

In vitro primary sensitization of hapten-specific T cells by cultured human epidermal Langerhans cells — a screening predictive assay for contact sensitizers

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Summary

Background The need to develop predictive tests which could identify potential allergens has been recognized for many years. There is as yet no accepted *in vitro* method for the assessment of contact sensitizers.

Objective We have tested the ability of a range of contact allergens to induce *in vitro* primary sensitization of autologous T cells.

Method T-cell proliferation induced by haptens using 2-day cultured human Langerhans cells as antigen-presenting cell was assessed by ³H thymidine incorporation. Antigen specific stimulation was calculated as stimulation indexes.

Results Strong allergens induced *in vitro* a primary T-cell response in all (trinitrophenyl, TNP: 13/13) or in the majority (fluorescein isothiocyanate, FITC: 7/10) of experiments. An irritant, sodium dodecyl sulfate (SDS), failed to generate a significant T-cell proliferation in any of the experiments (0/10). We obtained a significant lymphoproliferative response to weak sensitizers only in a limited number of experiments: (coumarin: 1/12, citronellal: 0/10, hydroxycitronellal: 2/8). *p*-Phenylenediamine (PPDA), a prohaptens and highly sensitizing chemical *in vivo*, generated primary sensitization *in vitro* in only one of six experiments, while Bandrowski's base (BB), a metabolization product of PPDA induced a significant T-cell response in all six experiments.

Conclusion The present *in vitro* model allows discrimination between two groups of substances: strong contact sensitizers (TNP, FITC, BB) on the one hand and weak sensitizers (coumarin, citronellal and hydroxycitronellal) and irritants (SDS) on the other hand. It could be used as a screening *in vitro* assay to eliminate strong contact allergens before further predictive animal tests have to be performed.

Keywords: contact sensitivity, Langerhans cell, *in vitro* primary sensitization, alternative test methods, contact allergens, irritants

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Introduction

Contact hypersensitivity is a delayed type T-cell mediated immune reaction occurring after epicutaneous immunization and challenge with reactive haptens. There is now evidence that Langerhans cells (LC), the dendritic

cells from epidermis, play a crucial role in the induction of this process by picking up the allergen in the epidermal layer and then migrating to regional lymph nodes where they trigger specific T-cell activation [1]. The need to develop predictive tests which could identify potential allergens and thus reduce the hazard resulting from occupational and consumer exposure to new products has been recognized for many years [2,3]. Seven *in vivo*

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guinea-pig methods are currently accepted by the regulatory authorities [4,5] and two mouse methods, the mouse ear swelling test and the local lymph node assay are recommended as screening tests by the Organization for Economic Cooperation and Development [4]. However, extrapolation from animal models to humans is not always possible. The animal welfare movement, as well as an increasing understanding of immunological mechanisms involved in contact sensitivity have prompted an accelerated development of *in vitro* testing methodologies. There is, as yet, no accepted method for the *in vitro* identification of skin sensitizing chemicals. Indeed, the use of full *in vitro* human assays for the assessment of contact sensitivity responses has been limited chiefly to the study of strong sensitizers and peripheral blood mononuclear cells have been used as antigen-presenting cells (APC) [6,7]. Hauser *et al.* first used murine cultured LC to generate *in vitro* hapten-specific T cells from non-sensitized mice [8]. In a previous study we provided the first direct evidence for the ability of human LC to induce *in vitro* primary sensitization of naive T cells to hapten [9]. We showed that 2-day cultured human LC (cCL) can generate primary sensitization of autologous T cells to trinitrophenyl (TNP). The proliferative T-cell response was hapten-specific, as shown in a secondary reaction by an accelerated response to TNP and by the absence of proliferation to an irrelevant hapten such as fluorescein isothiocyanate (FITC) [9]. The present study is aimed at testing the ability of a range of contact sensitizers to induce *in vitro* primary sensitization of autologous T cells using cultured human Langerhans cells as APC.

Materials and methods

Culture medium

The culture medium was RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated human AB serum, 10 µg/mL gentamycin, 2 mM L-glutamine and 1 µg/mL indomethacin (Sigma, St Louis, MO, USA): hereafter designated as complete medium.

