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Novel Predictive Assay for Contact Allergens Using Human Skin Explant Cultures

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Contact allergens sensitize the immune system by the binding to and subsequent activation of Langerhans cells (LCs), the antigen-presenting cells of the skin. At present, new chemicals are usually tested for their contact allergenicity in animal models. To develop an animal-replacing predictive* in vitro *assay for the identification of potential contact allergens, we compared the effects of epicutaneous application of six known contact allergens, five known irritants and two dermatologically inactive chemicals on LCs in skin biopsy cultures from seven healthy donors. Immunohistochemical analysis of cryostat sections of all the biopsies treated with contact allergens showed 1) a large reduction in the number of LCs in epidermis, as evaluated by a decrease in human leukocyte antigens (HLA)-DR-expressing cells, and CD1a-expressing cells and 2) accumulation of the remaining LCs at the epidermal-dermal junction. In contrast, the irritants, inactive chemicals, and solvents did not induce these changes. Morphometrical analysis indicated that the contact allergen-induced reduction in the number of HLA-DR$^+$ and CD1a$^+$ LCs per millimeter of epidermis was significant and was dependent on the concentration of the contact allergens. Flow cytometric analysis of isolated epidermal cells confirmed the immunohistochemical findings. In combination, these results suggest that the culture of* ex vivo *human skin explants provides a promising model to predict potential allergenicity of newly produced chemical compounds and can therefore replace current animal models. (Am J Pathol 1996, 149:337–343)

Allergic contact dermatitis is a cell-mediated delayed-type hypersensitivity that can occur after repeated skin contact with small chemically reactive molecules, the so-called contact allergens.[1] During sensitization, contact allergens are trapped by Langerhans cells (LCs), the principal antigen-presenting cells in the epidermis. These compounds subsequently induce the migration of LCs to the regional lymph nodes where the contact allergens are presented to residential T lymphocytes.[2–4] The most common contact allergen to cause allergic contact dermatitis in man is nickel, to which 10 to 20% of the Caucasian population is allergic.

Until now, *in vivo* and *in vitro* assays for the identification of contact allergenic compounds have been performed in animal models. The guinea pig maximization test predicts contact allergenicity by the ability of chemicals to induce sensitization in the guinea pig.[5–7] The local lymph node assay detects contact allergens by the proliferation rate of T lymphocytes in the draining lymph nodes after application to mouse skin.[8,9] Because of ethical objections and governmental restrictions on the use of animals in experimental models, the interest in alternative, animal-replacing test methods is considerable. However, only few (preliminary) studies have been reported on *in vitro* contact allergen screening methods.[10–13]

To study the possibility of using human skin for a reliable *in vitro* assay to detect potential contact allergens, we investigated the effects of a series of contact allergens, irritants, and non-immunogenic compounds on epidermal LCs in a human skin culture model previously established.[14,15] Cultured human skin biopsies were treated with the

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contact allergens dinitrofluorobenzene (DNFB), dinitrochlorobenzene, $NiSO_4$, oxazolone (OX), diphencyprone (DPC) and $K_2Cr_2O_7$, and the irritants sodium dodecyl sulfate (SDS), croton oil, nonanoic acid (NAA), propylene glycol, and benzalkonium chloride, all applied at previously optimized concentrations.[16] The effects of these compounds on LCs in human skin cultures were determined by studying the distribution of HLA-DR-expressing cells and CD1a-expressing cells in the epidermis by means of immunohistochemical analysis of biopsy sections and flow cytometric analysis of isolated epidermal cells.

Materials and Methods

Skin Organ Cultures

Human skin specimens were obtained from breast reduction surgery or abdomen reduction surgery. Skin specimens were washed in RPMI-1640 supplemented with 1% penicillin/streptomycin and processed for culturing according to our previous reports.[14,15] Briefly, skin biopsies of 6 mm radius were taken from the specimens using biopsy punches (Stiefel Laboratories, Maidenhead, UK). Skin explants were cultured on nitrocellulose filters (Millipore; pore size, 1.2 μm) placed on stainless steel grids at air-liquid interphase, epidermal side up. The grids were then placed in 6-well culture plates (Costar, Cambridge, MA) containing Iscove's modified Dulbecco's medium supplemented with gentamycin (40 μg/l, Life Technology, Breda, The Netherlands) and 10% pooled complement-inactivated normal human serum (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) and cultured at 37°C in 5% CO_2 humidified air.[14]

