



Chapter 8

Use of the LLNA:BrdU-ELISA for Skin Sensitization Hazard Assessment

David M. Lehmann

Abstract

Allergic contact dermatitis (ACD) continues to be an occupational and environmental health issue. Consequently, there is a need to employ predictive tests to reduce the incidence of skin sensitization leading to clinical manifestations of ACD. For more than a decade, the murine local lymph node assay (LLNA) has been the method of choice for the identification of skin sensitizers. While the original LLNA protocol has been extensively evaluated and subjected to exhaustive validation, the use of radioisotope (i.e., tritiated thymidine; 3HTdR) has discouraged utilization of this powerful assay in some countries. To promote further utilization of this method, the original LLNA protocol was refined to use 5-bromo-2'-deoxyuridine, a nonradioactive analog of 3HTdR. The LLNA:BrdU-ELISA has been reviewed, validated, and approved for use internationally, and its performance is regarded as equivalent to the traditional LLNA. Here, we provide guidance on how to perform and interpret data from this assay.

Key words LLNA, 5-Bromo-2'-deoxyuridine (BrdU), Skin sensitization, Allergic contact dermatitis (ACD), Hazard identification, Potency

1 Introduction

1.1 Skin Sensitization Background

Evaluating the potential to induce allergic contact dermatitis (ACD) is an integral element of the safety assessment process for chemicals that may come in contact with skin. ACD is a local skin reaction characterized by redness, swelling, and itching that may develop following exposure to skin-sensitizing substances [1]. ACD is one of the most commonly reported occupational diseases and can significantly impact quality of life, contributing to its importance as an occupational and environmental health issue [2].

Development and manifestation of ACD is a well-understood process that involves two stages: induction and elicitation [3]. In the induction phase of ACD, a susceptible individual is exposed topically to a skin-sensitizing substance, a low molecular weight chemical referred to as a hapten. Haptens are too small to be allergenic. However, as the hapten penetrates the

epidermis, it forms a complex with dermal proteins, becoming allergenic. Langerhans cells, immature dendritic cells in the skin, take up, process, and transport the hapten complex to the draining lymph nodes, where the complex is presented as a complete antigen to T lymphocytes. Activated T lymphocytes divide and differentiate to form allergen-specific T cells. The elicitation phase occurs when a previously sensitized individual is reexposed to the inducing allergen [4]. As before, the skin-sensitizing substance penetrates the epidermis, complexes with skin proteins, is processed by Langerhans cells, and is ultimately presented to T lymphocytes, promoting the release of cytokines and inflammatory mediators by antigen-specific T lymphocytes. This release produces an immune response in the skin leading to ACD [3, 5–7].

To reduce the occurrence of ACD reactions resulting from exposure to chemical substances, many regulatory authorities require that skin-sensitizing substances be labeled to identify the potential hazard posed by these items. The murine local lymph node assay (LLNA) is the preferred method for evaluating the sensitization potential of chemical substances to satisfy regulatory requirements [8, 9].

1.2 History and Evolution of the LLNA

1. In the standard LLNA, proliferation of T lymphocytes is quantified by measuring incorporation of radioactive 3HTdR. The magnitude of T-lymphocyte proliferation has been shown to correlate with the likelihood that skin sensitization will develop [10]. Since the assay measures events occurring during the induction phase and sensitized mice are not challenged with the allergen, mice used in the LLNA do not experience an allergic response. Compared with previously used guinea pig tests, the LLNA requires fewer mice, is faster to perform, and causes less pain and distress. In addition, the LLNA offers a quantitative endpoint that can be measured and used to make potency comparisons between different substances [7, 11, 12]. Given these attributes, the LLNA was globally adopted and, over time, has gained status as the “gold standard” method for evaluating substances for the potential to cause skin sensitization.
2. Although the advantages of the LLNA are widely acknowledged, the assay has been refined over the years to reduce the number of animals needed per study or to improve assay sensitivity and specificity [13, 14]. In addition, much effort has been devoted to developing LLNA protocol variants that do not require the use of radioisotope [14–23]. These particular protocols are necessary to facilitate sensitizer determinations in countries that restrict the use of radioisotope. The LLNA:BrdU-ELISA is a fully validated (e.g., ICCVAM, OECD, ECVAM)

radioisotope-free variation of the LLNA suitable for the identification of skin sensitizers.

