ACD) to natural compounds [1,3,5,6]. Alantolactone is a known contact allergen whose sensitizing properties are due to the presence of an α-methylene-γ-butyrolatone moiety [1] (Fig 1).

The guinea pig has been the model of choice for studying ACD since 1935 [7–9]. Several sensitization methods—open epicutaneous test (OET), Draize test (DT), guinea pig maximization test (GPMT), and optimization test (OT)—have been used [8,9]. In these experiments, alantolactone was found to be a moderate sensitizer with an average skin intensity of the test of 1 to 2 in a 0–3 scale [10]. However, in man, alantolactone is considered to be a strong allergen, even responsible for flare-up reactions [4].

Encouraged by the success of the murine model in the study of ACD to potent allergens, methyl alkanesulphonates [11], and in view of literature data [12], we have decided to use this model for the study of ACD to alantolactone. We have used four strains of mice: C3H/He, DBA/2, Balb/c, and Balb/b mice sensitized with different doses of allergens. We now report our results.

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Abbreviations:
ACD: allergic contact dermatitis
DT: Draize test
GPMT: guinea pig maximization test
LTT: lymphocyte transformation test
MEST: mouse ear swelling test method
OET: open epicutaneous test
OT: optimization test

MATERIALS AND METHODS

Animals Male C3H/He, DBA/2, Balb/c, and Balb/b mice were obtained from IFFA CREDO (France) and CSEAL-CNRS (France). Mice 5–6 weeks old were used in each experiment; they were maintained in an animal care facility at constant temperature (22°C) and received pelleted food and water ad libitum. Sensitization-induction treatment was achieved by the epicutaneous route, groups of 8 animals were used for each concentration, the experimental groups received 100 μL of alantolactone acetone:olive oil (4:1) solutions of 3%, 6%, 10%, 12% and 15% concentration on the shaved abdominal area. The control group received 100 μl of the vehicle alone.

Skin testing was effected using the previously reported mouse ear swelling test (MEST) method [13]. Four days after the beginning of sensitization, the animals were challenged on the ear ventral side by depositing 25 μL of a 1% acetone:olive oil (4:1) solution of the substance to be tested. Ear thickness was measured with an engineering micrometer (Oditest TM, West Germany). The thickness was recorded every day during 4 d after the application of the challenge dose. This was measured after depositing the hapten solution or vehicle on the right ear and by comparison with the opposite unchallenged ear, used as an internal standard. We have used as control groups non-sensitized and challenged mice. For purpose of comparison, a numerical average response value was calculated for each set of readings by summing up the individual rating and dividing the result by the total number of animals in the experimental or control group. The results are expressed in percentage in ear thickness increase of the tested ear over the unchallenged ear: % ear thickness increase = ear thickness of the challenged ear minus ear thickness of the unchallenged ear/ear thickness of the unchallenged ear × 100. Significances of the mean thickness increase of challenged ears over corresponding unchallenged ears controls were assessed by the two-tailed Student t test at the 0.05–0.001 level of significance.

Chemicals Helenin was purchased from Sigma products, St. Louis, MO. 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

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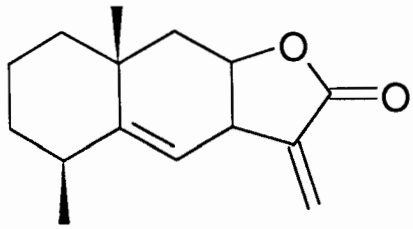

Figure 1. Alantolactone, a natural sesquiterpene lactone.

under reduced pressure and alantolactone was separated on a silica gel 10% silver nitrate column (500 × 25 mm, 250 g), eluted with an hexane-ethyl ether mixture (7:3). Alantolactone came first out of the chromatography column. Its purity was ascertained by vapor-phase chromatography (one peak), nuclear magnetic resonance spectra, and melting point: 80.5°C (identical to the reported 80.5°C) [14]. Isoalantolactone came out of the column afterwards. The criteria of purity used were the same (melting point: 113°C as compared to the 113°C reported).