Langerhans cell-enriched epidermal cell suspensions

Epidermal cell suspensions were obtained from normal human skin (plastic surgery) by trypsinization: 0.05% trypsin (Difco Laboratories, Detroit, MI, USA), 1 h at 37°C. They were enriched in LC by density gradient centrifugation on Lymphoprep (Flobio, Courbevoie, France). Cells from the interface containing 10–18% LC were incubated (10^6 /mL) for 48 h at 37°C in complete

medium supplemented with 200 U/mL human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; Genzyme, Cambridge, MA, USA). After incubation viable cells (cLC: 15–28% LC) were recovered by Lymphoprep density centrifugation. These LC were used as stimulating cells in the T-cell proliferative assays.

T cells

Autologous T cells were isolated from peripheral blood as previously described [10]. The T-cell population contained 95% or more CD3-positive cells, as assessed by FACS and was unable to proliferate in response to Concavalin A (Sigma, 5 µg/mL).

Test chemicals

The allergens chosen for the study were 2,4,6-trinitrobenzene sulfonic acid (TNBS, Sigma) and fluorescein isothiocyanate (FITC, Sigma), known as strong contact allergens; *p*-phenylenediamine base (PPDA, Sigma), a weaker but still highly sensitizing agent and its oxidation product Bandrowski's base (BB, ICN); several fragrance materials with low contact sensitizing capacity: citronellal (Sigma), hydroxycitronellal (Janssen), coumarin (Sigma). An irritant, sodium dodecyl sulfate (SDS, Sigma), was used for control of hapten specificity. All chemicals were used in aqueous solutions; citronellal, hydroxycitronellal, coumarin and dihydrocoumarin were previously dissolved in ethanol and BB — in dimethyl sulfoxide (DMSO, Sigma).

The working concentrations, including the highest non-toxic concentrations, were as follows: PPDA and BB 1–3 µg/mL; citronellal 5–50 µg/mL; hydroxycitronellal 5–10 µg/mL; coumarin 10–100 µg/mL; SDS 5–50 µg/mL. DMSO did not exceed 9 µg/mL and ethanol 25 µg/mL in the culture medium. To circumvent problems related to toxicity of TNBS and FITC, antigen presenting cells were haptenated before they were cultured with T cells. TNP modification was performed according to the method of Shearer [11]. Cell pellets were resuspended in Hank's balanced salt solution (HBSS) (pH 7.2) containing 5 mM TNBS. Cells were incubated for 10 min at 37°C and then washed out extensively. FITC modification was performed in the same way with HBSS containing 100 µg/mL of the hapten. The other chemicals were added directly to cell cultures.

Lymphoproliferative assays

The cells were cultured in round-bottomed microtitre plates (10^5 autologous T cells and graded numbers of

2-day incubated LC in a final volume of 200 μ L complete medium per well) at 37°C in a humidified atmosphere of 5% CO₂ in air. Triplicate cultures for each allergen concentration were maintained for 3–5 days. A kinetic study of T-cell proliferation was performed by pulsing the cells with 1 μ Ci of ³H-thymidine for the final 18 h of culture. Results from triplicate wells were expressed as mean cpm $\pm$ SD. Antigen-specific stimulation was calculated as stimulation indexes (SI = uptake of isotope in stimulated culture/uptake of isotope in non-stimulated culture). A chemical was regarded as a sensitizer in our test if at least one concentration resulted in a SI of 3 or greater. Statistical analyses were performed using Student's *t*-test.