Application of Chemical Compounds

Chemicals were applied epicutaneously onto the skin explants in pre-evaluated nontoxic concentrations (data not shown). Contact allergens used were 5% (w/v) aqueous $NiSO_4$, 2% and 1% (w/v) aqueous $K_2Cr_2O_7$, 0.2% (w/v) DNFB in acetone/olive oil (A/OO; 4:1), 0.2% (w/v) dinitrochlorobenzene in A/OO, 2% and 1% (w/v) OX in A/OO, 1% and 0.5% (w/v) DPC in A/OO, and 0.5% (w/v) fluorescein di-isothiocyanate (FITC; Nordic Immunologic, Tilburg, The Netherlands) in acetone/dibutylphtalate (1:1). Irritants used were 1% (w/v) aqueous SDS, 0.5% (w/v) aqueous benzalkonium chloride, 0.5% (v/v) croton oil in A/OO, 80% (v/v) non-anoic acid in propane-1-ol and 100% propylene glycol, all obtained from Sigma Chemical Co. (St. Louis, MO).[17] Structurally related non-immunogenic compounds used were 4% and 2% (w/v) 2,3- and 2,4-dichloronitrobenzene (DCNB) in A/OO. As controls, explants were topically treated with solvents. Untreated and treated skin explants were cultured for 24 hours at 37°C in 5% CO_2 humidified air. Cultures were harvested and either embedded in OCT compound (Miles Scientific, Naperville, IL), snap-frozen in liquid nitrogen and stored at −80°C for immunohistochemical analysis, or processed for the preparation of epidermal cell suspensions for flow cytometric analysis.

Immunohistochemical Analysis

Vertical cryostat sections (6 μm) were cut from the frozen skin explants, air dried at room temperature on slides coated with 2% (v/v) 3-aminopropyl-trimethoxysilane (Sigma). After fixation in acetone for 10 minutes at room temperature, sections were washed in phosphate-buffered saline (PBS) and incubated for 30 minutes with the primary monoclonal antibodies against CD1a (OKT-6; Ortho Diagnostics Systems, High Wycombe, UK) or HLA-DR (Beckton Dickinson UK, Oxford, UK), diluted 1:50 and 1:100, respectively, in PBS plus 0.5% bovine serum albumin (BSA). After washing in PBS, the sections were incubated with polyclonal goat anti-mouse Ig peroxidase-labeled antibodies (Dako, High Wycombe, UK) diluted 1:50 in PBS plus 0.5% BSA plus 1% human serum. Positive staining in the sections was detected by peroxidase activity with the substrate 3-amino-9-ethylcarbazole (Sigma). Staining for CD1a and HLA-DR was performed on six sections of each biopsy.

Quantification of LC Migration

The number of LCs in six sections of each cultured skin explant were calculated according to a method described by Bieber et al.[18] The number of LCs per millimeter length of the epidermal surface was measured by computer-assisted morphometrical analysis (Kontron MOP Videoplan). The results were statistically analyzed with a nonparametric analysis of variance, followed by Dunn's post-test in which results were considered significant when $P < 0.05$.

Flow Cytometric Analysis

To prepare epidermal cell suspensions, cultured skin explants were incubated with 0.25% (w/v) trypsin (Sigma) for 1 hour at 37°C and 5% CO_2 humidi-

