

Chapter 16

The Local Lymph Node Assay and Skin Sensitization Testing

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Abstract

The mouse local lymph node assay (LLNA) is a method for the identification and characterization of skin sensitization hazards. In this context the method can be used both to identify contact allergens, and also determine the relative skin sensitizing potency as a basis for derivation of effective risk assessments.

The assay is based on measurement of proliferative responses by draining lymph node cells induced following topical exposure of mice to test chemicals. Such responses are known to be causally and quantitatively associated with the acquisition of skin sensitization and therefore provide a relevant marker for characterization of contact allergic potential.

The LLNA has been the subject of exhaustive evaluation and validation exercises and has been assigned Organization for Economic Cooperation and Development (OECD) test guideline 429. Herein we describe the conduct and interpretation of the LLNA.

Key words: Contact allergy, Local lymph node assay, Hazard identification, Potency

1. Introduction

The mouse local lymph node assay (LLNA) was developed initially to provide an alternative to guinea pig tests for the identification of skin sensitization hazards (1). Descriptions of the development, evaluation and eventual validation of the LLNA for this purpose are available elsewhere (1–3), and a survey of that history is unnecessary here. The approach is based on an understanding that the acquisition of skin sensitization requires the induction of a cutaneous immune response to chemical allergen experienced at the skin surface. The immunobiology of skin sensitization is complex and requires an array of cellular/molecular interactions that are orchestrated in time and space. For the purposes of this article, however, it is possible to summarise the key events as follows. If an inherently susceptible subject is exposed

topically to a sufficient quantity of contact allergen then specific priming of the immune system will be provoked that results in sensitization. The inducing allergen is recognized in the skin by one or more of several populations of dendritic cells, including epidermal Langerhans' cells (LC). These cells have responsibility for internalization and processing of antigen (including chemical allergen) at skin surfaces and for delivery of that antigen to regional lymph nodes draining the site of exposure. Within nodes antigen is presented to responsive T lymphocytes that are triggered to divide and differentiate. The division of antigen-specific T lymphocytes results in selective clonal expansion and the immunological priming that is defined as sensitization. Central to the development of skin sensitization, therefore, is the activation and clonal expression of chemical (hapten)-specific T lymphocytes. We therefore chose to measure the ability of chemicals to cause skin sensitization as a function of their ability in mice to stimulate lymph node cell (LNC) proliferation following topical application (4, 5).

This is the approach that became known as the LLNA. In principle the assay is very straightforward. Groups of mice are exposed topically on the dorsum of both ears to various concentrations of the test chemical, or to an equivalent volume of the relevant vehicle alone. Exposure is performed daily for three consecutive days. Five days following the initiation of exposure mice receive a source of radiolabelled thymidine by intravenous injection. Thereafter animals are sacrificed, and draining lymph nodes excised and processed for determination of the vigour of mitosis. Results are recorded in the form of a stimulation index (SI) of LNC proliferation relative to values obtained with the concurrent vehicle control. Chemicals that induce, at one or more test concentrations, an SI of equal to or greater than 3 are classified as having skin sensitization potential. Chemicals that fail to elicit a threefold or greater SI at all concentrations are regarded as lacking significant skin sensitizing activity. Experience with this assay has now been extensive and it has been assigned an Organization for Economic Cooperation and Development (OECD) guideline method (guideline 429).

More recently attention has focused on the use of the LLNA not only to provide accurate hazard identification but also to produce an estimation of relative skin sensitization potency. This is predicated on an understanding that LNC proliferation not only has a causal relationship with the acquisition of skin sensitization, but it also correlates closely with the extent to which sensitization will be achieved (that is both a causal and quantitative relationship). For this reason the LLNA is now used also for hazard characterization with regard to relative potency; information that has proven invaluable for the derivation of effective risk assessments and risk management (1, 6).

