



Chapter 2

Skin Sensitization Tests: The LLNA and the RhE IL-18 Potency Assay

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Abstract

Contact allergy is of considerable importance to the toxicologist, and regulatory authorities worldwide require testing for skin sensitization potential and appropriate hazard labeling to enable management of the risk to human health. Although traditionally the identification of skin-sensitizing chemicals has been carried out using animal models, in Europe legislative changes have promoted, and now require, the use of non-animal methods (i.e., Cosmetic Directive, REACH). Several in vitro alternatives for hazard identification have now been validated, but do not provide information on the potency of a skin sensitizer. Here, we describe an animal model, the local lymph node assay (LLNA), and an in vitro model, the RhE IL-18 potency assay, in the context of the identification and potency classification of skin sensitizers. These two assays have been chosen among the different available tests as representative of an alternative in vivo model (the LLNA) and a promising in vitro method with the potential of both hazard identification and potency classification.

Key words Allergic contact dermatitis, Skin sensitization, In vivo test, Non-animal test, Skin, Cytokines, Local lymph node assay, Contact hypersensitivity

1 Introduction

Chemical allergy refers to immune-mediated adverse health effects caused by exposures exceeding the individual threshold to low molecular weight chemicals (<500 Da). Epidemiological studies suggest that the prevalence of contact allergy is ~15–20% [1], making allergic contact dermatitis (ACD) a major health problem. Chemical allergy is therefore of considerable importance to the toxicologist, who has the responsibility of identifying and characterizing the skin- and respiratory-sensitizing potential of chemicals, and estimating the risk they pose to human health. Regulatory authorities worldwide require testing for ACD and appropriate hazard labeling to enable management of the risk to human health.

The cellular and molecular events that are associated with the induction of skin sensitization and the elicitation of allergic contact dermatitis have been described elsewhere [2].

The preferred *in vivo* test for the evaluation of the contact sensitization potential of chemicals is the local lymph node assay (LLNA), as described in the OECD guideline 429, and its non-radioactive variants (TG442A, LLNA:DA and TG442B, LLNA:BrdU-ELISA). Older *in vivo* alternatives are the traditional guinea pig assays, the Buehler occluded patch test [3] and the guinea pig maximization test [4] as described in OECD guideline 406.

Although the identification of potentially skin-sensitizing chemicals is carried out using animal models, in Europe legislative changes (i.e., Cosmetic Directive, REACH) impose and promote the use of non-animal methods, with three *in vitro* methods now fully validated and implemented in OECD guidelines as well as in ECHA Guidance. In the last two decades, several non-animal tests, reflecting the major key events described in the OECD adverse outcome pathway for skin sensitization (OECD GD 168), have been developed and are at various stages of acceptance, validation, development, and use [5]. Among the non-animal tests recently validated, the KeratinoSens™ assay [6] which is based on a luciferase reporter gene under the control of an antioxidant response element of the human AKR1C2 gene stably inserted into HaCaT keratinocytes; the direct peptide reactivity assay (DPRA), which measures depletion of a cysteine- and a lysine-containing peptide in the presence of the test chemical [7]; and the human cell line activation assay (h-CLAT) [8], addressing CD86 and CD54 upregulation in THP-1 cells, can be mentioned.

For detailed information on the applicability domain and predictive capacities of the different methods, please refer to published articles [5, 9].

Here, we will describe in detail one *in vivo* method and one *in vitro* method, namely the local lymph node assay [10] and the RhE-IL-18 potency assay [11–15]. The LLNA was chosen as it is considered an alternative method to the guinea pig assays as it provides advantages over the OECD TG 406 (e.g., less animals, less pain, and distress). In addition, the LLNA was the first method to be fully and independently validated, as well as the first to offer an objective endpoint with a quantitative measure of sensitizing potency (in addition to hazard identification). While the RhE-IL18 potency assay is a recent *in vitro* method based on the use of commercially available reconstituted human epidermis and the production of interleukin-18 (IL-18) that we proposed for the identification and potency classification of contact allergens.

1.1 The Local Lymph Node Assay (LLNA)

The mouse LLNA involves stimulation of lymphocytes in local draining lymph nodes during the afferent or induction phase of skin sensitization as the endpoint, and provides quantitative data suitable for risk assessment. Following primary dermal exposure to skin-sensitizing chemicals, lymph nodes draining the site of application become activated. Initially, the response is located within the T-lymphocyte-dependent (paracortical) area of the node.

