

In vitro human T cell responses to diphenycyprone

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doi:10.1111/cod.12697

Key words: CD4⁺ T cell; CD8⁺ T-cell; diphenycyprone; experimental contact sensitivity; human; *in vitro*; lymphocyte proliferation.; T cell response; time course.

The experimental contact sensitizer diphenylcyclopropanone [diphenycyprone (DPCP)] is in clinical use for topical immunotherapy of various conditions, including

viral warts and alopecia areata (1–4). It is also used as an experimental tool for investigation of human immune responses; the contact allergic reactions elicited by DPCP patch tests can be quantified objectively, and the effects of stress, such as intense physical exertion (5), altitude (6), or coexisting human immunodeficiency virus infection (7), can be determined. In order to address possible mechanisms underlying altered immune responses, it is essential to have *in vitro* systems that allow analysis of the cellular elements involved in the response, such as effector/memory T cells and regulatory T cells. So far,

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Conflicts of interest: The authors declare no conflict of interest. Funding: This work was supported by internal departmental research funds. C. Lai is a Wellcome Trust Research Training Fellow.

no such *in vitro* system has been developed for DPCP, but we report here a pilot study in which we have elicited *in vitro* lymphocyte responses to this allergen, defining the effective stimulating doses of DPCP. We have used this system to show that reactivity in peripheral blood is slow to develop, and our preliminary evidence indicates that the response is predominantly mediated by CD8⁺ cells.

Methods

Patients/volunteers: 13 patients with verrucae or alopecia areata attending the dermatology department and 7 healthy volunteers were sensitized with DPCP. The research component of the study was conducted under local research ethics committee approval. A single application of 30 µg/cm² was applied to the upper inner arm on a Finn Chamber® (12 mm in diameter); the chamber was left in place for 48 h. Four weeks later, the degree of contact allergy was determined by the application of four or five doses of DPCP (0.48, 0.76, 1.22, 1.95 and 3.125 µg) on standard (8-mm diameter) Finn Chambers® to the upper inner surface of the opposite arm for 6 h. Reactions at each challenge site were measured with Harpenden Skinfold Calipers, as described previously (8, 9).

Patients received applications of DPCP as treatment for warts or alopecia once weekly to monthly. DPCP treatment was discontinued on clearance of warts or resolution of alopecia. Three healthy volunteers were rechallenged with four doses of DPCP at various intervals. Blood was taken at intervals of 4–8 weeks to examine *in vitro* reactivity to DPCP.

Peripheral blood mononuclear cells were isolated from venous blood by centrifugation over Lymphoprep. Cells were cultured at 10⁶ per well in 24-well plates for 6 days in the presence of a range of concentrations (2–128 µM) of DPCP dissolved in dimethylsulfoxide. Lymphocyte proliferation was quantified by measurement of [³H]thymidine incorporation, as described previously (10). Responses were determined at various times after induction of allergic sensitivity.

The phenotype of responding cells was determined in two individuals. After 6 days of culture with the optimal concentration of DPCP, cells were stained for Ki67 (proliferation marker) and CD4 or CD8. Staining was assessed by flow cytometry.

Results

All 20 subjects showed positive contact allergic responses to the DPCP patch test series 4 weeks after sensitization. Regarding lymphocyte responses, DPCP concentrations

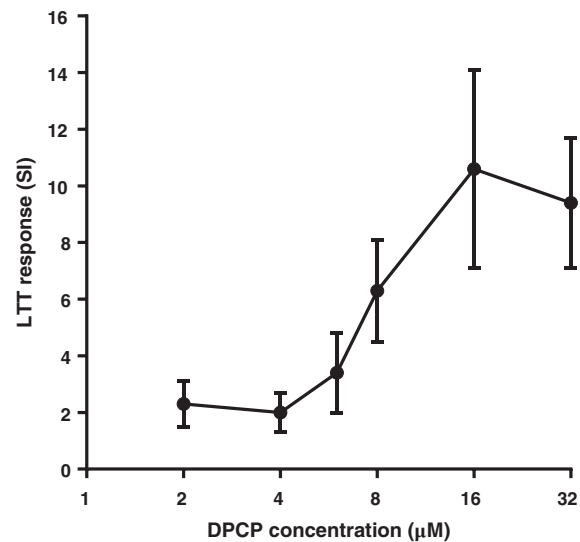


Fig. 1. Maximal lymphocyte transformation test (LTT) responses to diphenycprone (DPCP). The best response from each subject was selected ($n = 8$). The LTT response is shown as the stimulation index (SI) with the mean $\pm$ standard error of the mean at each concentration of DPCP.

