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## TITLE PAGE

# **Repeated monthly epicutaneous challenges with diphenylcyclopropenone result in a clinically reproducible level of contact allergy in de novo sensitized individuals**

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This study was performed in Odense, Denmark

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## CONFLICT OF INTEREST

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DEAR EDITOR, Diphenylcyclopropanone (DPCP) has been used as an experimental contact allergen in humans and for topical immunotherapy of patients with alopecia areata. However, the efficacy is mostly based on case series. Randomized controlled clinical trials are lacking<sup>1</sup>, as is detailed descriptions of how repeated topical exposure to DPCP affect the level of hypersensitivity. We studied the time-course of the developing cutaneous immune reactions to DPCP in 10 de novo sensitized volunteers using visual grading and caliper reading of skinfold thickness.

Ten healthy volunteers (1 woman and 9 men) between 20 and 41 years old (mean age: 24.5 years) were recruited. Exclusion criteria were atopic dermatitis or treatment with topical corticosteroids or other immunosuppressive medication. Written informed consent and Ethical Committee approval (Study-ID: S-20120085) were obtained.

Contact sensitivity to DPCP was induced by a single dose of DPCP (CAS no. 886-38-4; Alfa Aesar, Karlsruhe, Germany) of  $30 \mu\text{g}\cdot\text{cm}^{-2}$  (22.8  $\mu\text{l}$  of 0.125% DPCP in acetone) applied to a filter paper in a 12 mm Finn chamber (SmartPractice, Phoenix, AZ, USA), and placed on the upper buttock for 48 h<sup>2</sup>. Sensitization to DPCP was confirmed after 4 weeks by an elicitation challenge on the inner upper-arm consisting of a dose-series of 5 DPCP-loaded patches. The DPCP doses were applied to filter-paper-lined 8 mm Finn Chambers as 10  $\mu\text{l}$  of the appropriate concentration of DPCP in acetone to give the required dose range, increasing by 60 % increments. The doses were 1.24, 1.98, 3.17, 5.1 and  $8.12 \mu\text{g}\cdot\text{cm}^{-2}$ . In addition, a vehicle control patch loaded with 10  $\mu\text{l}$  of acetone was applied. Elicitation patches were removed exactly 6 h after application<sup>2</sup>. The responses were assessed visually at 24 h and 48 h after application and graded as negative (-), doubtful (?+), 1+, 2+, 3+ or 4+ using a modified version (based on our previous experience with DPCP) of the International Contact Dermatitis Research Group clinical scoring system. Reactions were also quantified as increase in skinfold thickness measured with a modified spring-loaded skinfold caliper (Harpenden skinfold caliper, British Indicators, England). Each individual received a series of four or five repeated challenges at 4-weekly intervals. Each rechallenge consisted of three challenge doses, which were applied with filter-paper-lined 8 mm Finn Chambers and removed after 6 h. Visual assessment and skinfold thickness measurements were done on the three challenge doses at 48 h. Mean skinfold thickness was determined from duplicate measurements at each vehicle control and challenge site. Vehicle control values were subtracted from the challenge site values and the results were summed ( $\Sigma$ ) giving an approximation of the area under the dose-response curve, representative of the overall reactivity to DPCP from each participant<sup>3,4</sup>. The data were analyzed using the Friedman test (level of significance:  $P < 0.05$ ). In order to avoid increased rechallenge reactivity at previously DPCP-

challenged sites<sup>5</sup>, patches were applied on previous non-challenged sites by using placement templates. Repeat challenges were also carried out alternately on the inner aspect of the left and right upper arm.