Results

Optimization of the primary T-cell response to TNP-modified LC

We first determined the optimal conditions for *in vitro* sensitization of T cells by TNP-treated cLC. To this end, we analysed the kinetics of the primary T-cell response induced by a graded number of TNP modified fresh or 2-day incubated LC. T lymphocytes (10^5) were cultured 3–7 days with 1.10^3 – 8.10^3 LC in a final volume of 200 μ L

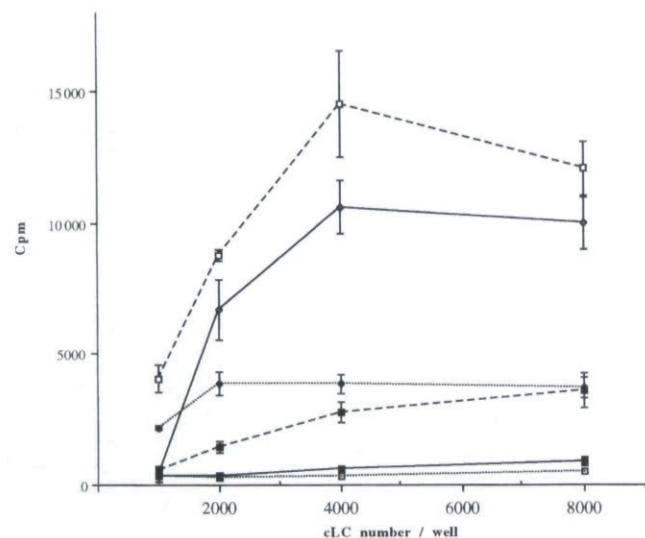


Fig. 1. *In vitro* primary T-cell response to a graded number of TNP-modified cLC. T lymphocytes (10^5) were co-cultured with 2-day cLC, treated or not with TNP. T-cell proliferation was assessed at day 4 (D4), 5 (D5) or 6 (D6) by ³H thymidine incorporation for the final 18 h of culture. Results are expressed as mean cpm $\pm$ SD of triplicate cultures. $\cdots\Box\cdots$, T cells + cLC D4. $\cdots\blacklozenge\cdots$, T cells + TNP-cLC D4. $\cdots\Box\cdots$, T cells + cLC D5. $\cdots\blacklozenge\cdots$, T cells + TNP-cLC D5. $-\Box-$, T cells + cLC D6. $-\blacklozenge-$, T cells + TNP-cLC D6.

complete medium per well. As previously reported [9], the addition of TNP-modified fresh LC (fLC) to autologous T cells did not induce detectable lymphoproliferative response (data not shown). By contrast, TNP-modified cLC generated a primary T-cell response which was the highest when 4.10^3 LC were cultivated with 10^5 T cells (Fig. 1). The autologous response to cLC was not significant and was proportional to the number of LC. The best stimulation indexes were recorded after 5 days of culture (SI = 20) but were significant at day 4 and 6 as well (SI = 13.3 and 6.2 respectively). Thereafter we studied the T-cell response to the other chemicals by