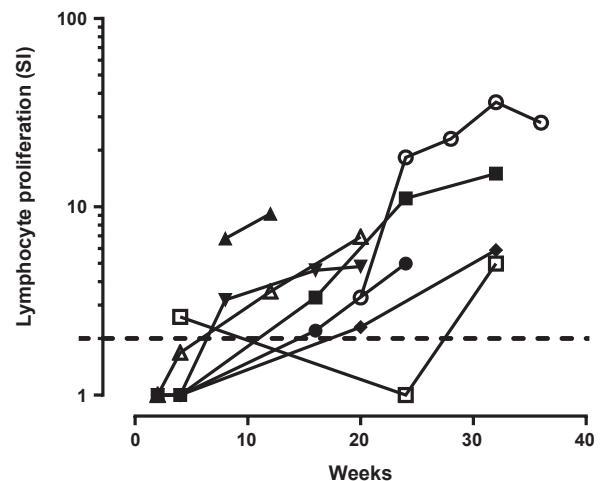


Fig. 2. The change in lymphocyte proliferation responses elicited by 16 µM diphenycprone over time. Data are from a total of 8 subjects who had positive lymphocyte transformation test responses. The results for each subject are joined. The dashed line at a stimulation index (SI) of 2 is the threshold for a positive response.

from 2 to 64 µM elicited positive lymphoproliferative responses in 6 patients and in 2 healthy subjects. Peak responses were ultimately seen at 16 or 32 µM in reactive subjects (Fig. 1); higher concentrations were toxic. However, in 12 subjects who gave repeated blood samples, negative or low responses were seen for up to 20 weeks

after sensitization. In the 8 subjects who developed positive lymphocyte proliferation assay responses over time, repeated tests beyond 20 weeks showed the time course of the appearance of positive lymphocyte reactivity in blood (Fig. 2). For 2 subjects, enough cells were available at the end of the culture period to allow immunophenotyping and flow cytometry. In both of these, a dominant proliferative response was seen in the CD8 populations (32.5% and 57.8% of total cells), whereas the response of CD4⁺ cells was minimal (1.5% and 4.7% of total cells).

Discussion

We have shown for the first time that it is possible to detect lymphocyte responses to DPCP *in vitro*. This is a small pilot study, but it establishes the methodological principles that can now be developed and optimized. Importantly, the responses to DPCP only become detectable in cultures containing quite large cell numbers. Usually, 2.5×10^5 cells cultured in 200 μ l in 96-well plates allow detection of lymphoproliferative responses to protein antigens and some water-soluble contact allergens such as nickel. However, we had observed in previous work developing lymphocyte proliferation assays with 2,4-dinitrochlorobenzene (DNCB) that larger cell numbers were required, and this was also the case for DPCP. This might indicate that, initially, there are very low

numbers of hapten-specific T cells in the peripheral blood, even though strong clinical contact allergy is detectable in the skin. The second observation was that reactivity in peripheral blood is slow to develop, really only becoming positive in the majority of tested subjects by 20 weeks after sensitization. In retrospect, a similar phenomenon was seen when we were developing lymphocyte proliferation assays for DNCB, but it was not analysed formally in terms of the time course. It is likely that it reflects the fact that, as hapten-specific effector/memory T cells are generated, they are initially recruited to the skin, where they take up residence as resident memory T cells – hence explaining why contact allergy is demonstrable in the skin within 7–14 days of sensitization (11). It is probably not until the skin compartment is fully populated that surplus cells are detected in the circulation. An additional observation in this pilot study is that, as has been shown for other contact allergens, the dominant response appears to be in the CD8⁺ T cell population (10, 12, 13). Clearly, our numbers are too small for us to be definite about this, but they do give an indication that fits with other work in the immunology of contact allergy.

In conclusion, this method can now be refined, optimized and taken forward for use in analysing the reasons for reduced contact allergic reactivity following various physiological stresses or the effects of different disease states in humans.

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