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2. Materials

1. Chemical to be tested, formulated in an appropriate vehicle and vehicle alone (see Note 1). Gloves should always be worn when handling suspect contact sensitizers. Solutions must be prepared freshly (within 1 h of dosing).
2. Female CBA strain mice (young adults); maintained in semi-barriered conditions (see Note 2).
3. Automatic pipet with disposable tips.
4. Tritiated thymidine: 2 Ci/mmol (80 μ Ci/ml). Store the stock solution at 4°C. Due to the potential for the tritium on the thymidine to exchange onto water molecules, the chemical expiry date provided by the manufacturer must be observed. Gloves must be worn, and the area in which thymidine is used must be swabbed and monitored regularly for radioactive contamination.
5. Phosphate-buffered saline (PBS); pH 7.2–7.4, stored at room temperature.
6. Filters (0.4 μ M).
7. Sterile 1 mL graduated syringes.
8. Sterile 25G 5/8 needles.
9. Temperature controlled "hot box" (see Note 3).
10. Mouse restrainer tube with outlet for tail.
11. 200 gauge stainless steel mesh, cut into approximately 3 cm squares, washed in 70% ethanol, autoclaved and edges turned up to form a box.
12. Sterile 5 mL syringes.
13. 5% w/v trichloroacetic acid (TCA) in water; stable at room temperature for 6 months.
14. Scintillation fluid (eg Hi-Safe Optiphase).
15. β -Scintillation counter.

3. Methods

After rigorous evaluation at an international level, the LLNA has become recognized as the preferred skin sensitization test method for the evaluation of chemicals. However, in common with all safety assessment methods, inappropriate conduct of the assay can lead to difficulties in data interpretation. Experience with the assay over recent years has demonstrated that strict adherence to

published procedures and guidelines are critical for the successful outcome of an LLNA. The standard LLNA identifies contact sensitization hazard potential as a function of radiolabeled thymidine incorporation into the lymph nodes draining the site of topical exposure to chemical. There are two protocols that are used routinely for this purpose. In both methods, mice are exposed topically on the dorsum of both ears to various concentrations of test chemical, or to vehicle alone, daily for three consecutive days. Five days after the initiation of exposure, animals receive an intravenous injection of radiolabeled thymidine. Five hours later, the draining auricular lymph nodes are excised, a single cell suspension of lymph node cells (LNC) prepared and thymidine incorporation measured by β -scintillation counting.

In the first method, four mice per group are used and lymph nodes are pooled on an experimental group basis. In the second method, five mice per group are used and lymph nodes are pooled for each individual animal. Processing of lymph nodes on an individual animal basis provides the opportunity to assess inter-animal variation in thymidine incorporation and for the application of statistical analyses.

The LLNA not only provides reliable data for hazard identification but also allows assessment of relative skin sensitizing potency for the purpose of risk assessment. It is recognized that contact allergens vary substantially (at least four orders of magnitude) with respect to the relative potency with which they are able to induce contact sensitization (6). An understanding of relative potency of contact allergens can provide useful information that can contribute to the risk assessment process. Data derived from the protocol using lymph nodes pooled on a group basis or data derived from the protocol using lymph nodes pooled per individual animal are both suitable for this purpose.

3.1. Topical Exposure to Chemical

1. Allow mice to acclimatize for at least 2 days after arrival in the facility in cages of four animals per group (see Note 4).
2. Identify animals individually (using an indelible marker), weigh and record individual weights (see Note 5).
3. Prepare dosing solutions. Three consecutive concentrations are generally selected from the following: 50, 25, 10, 5, 2.5, 1, 0.5, 0.25 and 0.1% (w/v). Also prepare the appropriate vehicle solution (see Note 6).
4. Dispense 25 μ L of chemical, or vehicle alone, onto the dorsum of both ears of each animal ($n=4$ per group) (See Note 4) using an automatic pipet with a disposable tip, ensuring an even distribution over the surface of the ear.
5. Perform identical treatment (steps 3 and 4) once daily for the next two consecutive days.
6. Rest the animals for 2 days.