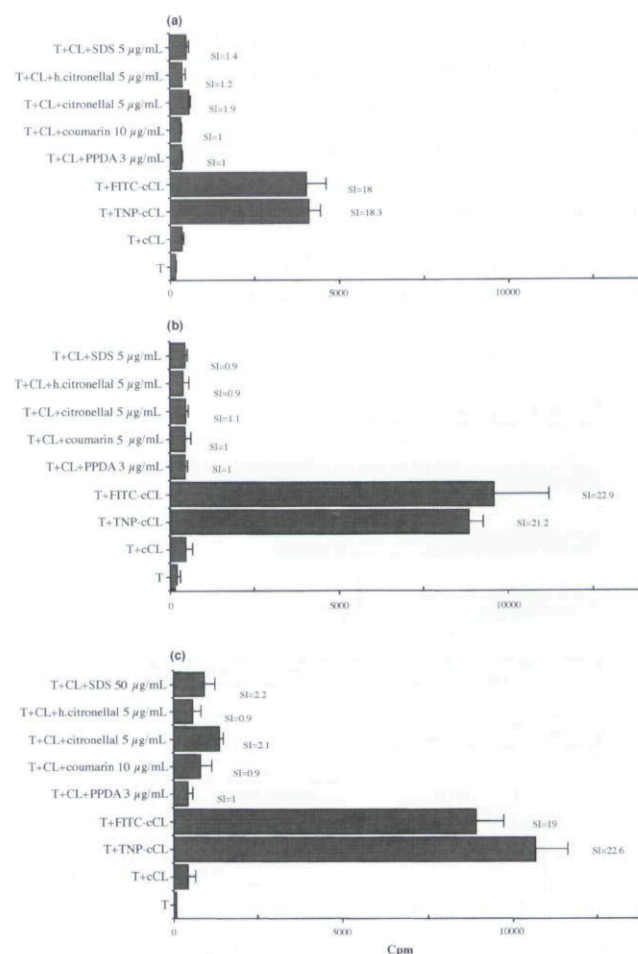


Fig. 2. *In vitro* T-cell proliferative responses to cLC modified by various chemicals. T-cell proliferation was assessed by ³H thymidine incorporation during the final 18 h of culture. Results are expressed as mean cpm $\pm$ SD of triplicate cultures. Antigen specific stimulation was calculated as stimulation indexes (SI = uptake of isotope in stimulated culture/uptake of isotope in non-stimulated control culture). (a) Day 4. (b) Day 5. (c) Day 6.

Table 1. Highest T-cell responses to chemical treated cLC. Antigen specific stimulation was calculated as stimulation indexes (SI = uptake of isotope in stimulated culture/uptake of isotope in non-stimulated control culture)

	TNP	FITC	Coumarin	Citronellal	Hydroxycitronellal	PPDA	SDS
Number of experiments	13	10	12	10	8	6	10
Number of experiments in which a primary T-cell response was generated	13	7	1	0	2	1	0
Range of highest stimulation indexes	3.5–25.4	2.4–23.8	0.9–3.2	1.1–2.2	1.0–8.0	1.3–2.7	1.3–2.7
Mean stimulation indexes $\pm$ SD	12.8 $\pm$ 7.4	10.1 $\pm$ 8	1.8 $\pm$ 0.7	1.6 $\pm$ 0.4	2.6 $\pm$ 2.3	1.6 $\pm$ 1.2	1.8 $\pm$ 0.4

applying the conditions in which we had obtained the highest responses to TNP, i.e. incubation of 10^5 T cells with 4.10^3 cLC for 4 to 6 days. This allowed us to test several concentrations of the chemicals in each experiment despite the limitations in the number of T cells and epidermal cells.

In vitro primary T-cell response to autologous cLC modified by various chemicals

We analysed the *in vitro* proliferative T-cell response to cLC after treatment of these cells with various chemicals.

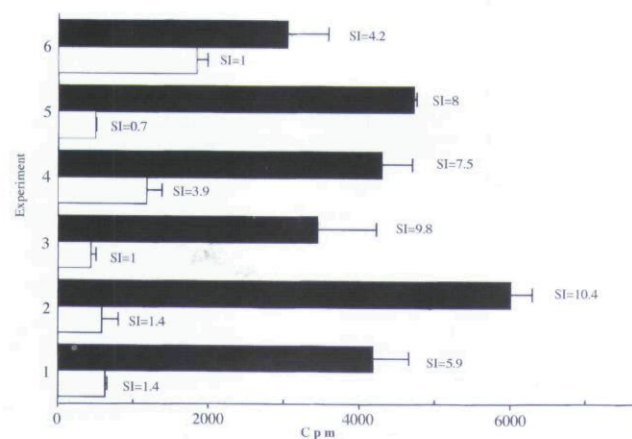


Fig. 3. Comparative *in vitro* T-cell proliferative responses to PPDA and BB. T lymphocytes (10^5) were co-cultured for 4–6 days with 2-day cLC. PPDA and BB were added directly to cell cultures in final concentrations of 1–3 $\mu\text{g}/\text{mL}$. T-cell proliferation was assessed by ^3H thymidine incorporation during the final 18 h of culture. Concentrations of chemicals inducing the highest proliferation in a given experiment are represented on the figure. Results are expressed as mean cpm $\pm$ SD of triplicate cultures. Antigen specific stimulation was calculated as stimulation indexes (SI = uptake of isotope in stimulated culture/uptake of isotope in nonstimulated control culture, i.e. T + cCL for PPDA and T + cCL + DMSO for BB). ■, T + cCL + BB. □, T + cCL + PPDA.