This T-cell response is essential for the development of contact allergy, where the vigor and duration of proliferation appears to influence directly the extent of sensitization achieved and the severity of dermal reaction that can be elicited [16].

Currently, the LLNA is the preferred and accepted method for assessing skin sensitization of most substances. LLNA that, in addition to the hazard identification, has proven very useful in assessing the skin-sensitizing potency of chemicals, based on the estimation of the concentration of chemical required to induce a stimulation index of three relative to concurrent vehicle-treated controls (EC3 value). Low EC3 values correlated well with sensitizers known to be potent in man, whereas high EC3 values were usually associated with weak human sensitizers [17].

Briefly, mice are exposed to various doses of the test chemical on the dorsum of both ears, daily for three consecutive days. Control mice receive equal volumes of the relevant vehicle alone. Two days following the final exposure mice are injected intravenously with ³H-thymidine (3HTdR), terminated 5 h later and the incorporation of 3HTdR into draining lymph node cells (LNC) measured by scintillation counting [18].

A chemical is classified as sensitizers if a threefold or greater elevation in ³H-TdR incorporation relative to vehicle control is recorded with at least one test dose. Compared to the pooled lymph nodes, the processing of lymph nodes from individual mice will allow for the assessment of inter-animal variability and a statistical comparison of the difference between test substance and vehicle control group. It is important to mention that recently two non-radioactive variants have been developed and accepted at the OECD level (TG442A, LLNA:DA and TG442B, LLNA:BrdU-ELISA).

1.2 The RhE IL-18 Potency Assay

Keratinocytes (KCs) play a key role in the activation of all skin immune responses. Among the different factors produced by KCs, IL-18 has been shown to play a key proximal role in the induction of allergic contact sensitization [19, 20], and recent evidence provided from our group has shown that IL-18 production in human KCs (NCTC 2544) can be used as a sensitive method to identify contact allergens, discriminating them from respiratory allergens and irritants [20, 21]. Replacing NCTC 2544 cell line with a standardized commercially available reconstituted human epidermis (RhE), as described in a previous

manuscript [13], made the assay extremely transferable and easy to perform [15], allowing to test chemicals of low water solubility or stability. A protocol was developed using different 3D-epidermal models including in-house VUMC model, epiCS[®] (previously EST1000[™]), MatTek EpiDerm[™], and SkinEthic[™] RhE. Briefly, following topical exposure for 24 h to contact allergens a robust increase in IL-18 release was observed only after exposure to contact allergens. Correlating the in vitro RhE sensitizer potency data (i.e., EC50 and IL-18 SI2) with human and animal data, a good correlation with both human DSA05 and LLNA EC3 values ($p < 0.001$) was observed.

The protocol is designed to identify contact allergens and to estimate the NESIL (no expected sensitization induction level) with the aid of reconstructed human epidermal (RhE) models. Using reference contact allergens, namely DNCB, isoeugenol, cinnamal, and benzocaine, standard curves can be created to predict in vivo data based on in vitro EC50 and IL-18 SI2, which is required for risk assessment. This protocol was adapted from the skin equivalent potency assay, which was modified to include the release of interleukin-18 (IL-18) as a means to identify potential contact allergens [11–15]. Following the protocol using the commercial available Epiderm[™] model is presented. The assay has proven to work with both commercially available RhE models as well as with in-house reconstructed epidermal equivalent made from primary human keratinocytes (VUMc-RHE).

2 Materials

2.1 Local Lymph Node Assay

1. Mice (*see Note 1*). Young female adult (8–12 week, nulliparous and non-pregnant) CBA/Ca or CBA/J mouse strains (*see Note 2*) are allowed to acclimatize for at least 5 days. Animals of a single sex must be used in each experiment. Experimental groups, each comprising minimum four mice, are caged separately. Animals should be housed at a temperature of $22 \pm 3^\circ\text{C}$, relative humidity not exceed 70%, 12 h light, 12 h dark. Conventional laboratory diets may be used as feeding with water ad libitum (*see Note 3*).
2. Vehicle. Vehicle selection is dictated by the relative solubility of the test materials. Vehicle is selected on the basis of maximizing the solubility in order to obtain the highest concentration achievable while producing a solution suitable for application (*see Note 4*). For many chemicals 4:1 acetone/olive oil (AOO) is suitable. For the preparation of AOO, add 160 ml of acetone to 40 ml of olive oil.