1.3 The LLNA:BrdU-ELISA

1. Like the standard LLNA, the LLNA:BrdU-ELISA (OECD 429B) involves quantification of cell proliferation in the lymph nodes draining the site of topical exposure [15]. In both assays, mice are dosed with the test substance for 3 consecutive days and then rested for 2 days. However, when using the LLNA:BrdU-ELISA, the mice are then injected intraperitoneally with 5-bromo-2'-deoxyuridine (BrdU) instead of radioactive 3HTdR [16]. BrdU is incorporated into the cellular DNA of locally proliferating lymphocytes and has been used as a substitute for 3HTdR in many proliferation assays [24]. Because BrdU is similar to 3HTdR, assay variations that utilize BrdU incorporation yield comparable results to the standard LLNA [14]. BrdU-based detection systems offer many advantages over those using 3HTdR including elimination of radioisotope (and associated use restrictions) and elimination of complicated handling and disposal of radioactive animals and contaminated cages/bedding and facilitate additional evaluation of isolated lymph node cells (e.g., counting, flow cytometry, and cytokine production). The sections that follow provide guidance on how to perform and interpret data from LLNA:BrdU-ELISA.

2 Materials

2.1 General

1. Personal protective equipment (lab coat, gloves, and respirator).
2. Datasheets for recording data.
3. Pipette aid.
4. Vortex sample mixer.
5. Balance and weighing standards.
6. Centrifuge.
7. Micropipettes and sterile, disposable tips.
8. Waste container and bags.
9. Laboratory timer.
10. Paper towels and wipes.
11. Tube racks.
12. Laboratory markers, assorted colors, permanent ink.
13. Serological strippettes.
14. Vacuum-filter flask apparatus.

2.2 LLNA:BrdU-ELISA

1. All studies involving animals must be performed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International-certified laboratory.
2. Test substance is formulated in a suitable vehicle and vehicle alone (vehicle selection discussed below).
3. Female mice (8–12 weeks of age) are group housed (4/cage) under constant environmental conditions with an ambient temperature of $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$, a relative humidity of 30–70% (optimal 55%), and a 12 h light/dark cycle. Provide mice a balanced diet of mouse chow and water ad libitum. While CBA/JN mice are the preferred strain for this assay, other strains have been successfully used (i.e., CBA, CBA/CaOlaHsd, BALB/cAnN, BALB/c) [8, 16, 18, 21, 23, 25]. Mice should be acclimated to these housing conditions for 5 days prior to use in this assay.
4. 70% (v/v) ethanol squirt bottle.
5. 5-Bromo-2'-deoxyuridine (BrdU).
6. Phosphate-buffered saline, pH 7.2–7.4, stored at room temperature.
7. 0.9% sodium chloride injection, USP, sterile.
8. Multichannel vacuum aspirator.
9. Styrofoam blocks and pins.
10. Electronic digital caliper, standards, and screwdriver.
11. ELISA plate reader and computer equipped with appropriate software.
12. Forceps.
13. Dissection scissors.
14. 70 μm cell strainers, sterile.
15. 1.5 mL microfuge tubes.
16. Reusable plastic pestles for 1.5 mL tubes, sterile.
17. Costar multichannel pipette.
18. 50 mL conical centrifuge tubes, clear and amber colored.
19. 15 mL conical centrifuge tubes, clear and amber colored.
20. 96-well flat-bottom microtiter plates with lids, tissue culture grade, sterile.
21. 1 mL tuberculin syringes.
22. 25-gauge 5/8" needles.