Histologic Evaluation Specimens of sensitized (10% alantolactone was used to induce sensitization) and control ears (painted with the vehicle only: acetone/olive oil 4:1) were obtained 24 h after the application of challenge dose (1% alantolactone) in Balb/c, Balb/b, DBA/2, and C3H/He strains. Biopsies were fixed in Bouin's liquid (an aqueous solution of picric acid, formaldehyde, and acetic acid). The specimens were processed in a Tissue-Tek Tissue Processor (Miles) and embedded into paraffin blocks. Four-micrometer sections were obtained (2030 Microtome, Reichert Jung) and after removal of the paraffin with xylene, followed by exposure to 100–70% ethanol, were stained using a Quadrichromic coloration technical (Varistain 24.3, Shandon) with Astra Bleu (Merck), Mayer's Hémalun, Erythrosine, and alcoholic saffron. The sections were exposed to 95%–100% ethanol, cleared in xylene, and mounted in Mounting Medium (Surgipath-Clearium). The tissues were examined using a light microscope (Labophot Microscope, Nikon).

RESULTS

They are shown in Fig 2*a–d*, and 3. All animals were tested with a 1% acetone: olive oil (4:1) alantolactone solution. The results clearly demonstrate the sensitizing capacity of alantolactone in mice. There is a significant difference between the ear thickening in the experimental and the control groups, and the histologic cuts confirm this (Fig 3). Figures 2*a–d* show the intensity of the response in each strain, expressed in percentage increase in ear thickness, using different sensitizing doses of alantolactone (from 3%–15%). A significant response in Balb/c, Balb/b (10%), and C3H/He (6% and 10%) mice occurred at 24 h. Clearly, the best responders were Balb/b and Balb/c strains. Figure 2*a,b* shows a gaussian distribution.