3. Positive control. If possible, positive control for each study, as also required by some regulatory authorities, should be included. Preferred positive control substances are 25% hexyl cinnamic aldehyde in acetone:olive oil (4:1, v/v) and 5% mercaptobenzothiazole in *N,N*-dimethylformamide.
4. Phosphate buffered saline (PBS). For 1 l of 1× PBS, add 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, 0.24 g of KH₂PO₄, adjust the pH to 7.4 with HCl and add distilled water to a total volume of 1 l. Dispense the solution into aliquots and sterilize by autoclaving (20 min, 121 °C, liquid cycle). Store at room temperature.
5. Trichloroacetic acid (TCA). Solution of 5% of TCA in distilled water.
6. ³H-methyl thymidine (³H-TdR, specific activity 2 Ci/mmol) or ¹²⁵I-iododeoxyuridine (UdR).

2.2 RhE IL-18 Potency Assay

1. Standard cell culture equipment.
2. CO₂ incubator (37 °C, 5% CO₂, 95% relative humidity).
3. ELISA microplate spectrophotometer equipped with specific filters (570 nm, 595 nm, 655 nm, and 450 nm).
4. Laminar flow hood for RhE model exposure.
5. Chemicals. All reagents and chemicals must be purchased at the highest purity available. Cell culture medium (EPI-100-MM-60) is supplied by MatTek Corporation.
6. Reconstituted human epidermis (RhE). EpiDerm™ (in Europe: MatTek In Vitro Life Science Laboratories, Bratislava, Slovak Republic; in the USA: MatTek Corporation, Ashland, MA, USA). EpiDerm™ is produced from normal human keratinocytes obtained from neonatal male skin donors. EpiDerm™ is maintained according to the suppliers' instructions at 37 °C, 5% CO₂, and 95% relative humidity (*see Note 5*). After 18–24 h of equilibration time, RhE is exposed to chemicals as described below.
7. Test substances are tested using two independent RhE batches in a dose-response starting with the highest soluble of 200 mg/ml, and decreasing with twofold serial dilutions until 0.10 mg/ml (or the closest lower concentration) is reached (i.e., each test substance should be tested in 6–12 concentrations). Whereas test substance dose-responses are tested in single fold in each independent experiment, control conditions (unexposed, vehicle(s), and positive control) should preferably be tested in duplicate per independent experiment.
8. Dulbecco's modified phosphate-buffered saline (dPBS). For 1 l of 1× PBS, add 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na₂HPO₄, 0.2 g of KH₂PO₄, adjust the pH to 7.4 with HCl and add

distilled water to a total volume of 1 l. Dispense the solution into aliquots and sterilize by autoclaving (20 min, 121 °C, liquid cycle). Store at room temperature.

9. MTT solution. Cell viability is assessed by the MTT reduction assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is used. The stock solution (5 mg/ml) of MTT in dPBS is prepared by dissolving 12.5 mg MTT in 2.5 ml dPBS. The solution is thoroughly vortexed and filtered using a sterile 0.22 µm filter. The stock solution can be stored frozen (−20 °C) up to 1 month. To prepare Final solution (1 mg/ml) of MTT for the cytotoxicity assay, add 2 mL of the stock solution to 8 mL of the RhE assay medium provided by the RhE model supplier. Keep the MTT solution in the dark. Final solution of the MTT should be prepared always freshly before the use and should not be stored for more than 4 h. This volume is sufficient for 24 tissues.
10. Coating buffer, 100 mM carbonate/bicarbonate buffer. For 1 l, add 1.59 g Na₂CO₃ and 2.93 g NaHCO₃, adjust pH to 9.6.
11. Blocking buffer and dilution buffer, PBS + 1% BSA.
12. Stop solution, 1 M H₂SO₄ in distilled H₂O.
13. Wash buffer, PBS + 0.01% Tween 20.
14. Anti-IL-18 antibody.
15. Detection biotin label antibody – Anti-IL-18 (Human) mAb-Biotin.
16. Streptavidin–HRP conjugate.
17. TMB (3,3',5,5'-tetramethylbenzidine).