2.3 BrdU-ELISA

1. Colorimetric BrdU cell proliferation ELISA assay kit.
2. Multichannel pipette.
3. Disposable troughs for dispensing reagents with the multichannel pipette.

3 Methods

3.1 LLNA:BrdU-ELISA

1. General considerations:

- (a) For animal welfare reasons, test articles known to be corrosive to the skin should never be tested in this assay.
- (b) Only mice that are in good health and free of skin lesions may be used in the assay.
- (c) Mice should be acclimated to the facility for 1 week prior to being randomly assigned to the control and the treatment groups.

2. Selecting appropriate doses for the assay:

- (a) The peak concentration tested should be the maximum dose that does not cause systemic toxicity or excessive local irritation following dermal application. In some instances, this information is not readily available. Consequently, the test article should be prescreened for acute toxicity and dermal irritation to identify appropriate doses for the assay.
- (b) Prescreening is conducted identically to the main LLNA:BrdU-ELISA. In this case, however, lymph node proliferation is not assessed, and fewer animals are used (1–2 mice are recommended per dose level). Mice in each dose group will be assessed for clinical signs of systemic toxicity and excessive local dermal irritation at the application site as described below.

- *Monitoring mice for signs of systemic toxicity after topical exposure*—a variety of clinical observations can be monitored that are indicative of systemic toxicity including changes in (1) survival, (2) nervous system function (e.g., piloerection, ataxia, tremors, and convulsions), (3) behavior (e.g., aggressiveness, change in grooming activity, activity level), (4) respiratory patterns (i.e., changes in frequency and intensity), (5) consumption of food and water, and (6) >5% reduction in body weight from experimental day 1 to day 6, and mortality should be considered in the evaluation. Moribund mice and those experiencing pain and distress should be humanely euthanized.
- *Monitoring mice for signs of excessive local dermal irritation following topical exposure*—not only can excessive local irritation cause undue pain and distress to laboratory animals, but dermal irritation has also been indicated as a potential cause of false-positive results in the standard LLNA (and presumably variations of the LLNA) [26–31]. Together, a strong case for assessing the potential for test substances to cause dermal irritation can be made.

Table 1
Erythema scoring system

Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema (beet redness)	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

- Excessive dermal irritation at the site of test article application can be indicated by visual inspection. Erythema (i.e., reddening of the skin) should be scored pretest, prior to dosing on each day of dosing and prior to termination. An erythema score ≥ 3 (Table 1) is indicative of excessive dermal irritation [32]. While useful, visual scoring is highly subjective, and, in some instances, colored test articles could corrupt the visual scoring system. Consequently, relying solely on erythema scores is not the preferred method for assessing local dermal irritation.
 - With practice, local dermal irritation can be quantitatively assessed by measuring changes in ear thickness. These measurements should be collected using a calibrated caliper prior to dosing on experimental days 1 and 3 and prior to termination on experimental day 6. To properly measure ear thickness, restrain the mouse (anesthesia is not necessary), place the caliper pads on the center of the apex of the ear with a margin of the ear showing on all sides, and, while taking care not to compress the ear, measure the thickness of each ear and record on a datasheet (Fig. 1). To minimize the risk of compression artifacts, alternate ears between measurements. A 25% increase in ear thickness is suggestive of excessive local skin irritation [32].
3. Prepare fresh dosing solutions daily. Select three consecutive concentrations from the following scheme: 50, 25, 10, 5, 2.5, 1, 0.5, 0.25, and 0.1% (w/v). The highest dose selected for the full assessment should be the highest dose that did not induce systemic toxicity and/or excessive local dermal irritation. The concurrent positive control (*see Note 1*) and dosing vehicle solutions should also be prepared at this point (*see Notes 1 and 2*).