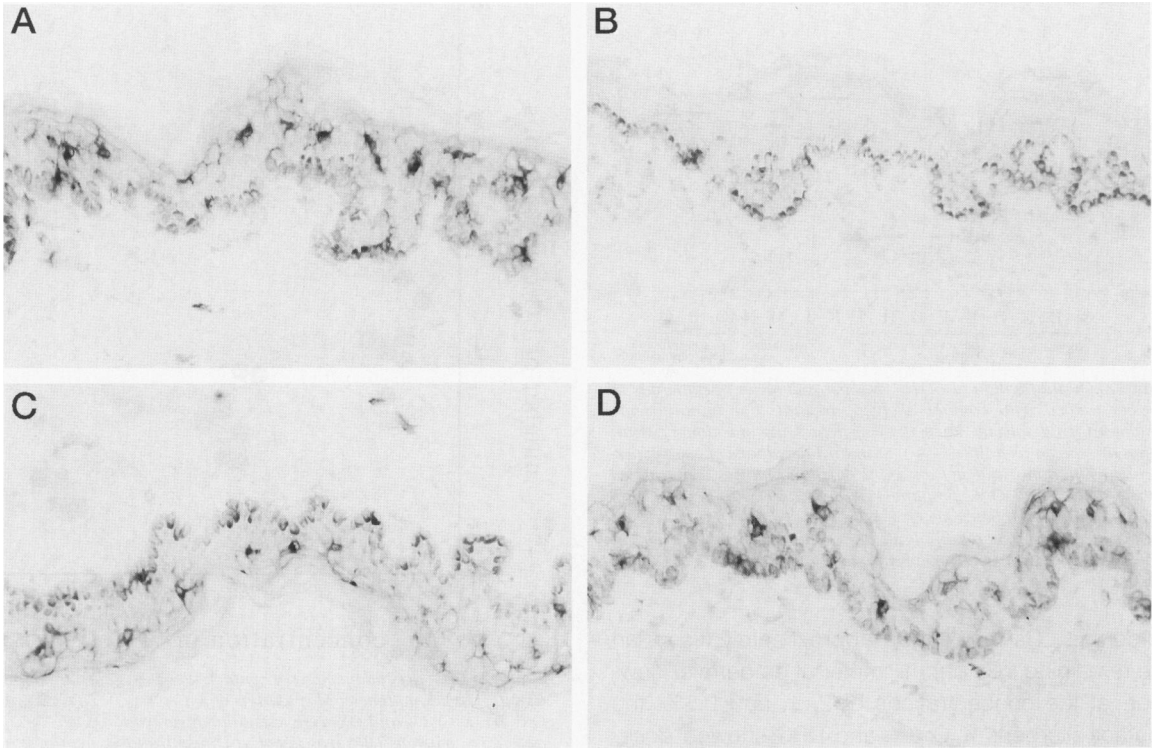

Figure 1. *Immunohistochemical analysis of CD1a$^+$ LCs in human skin cultures treated for 24 hours with solvent, contact allergen, irritant, or non-allergenic compound. The 6-μm sections of treated biopsies were stained with anti-CD1a monoclonal antibodies. Compared with biopsies treated with the solvent A/OO* (**A**), *a decreased number of LCs is observed in biopsies treated with the contact allergen $K_2Cr_2O_7$ (2% w/v;* **B**). *The irritant benzalkonium chloride (0.5%;* **C**) *and the non-allergenic chemical 2,3-DCNB (4% w/v;* **D**) *did not decrease the number of LCs.*

fied air. Epidermal sheets were collected and epidermal cell suspensions were prepared as previously described.[19] Epidermal cells were incubated for 30 minutes at 4°C with 1:20 diluted FITC-conjugated monoclonal antibody against CD1a (OKT-6-FITC; Ortho Diagnostics) and 1:50 diluted FITC-conjugated monoclonal antibody against HLA-DR (HLA-DR-FITC; Beckton Dickinson UK) in PBS supplemented with 0.5% BSA and 0.05% sodium azide. Fluorescence intensity of cells was detected by flow cytometric analysis using a FACScan (Beckton Dickinson UK). Dead cells were identified by propidium iodide uptake and gates were set so as to acquire 5000 living cells per measurement.

Results

After 24 hours of culture, cryostat sections were taken from contact-allergen-, irritant-, non-immunogenic-compound-, or solvent-treated human skin explants of seven donors and were investigated for the distribution of epidermal LCs by analysis of HLA-DR-expressing cells and CD1a-expressing cells. In immunohistochemically stained sections of untreated or solvent-treated skin, CD1a$^+$ LCs could be detected in the suprabasal regions of epidermis (Figure 1A). Application of contact allergens induced a large decrease in the number of CD1a$^+$ LCs in epidermis, the remaining LCs being positioned closely to the basal membrane probably due to migration within the epidermis (Figure 1B). Irritants and non-allergenic compounds did not decrease the number of CD1a-expressing cells in epidermis, which resembled the distribution of LCs in untreated or solvent-treated skin cultures (Figure 1, C and D, respectively). Identical results were obtained when cryostat sections were stained for HLA-DR (data not shown).