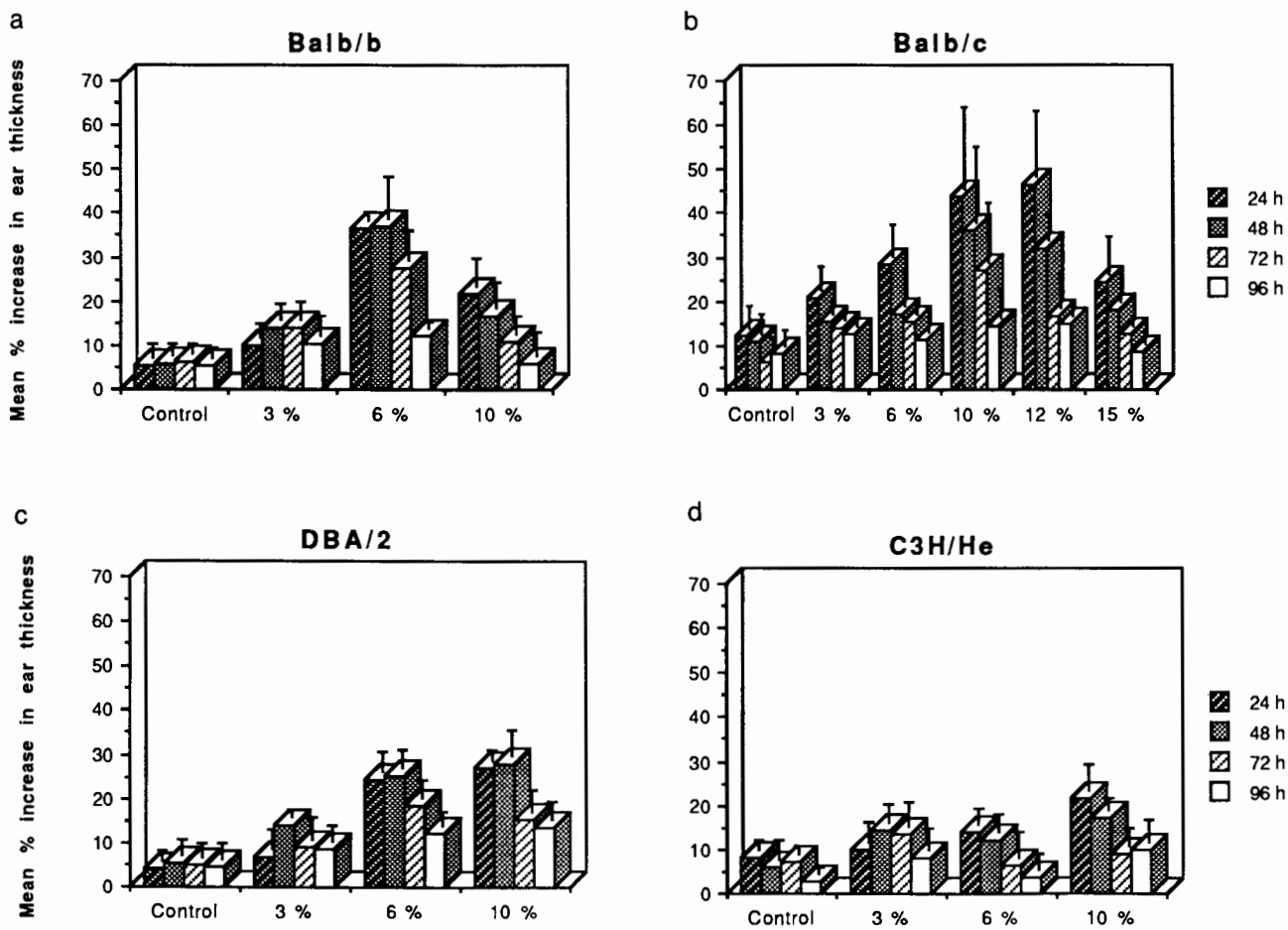

Figure 2. Percentage of ear thickness increase in different strains of mice sensitized to alantolactone at different doses. Ear swelling responses were measured 24, 48, 72, and 96 h after challenge using the MEST method [13]. *a*, Balb/b strain. Responses of the treated groups were significantly greater than the control group at 6% (24 h to 96 h) and 10% (24 h to 48 h) with a $p < 0.001$. *b*, Balb/c strain. Responses of treated groups were significantly greater than the control group at 3%, 6%, and 15% at 24 h ($p < 0.001$). At 10% and 12% concentrations (24 h to 96 h) the response showed a significant difference, too, with $p < 0.001$. *c*, DBA/2 strain. Responses of treated groups are significantly greater than control at 48 h, 3% ($p < 0.05$). On 6% concentration and 10% concentration (24 h to 72 h) we found significant responses with a $p < 0.001$ for each concentration. *d*, C3H/He strain. Responses of treated groups are not significant except for 10% concentration at 24 h and 48 h.