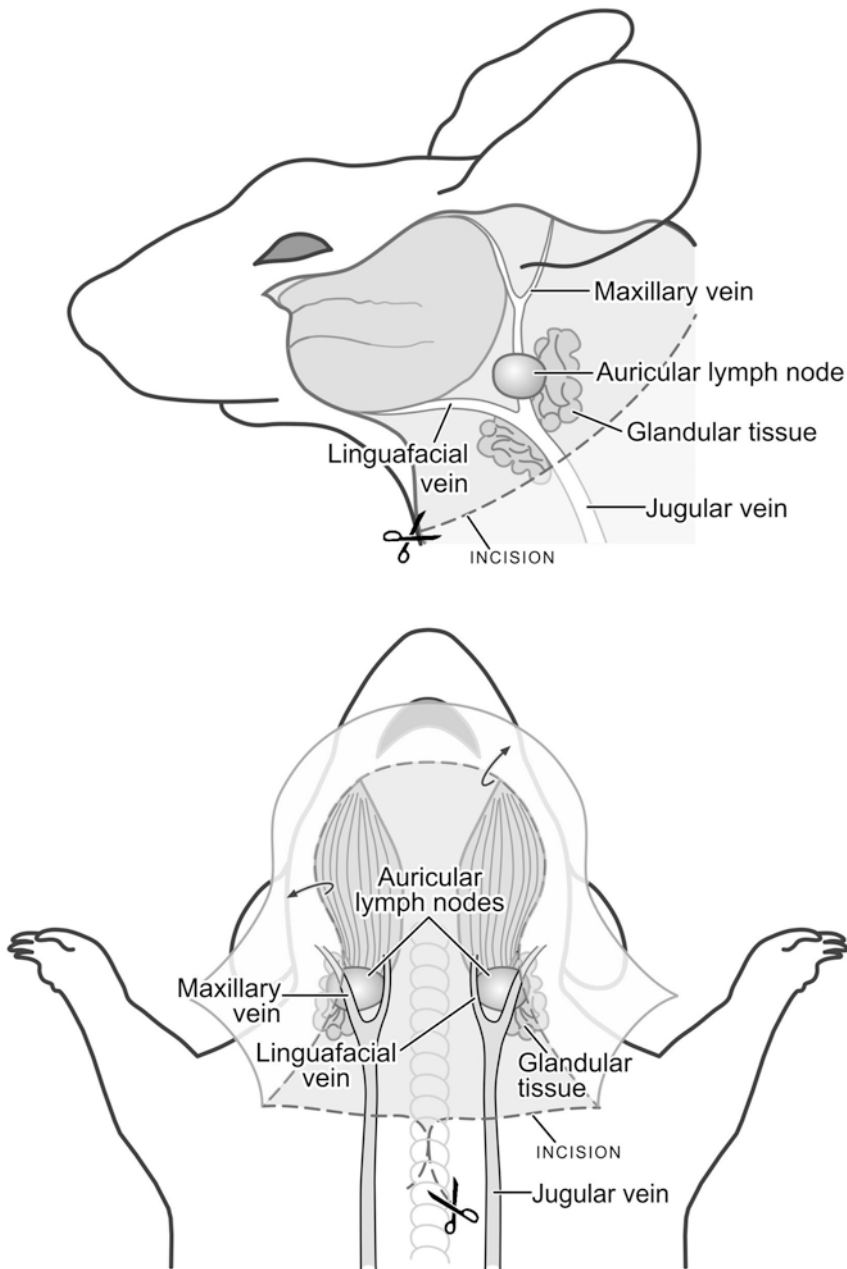


Fig. 1 Proper caliper placement for measuring ear thickness. Place the pads of the caliper on the center of the ear. Do not place the caliper pads on the tip of the ear or allow them to overhang the edge of ear. To promote accuracy and reproducibility, place the caliper pads on the same location for every measurement collected

4. LLNA:BrdU-ELISA procedure

(a) Experimental day 1

- Mark each animal with a unique identifier for tracking individual data (*see* **Note 3**).