3.2. Inject Thymidine Processing Nodes

3.3. Calculate and Interpret Results

3.2. Injection of Thymidine and Processing of Lymph Nodes

1. Weigh the animals and record individual weights (see Note 5).
2. Prepare a solution of tritiated thymidine in PBS (80 $\mu\text{Ci}/\text{mL}$) and filter it using a 0.4 μm filter until sterile.
3. Place the animals in a temperature controlled "hot box", one experimental group at a time, for 5 min to allow the veins to dilate. The temperature must not exceed 37°C.
4. One at a time, restrain each mouse using a restraining tube with an outlet for the tail. Inject the tail vein with 0.25 mL of radiolabeled thymidine (80 $\mu\text{Ci}/\text{mL}$), dispensed with a 1 mL graduated syringe and 25G 5/8 needle. Care must be taken to ensure syringe is free of air bubbles. (see Note 7).
5. Return the animals to their cages and allow to rest for 5 h.
6. Euthanize the animals, excise draining (auricular) lymph nodes and pool for each experimental group in a small volume (approximately 2 mL) of PBS. (see Notes 8 and 9).
7. Using tweezers, place the nodes onto a square of stainless steel gauze contained within a 60 mm plastic Petri dish with a small volume (approximately 2 mL) of fresh PBS. Prepare a single cell suspension of LNC by gently disrupting the lymph nodes and pushing them through the gauze using the plunger of a 5 mL syringe (mechanical disaggregation).
8. Transfer the LNC from the Petri dish into a 10 mL plastic centrifuge tube, rinsing the gauze and the Petri dish with fresh PBS to ensure that all of the cells are collected before disposal. Wash the LNC twice in fresh PBS by centrifugation at 100 $\times g$ for 10 min (see Note 10).
9. After the final wash, resuspend the cell pellet in 3 mL of TCA and store overnight at 4°C (see Note 10).
10. Centrifuge the cells at 100 $\times g$ for 10 min, pipette off the TCA with a disposable pastette and resuspend the cells in 1 mL of fresh TCA. Transfer the suspension to 10 mL of scintillation fluid (eg Hisafe Optiphase) and measure thymidine incorporation by β -scintillation counting. (see Note 11).

3.3. Calculation and Interpretation of Results

1. Record results as total disintegrations per min per node (dpm/node) for each experimental group. Use the vehicle control group as the comparator (see Note 12) in order to derive a stimulation index (SI) according to the following equation

$$\text{SI} = \frac{\text{testd pm / node}}{\text{vehicle dpm / node}}$$

2. The criterion for positivity in the LLNA is an SI of 3 or more at one or more test concentrations (see Note 13).

3. Typical results derived using the standard LLNA protocol are shown in Fig. 16.1 in which animals have been exposed to three concentrations of the potent contact allergen paraphenylenediamine (PPD), or to the non-sensitizing skin irritant methyl salicylate, both formulated in the vehicle of choice for the LLNA, AOO. Data are displayed as a function of dpm/node and as SI relative to concurrent vehicle-treated control values; the latter being the usual method of displaying the results. Exposure to PPD induced a marked dose-dependent increase in proliferation compared with vehicle treated control mice, with all concentrations tested provoking an SI of 3 or greater. This material is, therefore, classified as a skin sensitizer. In contrast, the non-sensitizing skin irritant methyl salicylate, despite testing at doses some 20-fold higher than those utilized for PPD, failed to elicit increases in thymidine incorporation at any concentration examined. This material is, therefore, classified as lacking significant potential to cause skin sensitization.

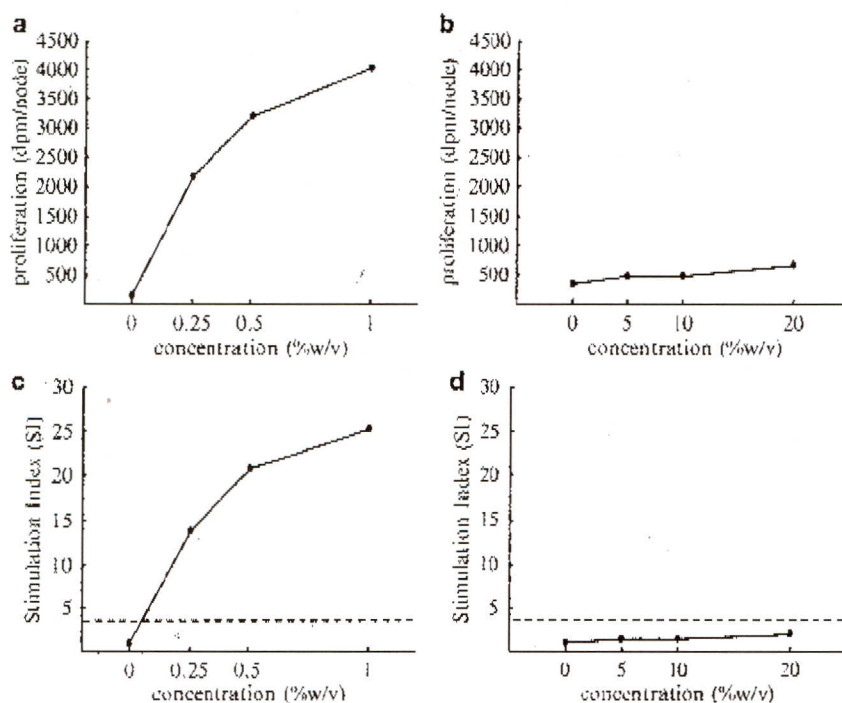


Fig. 16.1. Local lymph node assay responses to paraphenylenediamine (PPD) and methyl salicylate CBA strain mice ($n=4$) received 25 μ l of various concentrations of the contact allergen PPD (a, c) or the non-sensitizing skin irritant methyl salicylate (b, d), or vehicle (AOO) alone on the dorsum of both ears daily for three consecutive days. Five days after the initiation of exposure, draining auricular lymph nodes were excised, pooled on a treatment group basis and processed for β -scintillation counting. Results are expressed as mean dpm/node (a, b) or as a stimulation index (SI) relative to vehicle-treated control groups (c, d). An SI of 3 (current threshold value for a positive in the LLNA) is illustrated as a broken line.