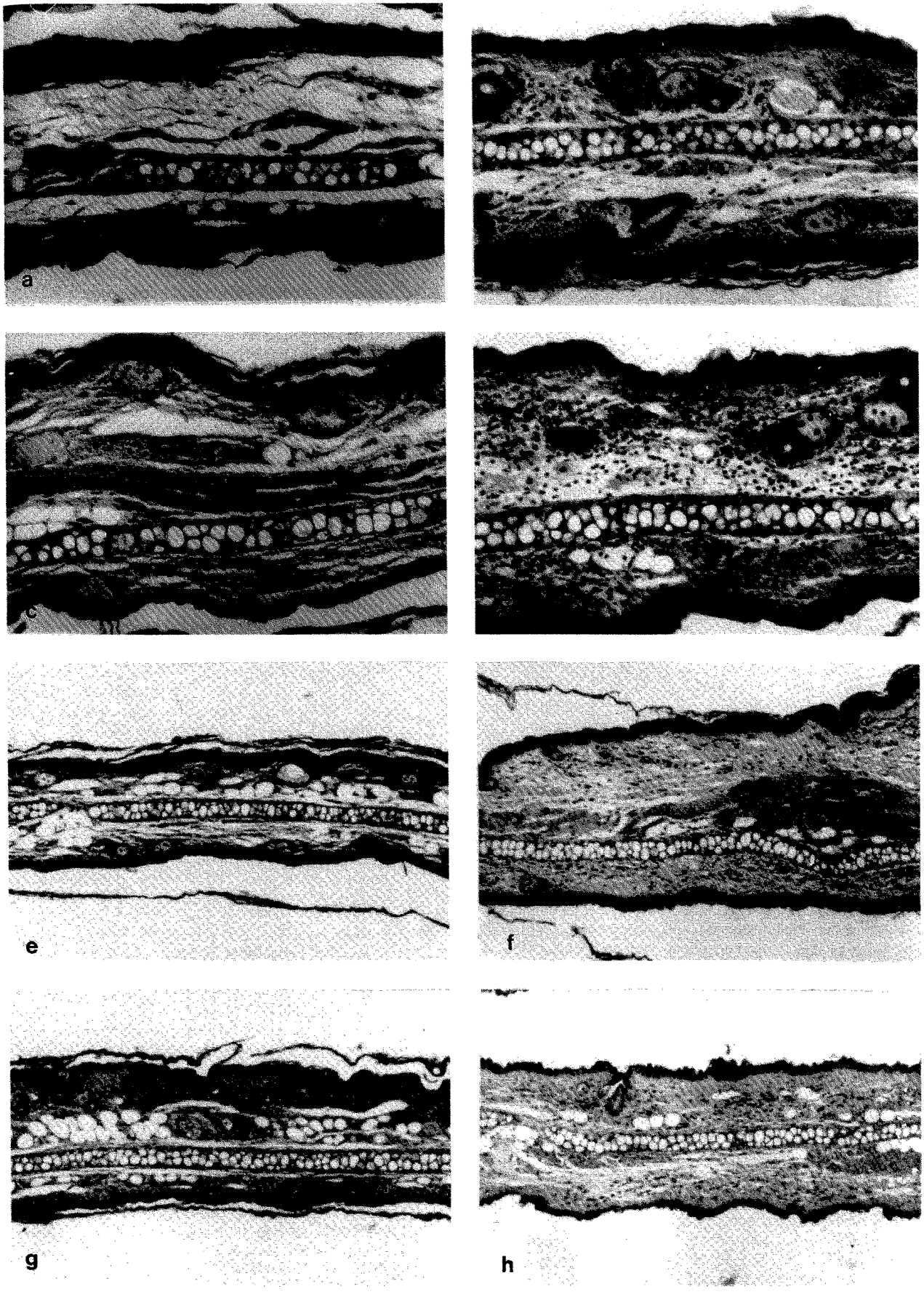

Figure 3. Quadrichromic coloration of technically stained sections of sensitized and control ears 24 h after challenge with alantolactone. *a*, control Balb/b mouse; *b*, sensitized Balb/b mouse; *c*, control Balb/c mouse; *d*, sensitized Balb/c mouse; *e*, control DBA/2 mouse; *f*, sensitized DBA/2 mouse; *g*, control C3H/He mouse; *h*, sensitized C3H/He mouse. The skin of mice sensitized to 10% alantolactone and challenged with 1% alantolactone showed a significant dermal edema, associated with proliferation of lymphocyte cells, compatible with allergic contact dermatitis. No basophil infiltrate was found. These changes are more pronounced in Balb/c strain.

24th-h Readings In Balb/c mice, this was the peak reaction with all alantolactone sensitizing doses. This was also true for Balb/b at 10% concentration. The reaction in the C3H/He strain was more erratic; a significant response ($p < 0.05$), however, was found at 10% concentration of alantolactone.

48th-h Readings Forty-eighth hour after challenge, we observed that in all strains at low concentration of alantolactone (3%), the ear thickness increase (15% – 18%) was larger than that of the control groups (5% – 10%). Control Balb/c mice showed a slight irritation reaction (thickness increase of 12% of the tested ear over the unchallenged ear). This was not observed in the other three control strains (around 5% increase). At 48 h, a response of low intensity was observed in Balb/c and C3H/He mice sensitized with alantolactone at 6% concentration (17% and 12% ear thickness increase, respectively), whereas 37% and 25% ear thickness increase was found in the other two strains (Balb/b and DBA/2).