- Weigh each animal and record weight on the datasheet.
 - Apply 25 μ L of test substance or vehicle onto the dorsum of both ears of each mouse ($n = 4$ successfully treated mice per experimental group) using a pipette and with a disposable tip. Being careful not to injure the skin, carefully spread the test article over the surface of the ear with the disposable pipette tip.
 - Return animals to their home cages (*see* **Note 4**).
- (b) Experimental days 2 and 3
- Repeat activities performed on experimental day 1.
- (c) Experimental day 4
- No activity.
- (d) Experimental day 5
- Prepare BrdU solution (10 mg/mL in sterile 0.9% sodium chloride for injection). Pass the solution through a 0.4 μ m filter to sterilize. Protect the BrdU solution from light.
 - Verify integrity of individual animal identification markings and collect body weight data.
 - Properly restrain the mouse by grasping the scruff of the neck and inject each mouse with 0.5 mL BrdU solution intraperitoneally (25-gauge 5/8" needle) (*see* **Note 5**). A fresh needle must be used for each mouse.
 - Return the mice to their home cages.
- (e) Experimental day 6
- Verify integrity of individual animal identification markings and collect body weight data.
 - While adhering to the American Veterinary Medical Association Guidelines for the Euthanasia of Animals, euthanize each mouse via CO₂ narcosis [33]. Ensure euthanasia by cervical dislocation.
 - To harvest the lymph nodes, place the mouse in a lateral position (i.e., on its back) and clean the cheek area with 70% ethanol. If desired, pin the mouse to a Styrofoam block to position it for dissection.
 - Using sterile scissors, make an incision across the chest and between the shoulders. Make a second incision up the midline perpendicular to the initial cut and then up to the chin area.
 - Using sterile forceps, carefully peel the skin away from the underlying tissue to expose the jugular veins in the neck area. Leave the fatty tissue intact and in place on the cheek.

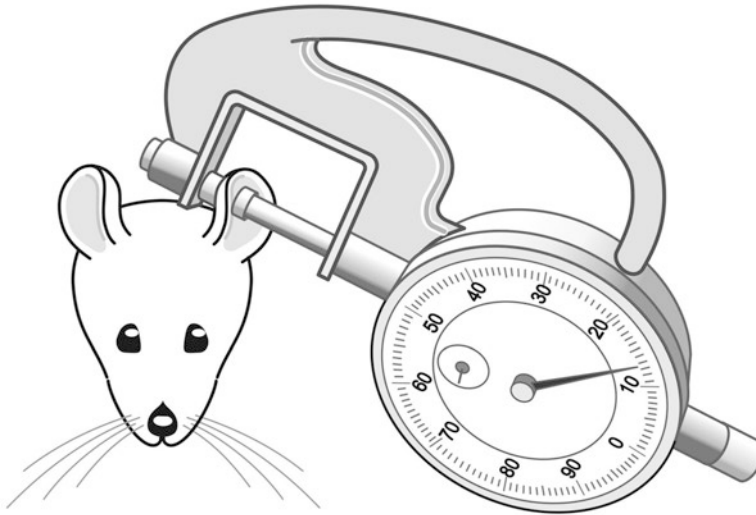


Fig. 2 Dissection approach and location of auricular lymph nodes. **(a)** Lateral dissection provides quick access to the lymph nodes. Place the euthanized mouse on its side and wet the face with 70% ethanol. Raise the neck skin between the front arm and below the ear using forceps and then carefully snip the skin with scissors. Avoiding the area below the ear and near the back of the jaw, extend the incision to create an arch across the neck. Angle the scissors slightly upward to prevent tissue damage. Gently peel the skin back and locate the lymph node draining the ear relative to the bifurcation of the jugular vein. Repeat the process to collect the other lymph node. **(b)** As an alternative to lateral dissection, the auricular lymph nodes can be harvested by ventral dissection. Place the euthanized mouse on its back and wet the abdomen, neck, and face with 70% ethanol. Using scissors and forceps, make an incision across the chest between the arms. Make a second incision up the midline. Carefully peel the skin back and locate the lymph nodes draining the ear (i.e., auricular) as described above. Adapted from ICCVAM 2010 [32]