3 Methods

3.1 LLNA

3.1.1 Determine Optimal Vehicle for Test Article (Solubility Testing)

Solid substances should be dissolved or suspended in the appropriate vehicles and diluted, if necessary, prior to application. Liquid test substances may be applied neat or diluted prior to dosing (*see Note 6*). Recommended vehicles are acetone:olive oil (4:1, v/v) (*see Note 7*), *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide [22].

3.1.2 Conduct Dermal Irritation Pre-screen to Determine Test Concentrations

As specified in the OECD guideline 429, an irritation pre-study should be performed under identical conditions as the main test in order to determine the maximum (irritation threshold) test concentration.

1. If no mouse ear irritation data are available, a quantitative range finder using 12 mice ($n = 1-2$) exposed to 6 concentrations (100%, 50%, 25%, 10%, 5%, 2.5%) should be performed first using conditions identical to the LLNA.

2. If the test article is irritating, a second range finder at lower doses (1%, 0.5%, 0.25%, 0.1%, 0.05%, etc.) may be necessary to select appropriate test article concentration for use in the definitive study.

Body weights are recorded pre-test and prior to termination on day 6. Ears are observed for erythema and thickness at pre-test and on day 3 and 6.

3. The highest dose selected for the LLNA will be the next lower dose in the pre-screen concentration series that does not induce systemic toxicity (e.g., neuronal or behavioral changes, changes in the respiratory pattern, changes in water and food consumption, changes in body weights, etc.) and/or excessive local skin irritation (*see Note 8*).

3.1.3 Exposure

1. All mice receive 25 μ l of the material or of vehicle alone on the dorsum of both ears. At least three concentrations should be used, so that the highest concentration maximizes exposure while avoiding systemic toxicity and/or excessive local.
2. The solution is delivered using an automatic pipet, with a disposable tip, ensuring even distribution over the surface of the ear. Identical treatment is performed daily for a total of three consecutive days (days 1, 2, and 3). Days 4 and 5—no treatment. Animals should be observed every day for any clinical signs, either of local or systemic toxicity. In case of excessive toxicity, animals should be terminated.

3.1.4 In Situ Labeling

1. On day 6 following the initiation of treatment, record the weight of all mice and inject i.v. (tail vein) 250 μ l of sterile PBS containing 20 μ Ci (7.4×10^5 Bq) of ^{3} H-methyl thymidine (^{3} H-TdR, specific activity 2 Ci/mmol) or 250 μ l sterile PBS containing 2 μ Ci (7.4×10^4 Bq) of ^{125} I-iododeoxyuridine (UdR). The use of ^{125} IUdR rather than ^{3} H-TdR as the isotope has been shown to be comparably robust in the LLNA (*see Note 2*). Mice are sacrificed 5 h later.

3.1.5 Preparation of Lymph Node Cells

1. After 5 h, humanely terminate the mice, and excise auricular draining lymph nodes.
2. Lymph nodes can be pooled or kept separated for each experimental group and collected into a small plastic screw-capped bottle of 5–7 ml capacity containing 1 ml of PBS.
3. Subsequently, excess PBS is decanted and the pooled lymph nodes placed onto a 4-cm² grid of stainless steel gauze (200 mesh), or another suitable technique for generating a single-cell suspension, within a 60-mm plastic Petri dish containing approximately 2 ml of PBS (*see Note 9*).

4. A single-cell suspension is prepared by mechanical disaggregation using the plunger of a 5-ml syringe.
5. Cell suspensions are transferred from the Petri dish into 16 ml capacity round-bottomed plastic tubes.
6. Lymph node cells are washed twice ($190 \times g$ for 10 min) with 10 ml of PBS.
7. After final wash, cells are resuspended in 3 ml of 5% trichloroacetic acid (TCA) and stored at 4 °C for 18 h for precipitation of macromolecules.
8. Precipitates are recovered by centrifugation, resuspended in 1 ml of 5% TCA, and transferred in a scintillation vial containing 10 ml of scintillation fluid for ^3H -counting, or transferred directly to gamma counting tubes for ^{125}I -counting.