Quantification of the number of CD1a$^+$ LCs after application of the various compounds on skin cultures of at least four donors revealed that all contact allergens significantly reduced the number of CD1a$^+$ LCs in epidermis. In contrast, irritants and non-immunogenic compounds did not (Figure 2). The irritant nonanoic acid (80% in propane-1-ol) was the only compound in the group of irritants to induce a decrease in the number of LCs. However, this decrease was not significant due to the decrease in LCs with the solvent propane-1-ol alone (Figure 2).

All contact allergens at highest concentration induced a comparable decrease in the number of

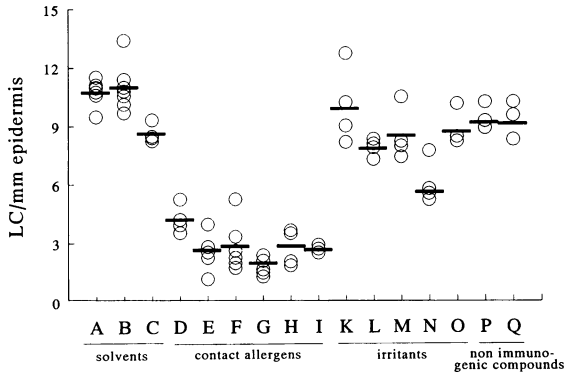

Figure 2. *Quantification of CD1a$^+$ LCs per millimeter of epidermis in biopsies treated with contact allergen, irritant, non-immunogenic compound, and solvent. Each dot (○) represents one donor. Mean values are shown as a line (——). Standard deviations of each experiment in one donor did not exceed 25%. Skin cultures were treated with: A, H_2O; B, A/OO; C, propane-1-ol; D, 0.2% DNFB; E, 6% $NiSO_4$; F, 0.5% dinitrochlorobenzene; G, 2% $K_2Cr_2O_7$; H, 1% DPC; I, 1% OX; K, 0.5% SDS; L, 0.2% croton oil; M, 0.5% benzalkonium chloride; N, 80% nonanoic acid; O, 100% propylene glycol; P, 4% 2,3-DCNB; Q, 2,4-DCNB.*

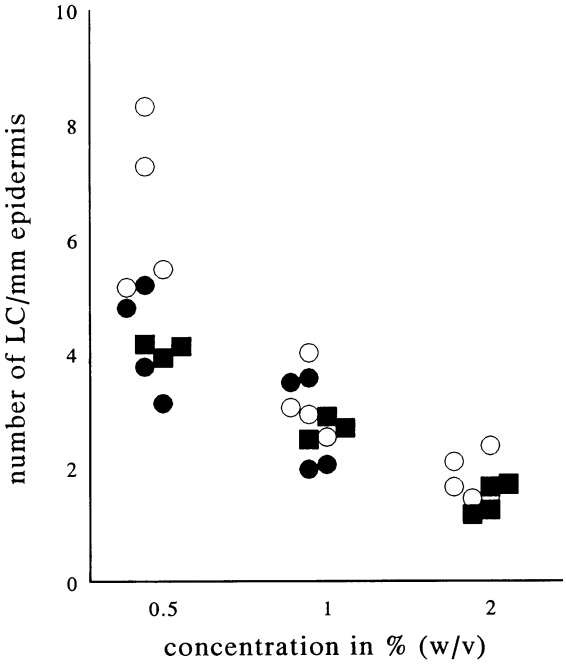

Figure 3. *Dose-dependent effect of contact allergens. Quantification of CD1a$^+$ LCs per millimeter of epidermis of $K_2Cr_2O_7$ (○), DPC (●), and OX (■). $K_2Cr_2O_7$ and OX were tested at concentrations (w/v) of 2, 1, and 0.5%, whereas DPC was tested at concentrations (w/v) of 2 and 1%. CD1a$^+$ LCs in 6-μm-thick sections stained with anti-CD1a monoclonal antibody were counted by means of the MOP videoplan (KONTRON), and the number of LCs per millimeter of epidermis was calculated. Each symbol represents one experiment. Standard deviations of each experiment did not exceed 25%.*

epidermal LCs resulting in approximately less than five remaining LCs per millimeter of epidermis. However, at the concentrations of 2, 1, and 0.5%, the contact allergens $K_2Cr_2O_7$ and DPC showed dose-dependent effects on the number of epidermal LCs (Figure 3). The contact allergen OX showed this effect at the concentrations of 1 and 0.5% (Figure 3). This implies that the application of unknown compounds should be performed in concentration ranges to detect the contact allergenic potential of such compounds.