- The lymph nodes draining the ears are located at the bifurcation point of the jugular veins (i.e., point where the maxillary vein and linguofacial veins diverge) (Fig. 2 and see Note 6).
 - Carefully remove each lymph node. Pool lymph node pairs from individual animals in a 1.5 mL microfuge tube containing 0.25 mL PBS at room temperature (see Note 7).
- Process pooled lymph nodes into cell suspensions by mechanically breaking them up using a reusable plastic pestle.
- Create single-cell suspensions by passing each cell suspension through a 70 μm cell strainer placed on a 50 mL conical centrifuge tube. To maximize lymph node cell recovery, rinse the pestle and 1.5 mL microfuge tube with 1 mL PBS. Next, wash the strainer with 5 mL PBS.
- Pellet the lymph node cells by centrifugation ($1000 \times g$ for 7 min) at room temperature.

- Carefully aspirate the supernatant leaving the cell pellet behind.
- Resuspend the cell pellet in approximately 15 mL PBS (*see Note 8*).

3.2 Plating Cells

1. Plate 100 μ L of each cell suspension into the wells (in duplicate) of a 96-well plate.
 - (a) Mix cells between every aliquot to promote consistency.
 - (b) Disperse cells evenly over the surface of the tissue culture plate using a tabletop shaker for 1 min.
2. Add 100 μ L of PBS to the appropriate wells of the 96-well plate(s) to serve as blanks (*see Note 9*).
3. Transfer cultures to a tissue culture incubator (37 °C, 5% CO₂, 95% humidity) for 30 min to allow the cells to settle to the bottom of the tissue culture plate.
4. After the 30 min incubation, distribute the lymph node cells to the bottom of the tissue culture plate by centrifugation (300 $\times g$) for 10 min at room temperature.
5. Using a multichannel aspirator fitted with disposable pipette tips, very carefully aspirate off 80–90% of the PBS. Do not disrupt the cells by touching the surface of the tissue culture plate with the tips of the aspirator.
6. Remove the residual PBS and dry the lymph node cells to the plate using a hot air oven (60 °C for 1 h).
7. After drying the plate, parafilm the top down and store at 4 °C in the cold box or refrigerator for up to 1 week.

3.3 BrdU-ELISA

1. Measure BrdU incorporation into the lymph node cells using a commercially available colorimetric BrdU cell proliferation ELISA assay kit and instructions.

3.4 Data Processing and Interpretation

1. Calculate the BrdU labeling index for each mouse as follows:
 - (a) The BrdU labeling index = $(\text{Abs}_{\text{em}} - \text{Abs blank}_{\text{em}}) - (\text{Abs}_{\text{ref}} - \text{Abs blank}_{\text{ref}})$ where Abs = absorbance, em = emission wavelength (i.e., 370 nm), and ref = reference wavelength (i.e., 492 nm, *see Notes 10 and 11*).
 - (b) Determine the mean BrdU labeling index for each test article and control group.
2. Calculate the stimulation index (SI) for each mouse by dividing the BrdU labeling index/mouse within each test article group and concurrent positive control by the mean BrdU labeling index for the solvent/vehicle control group.
 - (a) Stimulation index = $(\text{BrdU labeling index for test}) / (\text{mean BrdU labeling index for control})$.