In the Balb/c and Balb/b strains, at higher doses, a significant decrease of the response was observed, presumably because of an overload effect. This experimental effect was reported in several papers [15,16], although no clear-cut theoretical interpretation is presently available. In particular, in Fig 2*c,d* (DBA/2 and C3H/He strains), there is no gaussian distribution. For the latter strain, even if a 10% concentration corresponded to an increase in the ear thickness, severe signs of toxicity were observed and, consequently, no higher concentration was used. The Balb/c strain showed a maximum reaction when 10% – 12% alantolactone solutions were used for sensitization. In the C3H/He strain, there was no significant response at any concentration used (Fig 2*d*) except for 10% at 24 h and 48 h; we therefore conclude that this strain is not responsive to alantolactone. In the Balb/c strain, the alantolactone concentration used to obtain the maximum peak was higher (10% – 12%) than the ones corresponding to maximum peak in the Balb/b (6%) and the DBA/2 (6 – 10%) strains. At the 48th h, in the Balb/c strain sensitized with 10 – 12% alantolactone, an increase of the ear thickening was observed, whereas, at 15%, a significant decrease occurred, in agreement with an expected overdose effect. Figure 2 summarizes the results for the four studied strains with different concentrations. At 24 h, it can clearly be observed that at 6% alantolactone concentration, the strains most sensitive to alantolactone are the Balb/b and DBA/2 strains (37% and 25%, respectively); at the same concentration, Balb/c strain showed a 17% ear increase. The highest response for Balb/c-sensitized mice was a 10 – 12% alantone concentration (44% and 46%, respectively).

Histologic Evaluation Because the mouse skin only contains 2 to 3 epidermal cell layers, spongiosis, a clear clue to ACD, cannot be observed in this model. However, when compared to the skin of controls, the skin of mice sensitized and elicited with alantolactone showed an important dermal edema, provoking the dissociation of the conjunctive fibers associated with the proliferation of inflammatory cells of the lympho-histiocytary type. These are characteristics of ACD. The epidermis showed neither intracellular edema nor necrosis (Fig 3).

DISCUSSION

The above-mentioned results clearly show that sensitization to alantolactone not only depends on the concentration of the hapten but also on the mouse strain (this is evident in Fig 2). The sensitizing capacity of alantolactone demonstrated here suggests that the strain with the H-2^b and H-2^d haplotypes (Balb/c, Balb/b, and DBA/2) are high responders, whereas strain C3H/He, with H-2^k haplotype, is a low responder. Similar conclusions concerning the genetic dependence related to the H-2 complex and the sensitizing response to the other haptens, have been reported in murine model [17 – 21]. Genetic dependence of the sensitization to alantolactone was also studied in the guinea pig, where differences were also observed [9].

Toxicity to alantolactone also depends on the strain, the most sensitive one being DBA/2 (60% of the experimental group sensitized to 10% alantolactone solution died after 4 d). We do not know the nature of the toxic effect; nevertheless, in vitro, at high alantolactone concentration (up to 1×10^{-5}M), we observed that an important cell death occurred during the incubation in the LTT assay (unpublished results). The most resistant one seems to be the Balb/c strain.

Flare-up to alantolactone in man has been reported [4] and, for this reason, alantolactone is considered as a potent sensitizer.

In conclusion, the murine model has been successfully used for sensitization to a sesquiterpene lactone, alantolactone. As in the guinea pig model, it was found that alantolactone was a moderate sensitizer. However, it is interesting to note that no Freund's adjuvant was used for sensitization.

Among the four studied strains, two models were satisfactory: Balb/b and Balb/c strains. The maximum reaction in Balb/b mice was observed for a dose of alantolactone lower (6%) than in Balb/c mice (10 – 12%). The response at 24 and 48 h in this strain was typical of a type IV contact hypersensitivity. In Balb/c mice, the maximum reaction was observed for a 10 – 12% concentration of alantolactone at 24 h. We have verified that this reaction was an immunologic one as, in histologic cuts, no basophil infiltrate was found while an important lymphocyte infiltrate was present. In our hands, the most satisfactory model for alantolactone sensitization is the Balb/c strain.

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