Data from a representative experiment are shown on Fig. 2. The calculation of stimulation indexes was mandatory, as most of the allergens were not soluble in water and the corresponding control cultures contained different solvents (ethanol or DMSO) in equivalent concentrations. We chose a stimulation index of 3 or greater as an arbitrary criterion for the induction of primary sensitization. As shown in Fig. 2, significant stimulation indexes were obtained with highly sensitizing chemicals (TNP and FITC). Weak sensitizers (coumarin, citronellal, hydroxycitronellal) did not induce a significant T-cell response. A stimulation index lower than 3 was obtained with SDS, an irritant included in the study as a control of

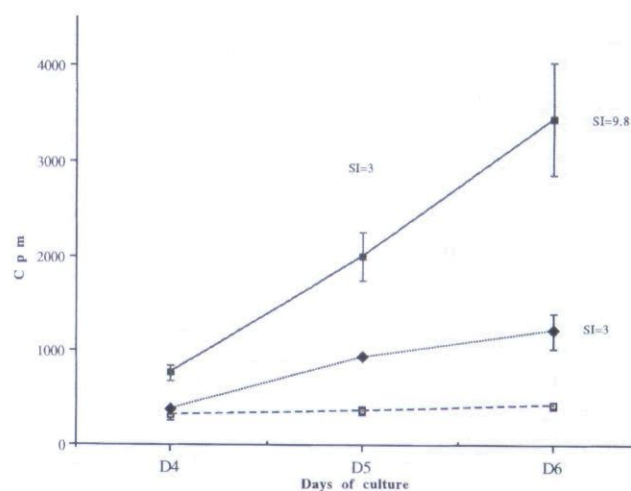


Fig. 4. Kinetics of primary T-cell response to various concentrations of BB. T lymphocytes (10^5) were co-cultured for 4–6 days with 2-day cLC in the presence of BB at 1–3 $\mu\text{g}/\text{mL}$. T-cell proliferation was assessed by ^3H thymidine incorporation during the final 18 h of culture. Results are expressed as mean cpm $\pm$ SD of triplicate cultures. Antigen specific stimulation was calculated as stimulation indexes (SI = uptake of isotope in stimulated culture/uptake of isotope in nonstimulated control culture). - - □ - - 1 $\mu\text{g}/\text{mL}$. ··· ◆ ··· 2 $\mu\text{g}/\text{mL}$. ··· ■ ··· 3 $\mu\text{g}/\text{mL}$.

happen specificity of the T-cell response. However PPDA, a strong contact sensitizer, did not induce a significant lymphoproliferative response.

The calculation of stimulation indexes makes possible the comparison of the primary T-cell response induced by the different haptens in several experiments carried out with different donors. We have shown on Table 1 the highest stimulation indexes registered within a single experiment. TNP induced primary sensitization in all 13 experiments and a dose response relationship was observed. FITC failed to induce primary *in vitro* response in only three of 10 experiments. The highest proliferative responses to TNP and FITC were usually obtained after 5 days of culture. T cells did not proliferate significantly to any working concentration of SDS and citronellal. The other haptens induced significant T-cell proliferation in a limited number of experiments. The responses were, however, dose-dependent and were the highest after 5 days of culture with coumarin (50 µg/mL) and PPDA (3 µg/mL) and after 6 days of culture with hydroxycitronellal (10 µg/mL).

In vitro primary T-cell response to BB-modified cLC

We have shown in a previous *in vitro* study that secondary proliferative responses of peripheral blood monocytes from patients with contact sensitivity to PPDA were higher when using as a stimulating agent BB, a metabolism product of PPDA than when using PPDA itself [12]. We therefore compared the ability of BB and PPDA to induce *in vitro* a primary T-cell response in the present sensitization assay. As shown on Fig. 3, BB generated significant T-cell proliferation in all experiments, while PPDA was effective in only one of 6 assays. BB generated the highest T-cell responses after 6 days of culture but in some experiments stimulation indexes greater than 3

were recorded after 5 days of culture as well. Furthermore, a dose response relationship was observed (Fig. 4). The best T-cell response was generated by BB at 3 µg/mL in four of the six experiments.