3. Test substances with a mean stimulation index ≥ 1.6 are generally regarded as positive. However, stimulation index values between 1.6 and 1.9 are regarded as borderline positive results. When borderline positive results are obtained, the final decision regarding sensitizer potential should include other factors that could impact the outcome of the assay (i.e., evidence of systemic toxicity, excessive local dermal irritation, statistical significance, strength of the dose response).
4. Typical results obtained when using the LLNA:BrdU-ELISA are shown in Table 2. Mice were exposed to a known contact allergen cinnamaldehyde (CA, formulated in AOO). Lymphocyte proliferation was determined using the Roche Cell Proliferation ELISA. The assay plate was incubated for 15 min prior to collecting absorbance values. For efficient presentation of data, optical absorbance values have been averaged for each experimental group. Data were processed using the formulas provided above to produce the BrdU labeling index and ultimately the SI. Topical exposure to CA induced a dose-dependent increase in proliferation of cells in draining lymph nodes at the site of application. The highest dose, 10% CA, induced an SI of 2.79, a positive threshold response. To confirm that the doses applied were not excessively irritating to the skin at the site of application, ear thickness and erythema were monitored before dosing on experimental days 1 and 3 and before necropsy on day 6. Application of CA resulted in thickening of the ears on experimental day 3 with edema largely subsided by experiment day 6 (Fig. 3). Importantly, the change in ear thickness did not exceed the threshold for excessive irritation (i.e., $\geq 25\%$ increase). Similarly, the maximum

Table 2
LLNA:BrdU-ELISA data and processing

Test article (% w/v)	Avg. Abs ₃₇₀ cells	Avg. Abs ₃₇₀ blank	Abs _{sample370} – Abs _{blank370}	Avg. Abs ₄₉₀ cells	Avg. Abs ₄₉₀ blank	Abs _{sample490} – Abs _{blank490}	BrdU labeling index (Abs _{sample370} – Abs _{blank370}) – (Abs _{sample490} – Abs _{blank490})	SI	SEM
AOO	0.67	0.34	0.32	0.07	0.05	0.01	0.31	NA	NA
10% CA	1.25	0.34	0.90	0.10	0.05	0.04	0.86	2.79	0.12
5% CA	0.85	0.34	0.51	0.08	0.05	0.02	0.49	1.57	0.10
0.5% CA	0.66	0.34	0.32	0.07	0.05	0.01	0.31	0.99	0.07

AOO acetone/olive oil (4:1), Avg. average, Abs absorbance, SI stimulation index, SEM standard error of the mean

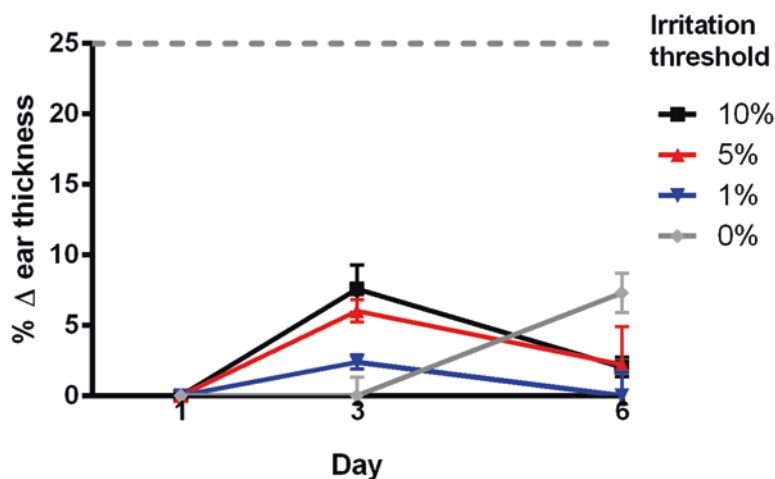


Fig. 3 Local dermal irritation assessment following treatment with cinnamaldehyde (vehicle = acetone/olive oil). Ear thickness was measured before dosing on experimental days 1 and 3 and before necropsy on experimental day 6. Representative data are shown ($\pm$ SEM, $n = 4$)

erythema score recorded for these test substances was 0 (i.e., no erythema; data not shown). Together, these data suggest that CA did not produce excessive dermal irritation at the concentrations tested.