To further substantiate the immunohistochemical findings in tissue sections, immunofluorescence studies were performed on isolated epidermal cells. To this aim, cells obtained from biopsies treated with the contact allergens DNFB, $NiSO_4$, OX, and DPC, the irritants croton oil and SDS, and the non-immunogenic compound 2,4-DCNB were stained for HLA-DR and CD1a. Flow cytometric analysis showed that the populations of HLA-DR$^+$ and CD1a$^+$ LCs in epidermal cell suspensions isolated from the contact-allergen-treated biopsies was largely reduced or even absent, whereas this reduction could not be observed in biopsies treated with irritants and non-immunogenic compounds (Figure 4). It should be noted that trypsinization of epidermal cells does not affect the expression of CD1a and HLA-DR on LCs, as described previously.[19] These findings confirmed the results of the immunohistochemical analysis.

Discussion

With the aim to develop an animal-replacing assay for the identification of contact allergens, the effects of contact allergens, irritants, and non-immunogenic compounds on epidermal LCs were determined in human skin cultures. Due to the low availability of human skin specimens, each compound was tested on biopsies of at least four donors within a panel of seven donors. Immunohistochemical and immunofluorescence analysis of CD1a- and HLA-DR-expressing cells in epidermis of 24-hour-treated biopsies showed that contact allergens, but not irritants and non-immunogenic compounds, significantly reduced the number of LCs. These results were consistently found for each compound tested in biopsies from at least four donors. The remaining LCs had accumulated at the basal membrane, indicating that the decrease in the number of LCs probably results from the migration of LCs. During migration from epidermis, the LC loses its expression of CD1a, hence the detection of LCs in the dermis is difficult. Furthermore, the process of migration of LCs through the nondense dermal matrix probably occurs much faster than the migration through epidermis, which might also contribute to not finding migrating LCs in the dermis. Nevertheless, the present findings highly suggest that the emigration of LCs, expressed as the decrease in the number of HLA-DR$^+$ and CD1a$^+$

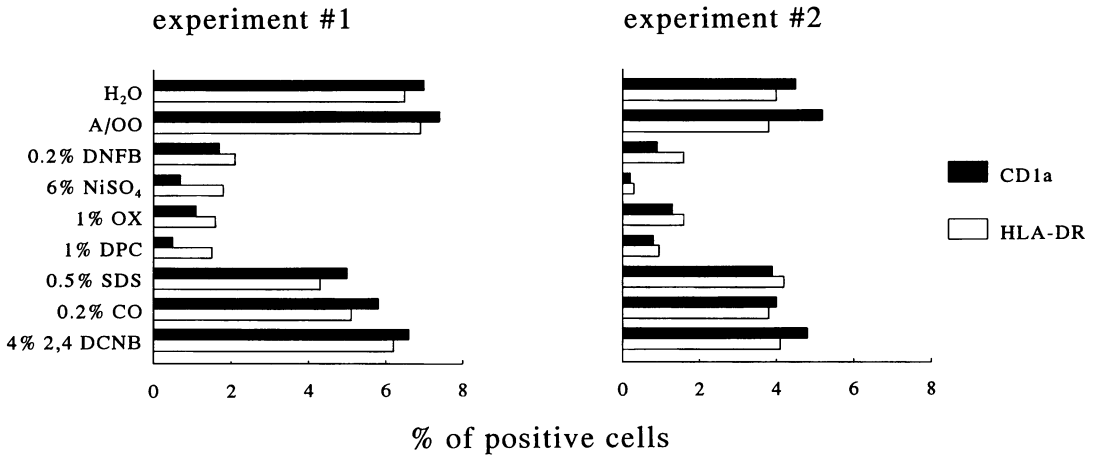

Figure 4. *Decreased numbers of CD1a$^+$ and HLA-DR$^+$ LCs in epidermal cell suspensions isolated from biopsies treated with the contact allergens $NiSO_4$ and DNFB but not with the irritant croton oil, the non-immunogenic compound DCNB, and solvent. Treated biopsies were incubated with 0.25% trypsin to isolate epidermal cell suspensions. Epidermal cell suspensions were incubated with anti-CD1a or anti-HLA-DR antibodies to determine CD1a$^+$ or HLA-DR$^+$ LCs in the fluorescence-activating cell sorter (FACS). Two representative experiments are shown.*

LCs in the epidermis, is a suitable parameter for the identification of contact allergens *in vitro*.