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4. Typical results derived using the LLNA protocol for the assessment of relative skin sensitizing potency are shown in Table 16.1. Animals have been exposed to five concentrations of the potent contact allergen PPD, or to the more moderate skin sensitizer hexyl cinnamic aldehyde (HCA), both formulated in the vehicle of choice for the LLNA, AOO. Data are displayed as dpm/node and as SI relative to concurrent vehicle-treated control values. Both chemicals provoked marked dose-dependent increases in proliferation compared with vehicle treated control mice. Both materials are therefore classified as skin sensitizers.

The EC3 value (Estimated Concentration of chemical required to induce an SI of 3 relative to concurrent vehicle treated controls) is derived by linear interpolation according to the following equation

$$EC3 = c + [(3 - d) / (b - d)](a - c)$$

where the data points lying immediately above and below the SI value of 3 have the co-ordinates (a, b) and (c, d), respectively

The vehicle-treated control (SI = 1) cannot be used for the (c, d) data point.

Table 16.1

Local lymph node assay responses to paraphenylenediamine (PPD) and hexyl cinnamic aldehyde (HCA): determination of EC3 values CBA strain mice ($n=4$) received 25 μ l of various concentrations of the contact allergens PPD or HCA, or vehicle (AOO) alone on the dorsum of both ears daily for three consecutive days. Five days after the initiation of exposure, draining auricular lymph nodes were excised, pooled on a treatment group basis and processed for β -scintillation counting. Results are expressed as mean dpm/node and as a stimulation index (SI) relative to vehicle-treated control groups

Conc. (%w/v)	PPD dpm/node (SI)	Conc. (%w/v)	HCA dpm/node (SI)
0	155 (1)	0	324 (1)
0.05	349 (2.2)	2.5	517 (1.6)
0.1	669 (4.2)	5	690 (2.3)
0.25	2,180 (13.7)	10	1,111 (3.7)
0.5	3,203 (20.8)	25	2,724 (9.1)
1	4,036 (25.3)	50	4,576 (14.2)

In each case, concentrations of chemical have been selected that elicit SI values either side of an SI of 3, thus the data are appropriate for the derivation of EC3 values.

$$\text{PPD EC3} = 0.05 + [(3 - 2.2)/(4.2 - 2.2)] \\ (0.1 - 0.05) = 0.07\%$$

$$\text{HCA EC3} = 5 + [(3 - 2.3)/(3.7 - 2.3)] \\ (10 - 5) = 7.5\%$$

According to the recommendations of Kimber et al. (6), PPD is categorized as an extreme contact sensitizer, whereas HCA is classified as a moderate contact sensitizer (see Note 14).

4. Notes

1. Many organic vehicles may be used. Water, however, is inappropriate as a result of its high surface tension that makes it impossible to apply evenly and to remain in contact with the surface of the skin for a sufficient period of time for absorption. Experience indicates that, in order of preference, the vehicles of choice are: 4:1 [v:v] acetone:olive oil (AOO), methylethyl ketone, dimethylformamide and dimethylsulfoxide. Vehicle selection is dictated by the relative solubility of the test material. For most purposes, AOO is suitable (7).
2. Young adult (6–12 weeks old) female CBA strain mice are used for regulatory LLNA studies. For non-regulatory studies, mice up to 20 weeks old may be used.
3. An alternative approach to improve tail vein dilation is to hold the tails under warm running tap water.
4. In the alternative protocol in which lymph nodes from individual animals are processed, $n=5$.
5. Body weight changes of the animals are monitored over the course of the study to confirm their health status. A decrease in body weight of 10% or greater may be an indication of systemic toxicity and should call into question the validity of the data for that group (8).
6. The dose selection is based on providing the highest possible test concentration to maximize the potential for hazard identification. Test concentration selection will be limited by compatibility with the vehicle chosen and the suitability of the resultant preparation for unoccluded dermal application and should preclude local (dermal) trauma or systemic toxicity. Dosing levels may be set on the basis of oral toxicity data, but

when dealing with a new chemical, it is advisable to perform preliminary sighting studies using limited numbers of animals (1 per dose group is sufficient). In the sighting study, the health status and weight of the animals is recorded, on the fifth day the animals are terminated and the draining (auricular) lymph nodes examined visually for signs of activation (increase in size).