4 Notes

1. Proper study design dictates that positive and negative control groups be included in each test. Inclusion of these substances is necessary to demonstrate proficiency with the assay and to also verify that the assay is being performed correctly (i.e., achieving reproducible assay sensitivity and magnitude of response). A negative (solvent/vehicle) control is required for determining the background stimulation index. Positive controls are substances with well-characterized assay responses that generate a positive response when tested at a concentration known to yield a stimulation index of ≥ 1.6 over the negative control group. Recommended positive control substances are 50% hexyl cinnamic aldehyde (HCA; CASRN 101-86-0) and 50% eugenol (CASRN 97-53-0) in AOO. In some situations (e.g., when using a novel solvent/vehicle), a naïve untreated control group should be included. Mice in the negative and naïve control groups should be handled and processed identically to the mice in the test article treatment groups.
2. Selecting an appropriate solvent/vehicle is a key consideration when using the standard LLNA or variations of the LLNA. The solvent/vehicle should be selected based on maximizing test

article solubility in order to produce a solution/suspension containing the highest concentration achievable without interfering with test results. Recommended vehicles include acetone/olive oil (4:1 v/v) (AOO), N,N-dimethylformamide (DMF), methyl ethyl ketone (MEK), propylene glycol, and dimethyl sulfoxide (DMSO) [34, 35]. Due to its high surface tension, which limits distribution and contact time with the skin, water is not recommended for use in this assay. Consequently, hydrophilic test articles are often tested using a vehicle system that incorporates solubilizing agents that wet and adhere to the skin [36].

3. Mice should be marked to facilitate individual identification. However, because the ears are the site of topical application and are also critical to assessing test substance-related dermal irritation, they should not be adulterated in any way.
4. Some investigators may find it useful to use temporary mouse holding cages to help organize processing steps and to eliminate the possibility of accidentally duplicating experimental procedures.
5. Although intraperitoneal injection is a widely used technique, it can be unreliable because the material may be inadvertently introduced into the gut, abdominal fat, and/or subcutaneous tissues. Proper injection technique can minimize issues associated with this technique. Mice should be scruffed, and, with the mouse's head tilted slightly downward, the needle should be inserted into the mouse's right side (to avoid the cecum) at a 30° angle to a depth of about half a centimeter. Before injecting the BrdU solution, the technician should verify that the needle has not penetrated the intestines or urinary bladder and check for a yellow aspirate by gently pulling back on the syringe plunger. If any fluid is aspirated, the BrdU solution must be discarded and the procedure repeated using a new syringe and needle.
6. Experience is required to correctly identify and isolate the lymph nodes. Investigators are encouraged to practice the procedure on naïve mice and mice topically exposed to a positive control substance prior to conducting any investigations.
7. Some LLNA protocols recommend pooling all lymph nodes from a single experimental group and processing them as a single entity. While this approach saves a small amount of time, pooling lymph nodes on an individual animal basis facilitates assessment of inter-animal variability and statistical analysis.
8. The target volume of the lymph node cell suspension should be adjusted to a predetermined optimized volume (approximately 15 mL) based on achieving a mean absorbance value of the negative control group within 0.1–0.2. However, because absorbance values depend on the assay apparatus and the target volume of cell suspension, every laboratory should decide their own optimal volume of LNC suspension in advance.

9. Blanks are necessary to determine non-specific binding of BrdU and BrdU detection antibody to the plate. The absorbance of the blanks has to be subtracted from all other values.
10. Cellular debris can interfere with assay readings. To correct for this, a reference wavelength is used to determine how much of the signal is attributed to noise. The noise can then be subtracted out from the test wavelength by using a reference wavelength.
11. Stop solution (25 μ L 1 M H_2SO_4) may be used to halt development of the ELISA plate to prevent saturation. However, the use of stop solution will only allow for reading the plate at a single time point. Also, if stop solution is added obtain ABS_{450} (reference wavelength 690 nm).

Disclaimer

This chapter has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the agency, nor does the mention of trade names of commercial products constitute endorsement or recommendation for use.

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