Previous studies provided indirect evidence supporting migration of LCs after epicutaneous application of contact allergens. In mice it was shown that the number of dendritic cells in the lymph nodes increases after application of contact allergens, suggesting LC migration to the lymph nodes.[3,20] It was also reported that skin application of the contact allergenic fluorochrome FITC led to an increased number of FITC$^+$ LCs in the lymph nodes, suggesting migration of antigen-laden LCs.[4,21]

Patch-test studies on the effects of contact allergenic and irritant compounds on epidermal LCs revealed contradictory data. The various studies on patch-test reactions showed either a decrease in the number of CD1a$^+$ LCs in allergic[22,23] and irritant[17,24] contact dermatitis or an increase in CD1a$^+$ LCs in both reactions.[25,26] In contrast with the present study, the number and function of LCs in *in vivo* patch-test reactions will be modulated by the infiltration of inflammatory cells, which may explain the variable results. As compared with patch-test biopsies, in human skin cultures, infiltration of inflammatory cells will not occur, allowing the detection of the mere effects of chemicals on pre-existing LCs.

In this study, the 24-hour application of irritants did not induce a significant reduction in the number of LCs. However, recent reports have shown that LCs migrate from the epidermis during irritant contact dermatitis.[27–29] It has also been indicated that contact-allergen-induced migration starts earlier than irritant-induced migration,[29–32] which would explain the results of the present study. Topical application of carcinogenic chemicals, such as 7,12-dimethylbenz(a)anthracene, on sheep skin has been shown to decrease the number of LCs in the epidermis.[33,34] However, the migration of LCs had a much slower onset as compared with contact allergens in these studies.[33] Chemical carcinogens were not tested in this present model. In this *in vitro* model, contact allergens are likely to be discriminated from carcinogens based upon the different kinetics of carcinogen-induced LC migration as compared with contact allergens. The difference in kinetics of allergen-induced and irritant- or carcinogen-induced LC migration might depend on the differences in the levels and types of cytokines produced in the epidermis. Epidermal- or dermal-derived tumour necrosis factor-α has been shown to induce the migration of murine LCs from epidermis[35] and was reported to be a critical mediator of both allergic and irritant contact dermatitis.[36] Interleukin-1β, which is rapidly produced after application of contact allergens, induces migration of LCs.[37–39] However, interleukin-1β is produced only after activation of epidermal cells with contact allergens and not with irritants,[40] possibly explaining the difference between the effects of contact allergens and irritants observed in this study.

The results obtained in this study probably did not depend on the allergic state of the skin donors. In a previous study (unpublished data) with skin biopsies obtained from 37 females with unknown nickel-allergic state, a consistent significant decrease in the number of LCs after treatment with 5% $NiSO_4$ could be detected. As 20% of all women are sensitized to $NiSO_4$, this finding highly suggests that the allergic state of the individual donor did not interfere in this study.

In the present report, we show that the reduction in the number of LCs in human skin cultures after treatment with contact allergens might be a suitable model to identify potential contact allergens. This model has important advantages compared with other contact allergen screening methods. First, no animals are needed for this predictive assay. Second, the model can be used for various types of chemicals, whereas *in vitro* studies with isolated cells are largely obstructed by the water insolubility of many contact allergenic chemicals.

In this new assay, we have used strong contact allergens that induced a comparable reduction in the number of LCs. In additional studies, the effects of classes of weak to strong contact allergens will be tested in the current *in vitro* model. Simultaneously, the study of new suitable parameters for the identification of contact allergens might further improve the sensitivity of this *in vitro* model. The measurement of contact-allergen-induced production of epidermal cytokines by means of *in situ* hybridization in sections and extraction of epidermal mRNA is one of these parameters to be investigated.

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