3.1.6 Analysis of Results

1. Results are recorded as total disintegration per minute (dpm) per node for each experimental group or individual animal, or as stimulation index using the values from vehicle control as the comparator. The processing of lymph nodes from individual mice allows for the assessment of inter-animal variability and a statistical comparison of the difference between test substance and vehicle control group (*see Note 10*).
2. Results are expressed as stimulation index, derived by dividing the mean DPM within each test group for the mean DPM for the vehicle control group.
3. Calculate, if possible, the EC3 (minimal sensitizing concentration; where $\text{SI} = 3$). A chemical is classified as sensitizers if a threefold or greater elevation in ^3H -TdR incorporation relative to vehicle control is recorded with at least one test dose (*see Note 11*).

3.2 RhE IL-18 Potency Assay

3.2.1 Determine Chemical Solubility

The maximum solubility of the test substance is identified by dissolving the compound in PBS (phosphate buffered saline) containing Ca^{2+} and Mg^{2+} (for water-soluble substances), and for less soluble substances in 1% DMSO in PBS, or mixture 4:1 of acetone/olive oil (AOO, 4:1 v/v). For the majority of chemicals, AOO is suitable. For the preparation of AOO, add 160 ml of acetone to 40 ml of olive oil (*see Note 7*).

Dilutions are made in the following order: 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 1.56 mg/ml, 0.78 mg/ml, 0.39 mg/ml, 0.20, and 0.10 mg/ml until a clear solution is reached. The vehicle with the highest dissolving capacity and lowest cytotoxicity should be chosen. All solutions should be prepared at room temperature (20–26 °C) or max. Warmed to 37 °C, if necessary. When a liquid compound is viscous, use positive displacement pipet to avoid pipetting errors that could be introduced by regular pipettes.

Poor solubility of a solvent is indicated by:

1. Formation of two layers or separation into two layers shortly after vortexing.
2. Formation of a milky suspension.
3. Visible precipitation of the test substance (e.g., after 10 min waiting time at RT).

If any of these effects occurs then test substance concentrations have to be lowered in the vehicle(s) or different vehicle should be chosen for testing (*see* **Notes 12–14**).

3.2.2 Protocol Scheme

Day 1:

1. Receipt of the tissues.

Upon arrival, the RhE-containing package should be checked for breaches. Subsequently, the integrity of the tissue should be confirmed.

RhEs and the other components (e.g., media) provided should be free of contamination. Any abnormalities related to the color of the medium, shipping agar, and the presence of unusual effects on the tissue surface (like blisters) should trigger caution. If you observe any of these irregularities before or during the assay, contact immediately the tissue model supplier and provide photo-documentation of the effects observed.

Only properly transported and entirely intact tissue without any evidence of contamination and defects should be used in the experiments.

2. Pre-incubation of the RhE models.

(a) Transfer the transwells containing the RhE models into a sterile 6-well culture plates prefilled with 0.9 ml of the culture medium provided by the RhE manufacturer. Each batch of RhE should contain the following controls: unexposed tissues ($N = 2$), vehicle control ($N = 2$), and positive control ($N = 2$).

(b) The plates are incubated overnight at 37 ± 1 °C, $5 \pm 1\%$ CO₂, $90 \pm 10\%$ relative humidity, before the test substance exposure.

Day 2

1. Chemical exposure.

All solutions, including stock solutions of test substances must be made on the day of the RhE exposure. Vortexing is required after the preparation, and also shortly before the application to the RhE to assure homogeneity (*see* **Note 15**).

In order to determine the RhE-EC50 and IL-18 SI2 values, test substances are tested using two independent RhE batches in a dose-response, starting with the highest soluble concentration and decreasing in twofold dilutions.

Test substances are tested using one tissue replicate per concentration. All controls (i.e., unexposed controls, vehicle controls, and positive control), are tested in duplicate per independent experiment. DNCB 3 mg/ml in AOO is used as a positive control.

- (a) 25 μ l of the testing samples are pipetted directly onto RhE. If needed improve the spreading using a bulb-headed Pasteur pipette.
- (b) Incubate exposed cultures for 24 h (37 ± 1 °C, $5 \pm 1\%$ CO₂, $90 \pm 10\%$ relative humidity).

Day 3

1. Termination of the test substance exposure.
 - (a) After 24 h of incubation, take exposed cultures out of the incubator and wash three times in dPBS by submerging and gentle swirling the tissue in a beaker filled with 100 ml dPBS.
 - (b) Blot the tissues on the absorbing paper and perform MTT assay to assess cell viability.
 - (c) Transfer the culture medium to 1.5 ml Eppendorf tubes for IL-18 assessment. Store at -80 °C until ELISA testing.