Statistical analysis

The results from the statistical analysis of stimulation indexes obtained with each of the chemicals are shown on Fig. 5. The present *in vitro* model allows to differentiate strong contact allergens (TNP, FITC, BB) from weak allergens (coumarin, citronellal, hydroxycitronellal) and irritants ($P < 0.05$). However, no significant differences were found between the stimulation indexes of weak allergens and an irritant (SDS).

Discussion

The complexity of cellular and molecular interactions which characterize the induction and elicitation phase of a contact allergic reaction has hampered the development of alternative assays to animal testing methods. Efforts have concentrated on several areas of investigation. Quantitative structure-activity relationships models have been successfully applied to predict the sensitizing potential of homologous series of chemicals but are inefficient with molecules that undergo metabolism in the skin [13]. Evaluation of epidermal cytokines is a promising approach to *in vitro* assessment of sensitizing potential but the observation that the production or increased production of some keratinocyte- or LC-derived cytokines is induced only by sensitizers and not by irritants, should be confirmed [14,15].

The present study was aimed to develop a model of *in vitro* priming of human naive T cells to contact allergens with different sensitizing potential. The measurement of *in vitro* antigen-induced primary proliferative responses in man provides an interesting alternative to animal testing, as it obviates the necessity of intraspecies extrapolation. We have used in our model the cells that are physiologically involved in the generation of contact sensitivity response, i.e. T cells and LC. Furthermore we have reproduced, as far as possible the conditions necessary for a functional *in vitro* maturation of LC. While the role of LC as APC is now firmly established [16,17], the importance of a short-term incubation of LC with GM-CSF has been reported only recently. It has been found that after 2–3 day *in vitro* incubation both murine and human LC undergo profound phenotypic changes, as an enhancement in the expression of MHC class I and II (Ia) antigens, LFA-3, ICAM-I and B7 molecules, a decrease of CD1a antigens and a loss of FcγRII [18–20]. Therefore after incubation LC seem to

TNP	NS	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
	FITC	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
		BB	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
			PPDA	NS	NS	NS	NS
				Coumarin	NS	NS	NS
					NS	NS	NS
					Citronellal	NS	NS
						Hydroxycitronellal	NS
							SDS

Fig. 5. Statistical analysis of mean stimulation indexes obtained with each of the chemicals. The degree of significance P was calculated using Student's t -test. (NS, not significant.)

acquire most of the features of lymphoid dendritic cells suggesting that cultured LC may represent the *in vitro* analogues of antigen-bearing LC that have migrated to regional lymph nodes [18,21]. GM-CSF was reported to enhance murine LC viability during *in vitro* incubation [22] and by adding it to culture medium we increase the yield in viable human LC from 10 to 20% [9].

In a previous study we obtained for the first time an *in vitro* T-cell primary proliferative response to a hapten (TNP) using human LC as APC [9]. We found that LC incubated 2 days in the presence of GM-CSF, but not fresh LC were able to sensitize T cells to TNP, thus confirming the results obtained by Hauser and Katz in a murine model [23]. In the present study we induced primary sensitization to TNP in all 13 experiments. We used the same model to investigate the ability of other strong allergens to sensitize T cells *in vitro*. FITC has been shown to induce *in vitro* primary sensitization of murine responder lymphnode cells using as APC syngeneic dendritic cells [24]. More recently Hauser *et al.* reported *in vitro* priming of TH cells by FITC-modified murine LC [25]. We were able to generate a primary response to FITC in seven of 10 experiments. To our knowledge *in vitro* sensitization to FITC using human LC as APC has never been generated before.