Nine of the 10 volunteers sensitized with DPCP gave positive responses to the initial elicitation challenge at 48 h (the non-respondent volunteer reacted at the next challenge (i.e. rechallenge 1)). The visual scores at the 5 challenged sites ranged from no reaction to 3+ and the summed scores for the 5 sites were mean  $\pm$  SEM:  $8.0 \pm 1.45$  ( $n=10$ ). Due to the overall weak responses to the initial elicitation challenge and because of space limitations on the inner upper-arm, the top 3 challenge doses were subsequently used in repeat challenges for 9 of the participants, but for the 10th who gave strong responses, 3 intermediate doses were used. The summed visual scores for the reactions at the top 3 challenge doses and the 3 intermediate doses for the 10th volunteer were mean  $\pm$  SEM:  $6.2 \pm 0.99$  ( $n=10$ ) and the summed increase in skinfold thickness was  $0.96 \pm 0.2$  mm ( $n=10$ ) (Fig. 1a and b). Four weeks later, responses to the first repeat challenge with 3 doses of DPCP showed a significant increase in the strength and grade of the reactions, some individuals developing vesicles or bullae (Mean  $\pm$  SEM:  $10.5 \pm 1.44$ ;  $n=10$ ). The increased clinical reactions were reflected in the increased skinfold thickness measurements (Mean  $\pm$  SEM:  $3.55 \pm 0.7$  mm;  $n=10$ ). At subsequent repeated challenges at 4-weekly intervals, there was no further increase in the visual grades of the responses – the magnitude of the response having reached a plateau. The skinfold thickness reached a plateau at rechallenge 2 (Fig. 1b). The almost identical time-course generated by caliper responses and visual scores was ascertained by measuring the correlation coefficient in a scatter plot. A strong positive correlation ( $R^2 = 0.9337$ ) was identified between the skinfold thickness measurements and the visual scores (Fig. 2).

We have shown that immune responses to repeated monthly challenges with DPCP in de novo sensitized individuals reach a plateau after two (by visual grading) and three challenges (by skinfold measurements), respectively. These findings indicate the presence of regulatory control mechanisms that prevent progressive augmentation of immune responses by repeated antigen exposure. However,

the putative control mechanism(s) whereby the repeated challenge responses are regulated remains to be determined. Few studies have attempted to quantify the time-course of CA. Of the few published studies, the majority have investigated the time-course of repeated nickel challenges by assessing the visual grading of responses. It has been demonstrated that individuals with established CA to nickel exhibit a reproducible and relatively constant level of reactivity after 6-months<sup>6</sup>. This observation was concordant with another study in which 30 nickel-allergic patients were tested with a nickel dilution series on 4 separate test occasions over 7 months. No upgrading or downgrading of the patch test reactivity was seen by visual scoring, however, a large intra-individual variation in threshold responses was observed<sup>7</sup>. The experimental model presented here uses a continuous variable (skinfold thickness) that more accurately can detect differences in the level of immune response to repeated challenges with a contact allergen as opposed to relying only on subjective (e.g. visual grading) forms of assessment. This can be derived from the fact that the visual scores reached a maximum after 8 weeks and caliper readings after a further 4 weeks (12 weeks after sensitization) indicating that the skinfold measurements were more sensitive compared with visual grading. In addition, we can conclude that repeated applications with an appropriate dose-series of DPCP at a fixed time interval ultimately result in cutaneous immune responses with constant levels of reactivity over time.

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## FIGURE LEGENDS

### FIGURE 1

**Initial amplification of the immune response to DPCP followed by a constant level of immune reactivity.** Sensitized volunteers received an initial elicitation challenge with DPCP followed by four or five repeated monthly challenges. (a) Visual assesment (numerical values) and (b) skinfold thickness measurements of responses at 48 h relative to a vehicle control site, were summed ( $\Sigma$ ) for the three challenge sites (Mean  $\pm$  SEM;  $n = 10$ ;  $^{\vee}$ Rc5:  $n = 5$ ). The SEM was calculated using the mean values for area under the dose response curve (AUC) for the included volunteers. Significant changes from initial elicitation challenge are designated by: \*\*P < 0.01; \*\*\*P < 0.001. VC: vehicle control; IEC: initial elicitation challenge; Rc1-5: rechallenge 1-5.

### FIGURE 2

**The strength of the linear association between skinfold thickness measurements and clinical scores.** A strong positive correlation ( $R^2 = 0.9337$ ) was identified between the caliper responses and the clinical scores. IEC: initial elicitation challenge; Rc1-5: rechallenge 1-5. IEC-Rc4:  $n = 10$ ;  $^{\vee}$ Rc5:  $n = 5$ .