3.2.3 Exposure of the RhE to MTT and Isopropanol Extraction

To perform the MTT assay, follow the steps below (*see Note 16*):

1. Pipette 300 μ l per well of the MTT solution in each well of a 24-well plate.
2. Pick up the RhE test cultures with a tweezer and tap the cultures gently on a paper to dry the bottom of the culture.
3. Place the cultures into the 24-well plate previously filled with the MTT solution.
4. Once samples are in contact with MTT solution, incubate for 3 h in the dark at 37 ± 1 °C, $5 \pm 1\%$ CO₂, $90 \pm 10\%$ relative humidity. Make sure that no bubbles are present underneath the bottom of the cultures and the MTT solution in the well. If so, tap the plate gently to remove the bubbles present underneath the cultures.
5. Shortly before the end of MTT assay, prepare new 24-well plate filled with 2 ml isopropanol.

6. Pick up the RhE cultures from the plates with MTT solution. Tap the cultures gently on a paper until the bottom of the culture is dry.
7. Place the cultures into the 24-well plate previously filled with isopropanol. Make sure that no bubbles are present between the bottom of the cultures and the isopropanol in the well. If so, tap the plate gently to remove the bubbles present underneath the cultures.
8. Cover the plate with Parafilm to avoid evaporation of the isopropanol.
9. Incubate the plates with cultures in isopropanol at least for 2 h (at room temperature on a plate shaker) or overnight (at 4 °C), covered with Parafilm and protected from the light.
10. Once extraction is completed, discard the cultures and transfer 200 µl supernatant from each well of the 24-well plate into a 96-well plate.
11. Read plates using a spectrophotometer at 540–570 nm wavelength using isopropanol as blank. If the OD is above the limits of the plate reader (e.g., outside the linear range of the plate reader), dilute all the isopropanol solutions with a constant volume of additional isopropanol. Proceed as follows: for ALL samples within a given experiment transfer 100 µl to a new plate and add 100 µl isopropanol.
12. Calculate cell viability: absorbance of the wells from the vehicle-exposed cultures is set to be 100% cell viability. The RhE-EC50 values are obtained by linear regression analysis based on changes in metabolic activity (MTT).

3.2.4 Determination of the IL-18 Release Using In-House ELISA

This sandwich ELISA is based upon consecutive incubation steps with different antibodies and the samples of interest, resulting in the visualization via a substrate reaction of the amount of IL-18 protein released in the culture media. Refer to Galbiati and Corsini [23] for details (*see Note 17*).

When using the antibodies in an in-house ELISA, proceed as follows:

Day 1:

1. Coat wells with capture antibody: dilute (1/1000) the IL-18 capture antibody in coating buffer: 10 µl capture antibody +10 ml coating buffer (1 µg/ml).
2. Pipet in each well 100 µl of the diluted IL-18 capture antibody.
3. Seal the 96-well plate with a plate sealer or adhesive sealing film and incubate overnight at 4 °C.

Day 2:

1. Bring all reagents (except antibodies) to RT and put the plate at RT. Thaw samples and the IL-18 standard on ice (*see* **Note 18**).
2. Remove the capture antibody and wash 3× with wash buffer using a multiwell pipette. For the last wash, remove any residual buffer by tapping on paper towel.
3. Block plate with blocking buffer. Pipet in each well 200 µl of blocking buffer. Seal plate with a plate sealer or adhesive sealing film and incubate 1 h at RT.
4. Prepare standards, quality control samples, and dilutions for samples.
 - (a) During the 1 h incubation with the blocking buffer (=dilution buffer), samples are thawed on ice and kept on ice.
 - (b) The recombinant IL-18 for the standard curve is diluted in dilution buffer. The following standard dilutions should be used: 1000, 500, 250, 125, 62.5, 31.25, and 15.6 pg/ml.
5. Remove the blocking buffer (=dilution buffer) and wash 3× with wash buffer using a multiwell pipette. For the last wash, remove any residual buffer by tapping on paper towel.
6. Incubation of standard dilutions, quality controls, and sample dilutions. Pipet 100 µl of standards dilutions from 0 pg/ml (= dilution buffer) to 1000 pg/ml, and samples per well. Seal plate using plate sealer or adhesive sealing film and incubate for 2 h at RT.
7. Remove standards, quality controls, and samples, and wash 3× with wash buffer. For the last wash, remove any residual buffer by tapping on paper towel.
8. Incubation with detection biotin label antibody. Prepare the detection antibody: dilute the detection antibody (biotin) at 1:2000 in dilution buffer: 5 µl detection antibody + 10 ml dilution buffer. Pipet 100 µl of detection antibody each well. Seal plate and incubate 1 h at RT.
9. Remove the detection antibody and wash 3×. For the last wash, remove any residual buffer by tapping on paper towel.
10. Incubation with streptavidin–HRP conjugate. Prepare the streptavidin–HRP: dilute the streptavidin–HRP conjugate 1:5000–1:500 in dilution buffer: 2 µl–20 µl streptavidin-conjugated + 10 ml dilution buffer. Pipet in each well 100 µl of streptavidin–HRP conjugate. Seal plate and incubate 30 min at RT.