PPDA is ranked as a strong to extreme sensitizer by all animal and human predictive tests [26–28]. It produced a significant proliferative response in our *in vitro* model in only one of six experiments. However BB, an oxidation product of PPDA induced primary sensitization in all experiments. We have previously shown that BB induced a dose-dependent secondary proliferative response of peripheral blood mononuclear cells obtained from patients contact sensitive to PPDA which was higher than the response to PPDA itself [12]. We thus provided experimental evidence to the concept of PPDA being the prohaptens in PPDA contact sensitivity [29,30] and suggested that BB could be the immunogenic hapten. The failure to induce reproducible primary sensitization to PPDA in the present *in vitro* system confirms that assumption. We are aware that contact allergy is a reaction involving several organ systems and that it is impossible to develop an *in vitro* model that could reflect all the complex reactions taking place from the moment in which the allergen is applied on the skin to the appearance of skin lesions. A limitation of our model is that it cannot reproduce the metabolism in the dermis of certain chemical compounds, designated as prohaptens [29] to immunogenic moieties. Therefore, it is representative of phenomena occurring in a definite compartment of the contact sensitivity reaction, after the chemical has penetrated in the skin and has undergone modification by enzyme systems in as yet unknown compartment.

Coumarin, citronellal and hydroxycitronellal are widely employed fragrance materials. The failure of citronellal to induce primary T-cell sensitization in the present *in vitro* model is in accordance with the negative results from human predictive testing [31] and reported epidemiological data. Coumarine induced a significant lymphoproliferative response in one out of 12 experiments. Although a human maximization test with coumarin did not sensitize anyone in a group of 25 volunteers [32], case reports of contact sensitization can be found in the literature and therefore we consider the results of our *in vitro* test concerning coumarin as relevant to its capacity to generate contact sensitization *in vivo*. Hydroxycitronellal has been identified as one of the causes of allergic contact dermatitis from household and personal care products and has been included in the fragrance mix of the European standard patch test series. However, the incidence of contact sensitivity to hydroxycitronellal in the general population has been found to be very low, as it failed to elicit a positive patch test reaction in a panel of 9300 healthy volunteers [33]. There is a discrepancy between the results from a number of predictive studies carried out on humans revealing a very low potential for human sensitization [33,34] and the results obtained in animal models which rank it as a moderate sensitizer [30]. It induced a significant T-cell response only in a couple of experiments in our study. This reflects the conflicting results from human and animal predictive tests and patch test data. Two possibilities could be considered to explain that coumarin and hydroxycitronellal induce *in vitro* primary sensitization only in a limited number of experiments. First of all, in the light of the prohaptens concept, they might be metabolized, like PPDA, to other products which would be able to induce a constant and dose dependent T-cell response. Their failure to induce primary sensitization in the majority of the experiments might be also attributed to MHC restriction of the immune response.

SDS which is exclusively an irritant in human pathology, has been used as a model chemical for studying skin response to irritant insult [35,36]. It has been the negative control essential for the validation of a number of human and animal predictive methods aimed at detecting the sensitizing potential of chemical substances. While it has never been shown to elicit contact sensitivity in human volunteers or in the guinea-pig model [2,3], it was reported to produce false positive results in the local lymphnode assay [37–39]. Not only stimulation indexes were higher than 3 but there existed a clear dose-response relationship as well [39]. SDS has been shown to cause LC migration from the epidermis to the draining lymphnodes, presumably by stimulating the production of TNF α by keratinocytes and it has been suggested that

these migrating LC bear 'environmental antigens' which induce a proliferative response [40,41]. In the present *in vitro* model SDS did not induce a significant T-cell proliferation in any of the experiments. This assay provides therefore better control of test conditions, with no interference of extrinsic factors such as 'environmental allergens'.

In conclusion, we presented here an *in vitro* model of human T-cell sensitization by hapten treated cLC. This model allows discrimination between two groups of substances: strong contact sensitizers (TNP, FITC, BB) on the one hand and weak sensitizers (coumarin, citronellal, hydroxycitronellal) and irritants (SDS) on the other hand. It cannot discriminate between weak sensitizers and irritants, nor can it predict the sensitizing potential of prohaptens, such as PPDA. An important advantage of our *in vitro* model is that it does not overestimate the sensitizing potential of irritant substances. It could be used as a screening *in vitro* assay to eliminate strong contact allergens before further predictive animals tests have to be performed.

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