11. Remove the streptavidin–HRP conjugate and wash 3×. After last wash, remove any residual buffer by tapping on paper towel.
12. Add TMB (3,3',5,5'-tetramethylbenzidine) substrate solution. Pipet in each well 100 µl of TMB substrate solution.
13. Incubate plate (without plate sealer) ~3–30 min at RT.
14. One can read the plate, without stopping the reaction, at 595 nm, or stop the reaction by adding 50 µl of stop solution to each well and read at 450 nm.
15. Record all data. From the raw data (measured absorbance values) the analysis program calculates the mean absorbance for each set of duplicate standards and samples. Sample concentrations (pg/ml) are then computed in the linear section of the recombinant IL-18 standard curve by using a log/log interpolation.

For the assessment of allergenicity (YES/NO), the relative amount of IL-18 protein present in supernatants is given in pg/ml. The result is then expressed as fold change, calculated by the following equation:

$$\text{IL-18 SI} = \frac{\text{IL-18 pg/ml in test substance-treated RhE}}{\text{IL-18 pg/ml in vehicle-treated RhE}}$$

3.2.5 Data Analysis

Statistical analysis is performed using GraphPad InStat (GraphPad Software, San Diego, CA, USA). Using EpiDerm™ the following provisional prediction model is used.

For Allergenic Potential: if the fold increase in intracellular IL-18 is ≥ 2.0 in at least one of the concentrations tested, the chemical is classified as contact sensitizer (H317). In case the two experiments gave inconsistent results (e.g., one positive and one negative), a third experiment should be done, and the majority used for classification (e.g., if two positives and one negative, the classification will be contact allergen).

For Potency Assessment: the arithmetic means of the values obtained in the two experiments for both cytotoxicity (MTT assay) expressed as of EC50 and IL-18 SI2 (chemical concentration resulting in twofold increases in IL-18 release) is considered. Using reference contact allergens, namely DNCB, isoeugenol, cinnamal, and benzocaine, standard curves can be created to predict in vivo LLNA EC3 and human NOAL data based on in vitro EC50 and IL-18 SI2. Refer to Galbiati et al. [15].

3.3 General Discussion

A combination of reliable, validated predictive in vivo and in vitro test methods for identification of skin-sensitizing chemicals are available [9, 24], as well as clearly documented and transparent

approach to risk assessment [25]. A key component of this work is the predictive identification of relative skin-sensitizing potency, achieved normally by the measurement of the threshold (the “EC3” value) in the LLNA, which shows a good correlation with human data with three-quarters of the dataset, helping particularly to identify stronger sensitizers [25, 26].

Here, we have described in detail one *in vivo* method and one *in vitro* method, namely the local lymph node assay [10] and the RhE-IL-18 potency assay [11–15]. The first was chosen, as it is an alternative method to the Guinea pig assays still based on the use of animals. While the RhE-IL18 potency assay is a recent *in vitro* method, currently under validation, based on the use of commercially available reconstituted human epidermis and the production of interleukin-18 (IL-18) that we proposed for the hazard identification and potency classification of contact allergens.

However, no method is perfect: both *in vivo* animal models as well as *in vitro* methods have advantages and disadvantages, with false-positive and false-negative results [5, 9, 24, 25]. Analysis of the correlation [25, 26] between the LLNA and human data identified potential outlier types of (fragrance) chemistry, exemplified by hexyl and benzyl salicylates (an over-prediction) and trans-2-hexenal (an under-prediction). It is intriguing to know that, where metabolism is often the bottleneck of many *in vitro* methods, the analysis of the data generated using *in vitro* methods to assess skin sensitization showed that skin metabolism was not likely to be a major consideration for assessing sensitization potential, as sensitizers requiring activation could be identified correctly using one or more of the current non-animal methods [27]. Regarding *in vitro* methods, it is foreseen that the validated assays and relevant QSAR models can be organized into formal testing strategies to be applied for regulatory purposes by the industry [28].

4 Notes

1. Ethical approval on the use of animals is required.
2. Strain: based on comparative studies, the CBA/Ca or CBA/J mice are considered the strains of choice. Alternatively, other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA response do not exist (OECD TG 429).
3. Safety and precautions: protective clothing, gloves, and safety spectacles are required for animal work as well as for handling ^3H -thymidine and radiolabeled tissue and cells. TCA can cause chemical burns and is harmful if inhaled.

4. Vehicle: many organic vehicles can be used. Care should be taken to ensure that hydrophilic substances are incorporated into a vehicle system, which wets the skin and does not immediately run off. Water, therefore, is inappropriate. In order of preference, the vehicles of choice are 4:1 acetone/olive oil (AOO), methyl ethyl ketone, N,N-dimethylformamide, propylene glycol, and dimethyl sulfoxide.
5. Gloves should be worn at all times when handling human tissues and cells. Safety precautions when handling test substances should conform to those recommended by the supplier.
6. Test substances should be prepared daily unless stability data demonstrate the possibility of storage.
7. Since acetone dissolves some plastic devices including polystyrene test tubes, it is recommended to use glass or polypropylene tubes to prepare the test substance solutions dissolved in AOO.
8. Test doses: in dose-response experiments, at least three doses are tested together, with the appropriate vehicle control group (i.e., 50%, 25%, 10%, 5%, 1%, 0.5%, 0.25%, and 0.1% w/v). It may be desirable to select the highest test concentrations possible. Poor solubility and/or acute local or systemic toxicity may dictate a more conservative approach.
9. It is normal practice for the edge of the gauze square to be turned up to contain the nodes.
10. Borderline results: the strength of the dose-response, the statistical significance, and the consistency of PC responses should also be taken into account when determining whether a borderline result is declared positive.
11. Applicability domain: it must be recognized that there are certain limitations in the applicability domain that require the use of guinea pig tests. These include false negative with some metals and false positive with some surfactants [19].
12. The vehicles that were successfully evaluated on several RhE models include water, PBS, DPBS, acetone: olive oil (AOO) in a ratio of 4:1, absolute ethanol and DMSO (maximum 1%), other vehicles have not been tested. Vehicle controls are cultures that are treated with the solvent used to dissolve the test material. It is used for the calculation of the viability of the treated tissues and therefore it is important that it does not exhibit cytotoxicity. If there is a significant decrease in viability in comparison to the untreated control (more than 20%) it should be considered as cytotoxic.
13. Water-insoluble test substances are applicable, but care should be taken that the selected vehicles are not used at cytotoxic concentrations. It is important that the test substance is homogenous when applied on the RhE.

14. Volatile test substances and aerosols are considered outside the applicability domain of this assay.
15. If AOO is the vehicle of choice for the test substance, take into account that acetone is a volatile liquid. Therefore, open and close tubes with AOO with or without a test substance rather quickly and prepare more than 300 µl in order to limit evaporation.
16. Chemicals which interfere with the readout of the MTT assay should be considered with care and excluded since they fall outside of the applicability domain of the assay. To assess possible interference the following should be performed:
 - (a) MTT assay: the highest soluble chemical concentration should be tested in the absence of RhE. If a color change is observed, then the chemical is excluded.
17. Chemicals which interfere with the readout of the IL-18 assay should be considered with care and excluded since they fall outside of the applicability domain of the assay. To assess possible interference the following should be performed:
 - (a) IL-18 ELISA: a known amount of human recombinant IL-18 (500 pg/ml) is used to spike directly culture supernatant from RhE exposed to the highest soluble chemical concentration. If the calculated value obtained from the ELISA differs from 500 pg/ml, then the compound interferes with the ELISA and is excluded.
18. In order to avoid protein degradation, it is recommended to keep the samples on ice during the procedure of thawing and plating for IL-18 assessment. Avoid repeated freeze-thaw cycles. If possible, store samples at -80°C .

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