7. There are two appropriate veins on the tail of the mouse. The needle should be introduced at a shallow angle (less than 45°) about halfway down the tail and should be tracked up the vein for most of the length of the needle. Once inside the vein, less resistance should be felt.
8. Quiescent (vehicle-treated) lymph nodes can be difficult for the novice to find. Exposure to a potent contact sensitizer such as 2,4-dinitrochlorobenzene results in a marked increase (approximately tenfold) in lymph node size, thus, it is helpful to identify these activated lymph nodes initially. The lymph nodes are found with surrounding adipose tissue, the lymph nodes can be teased away from the adipose tissue using a pair of forceps. In some animals the auricular lymph node may appear to be a chain of several lymph nodes rather than a single lymph node; the whole lymph node chain should be isolated in this case.
9. In the alternative protocol in which lymph nodes from individual animals are processed, each pair of lymph nodes are pooled on an experimental animal basis and processed.
10. Clumping of LNC is avoided by ensuring the pellet is first completely resuspended in a small volume of liquid before making up to final volume. Round bottomed test tubes are preferable as these allow a more even resuspension of the cell pellet. When washing the LNC by centrifugation, it is advisable to pipette off the liquid from the cell pellet rather than tipping off the waste PBS as this may disturb the cell pellet. Even for vehicle-treated control lymph nodes, the cell pellet should be clearly visible at the bottom of the test tube after every centrifugation step. Loss of the cell pellet may indicate a cracked test tube or that the centrifugation step has been compromised.
11. It is possible that the TCA solution may become contaminated and may react with the scintillation fluid giving unacceptable backgrounds. It is good practice to incorporate concurrent controls of scintillation fluid alone and scintillation fluid plus 1 mL of 5% TCA and process these for β -scintillation counting along with the experimental samples. Disintegrations per minute (dpm) recorded for these controls should be less than 100 dpm.

12. The interpretation of LLNA data requires that the proliferation achieved in test groups compared with the background activity in concurrent vehicle-treated controls so that the SI can be derived. Clearly, the use of the vehicle treated control value as the denominator in the calculation for deriving the SI means that if this value is abnormally high or abnormally low, it will impact markedly on the calculated SI. An abnormal level of thymidine incorporation may be due to technical difficulties with the assay (such as failed intravenous injections) or may reflect the health status of the animals; for example relatively high levels of basal proliferation accompanied by increased lymph node size in the vehicle-treated control group can be indicative of a viral infection or a lymphoproliferative disorder. It is apparent that for experienced competent laboratories, control levels of proliferation in the LLNA are relatively consistent, although the absolute values vary according to the testing laboratory (9). Some biological variation and inter-laboratory variation is to be expected in basal levels of thymidine incorporation. However, in the hands of experienced technicians, very little inter-experimental variation in derived SI values is observed generally for a given chemical (10). It is necessary therefore to establish a normal range of control values for a given vehicle and to monitor any excursions from this range in any laboratory conducting the LLNA.
13. In many experiments, a typical dose response curve will be observed for chemical allergen-induced proliferation, with dose-dependent increases in proliferation that may or may not reach a plateau. In some cases, a bell shaped curve is recorded, with thymidine incorporation reaching maximal levels at intermediate doses and at higher doses, less vigorous proliferation observed. This effect may be due to systemic toxicity at high doses, which may be monitored by general health checks such as the daily recording of weight gain on an individual animal basis. The existence of a bell-shaped curve should not affect the interpretation of the LLNA with respect to hazard identification or relative potency assessment. In some cases, the proliferative response to chemical may show a dose-dependent increase in thymidine incorporation that does not attain an SI of 3 at the maximum dose tested. In this case, the most judicious interpretation is that the chemical has potential to cause contact allergy and, if possible, a repeat assay using higher concentrations of material or an alternative vehicle should be performed.
14. It is advisable for a laboratory to demonstrate regularly that competence has been achieved with the assay. This can be effected by the use of a positive control material such as HCA, which is recommended currently by the OECD as a positive

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control for tests for contact sensitization. This chemical has been evaluated thoroughly in the LLNA, both in the context of inter-laboratory trials and with respect to the stability of responses with time and the comparative data are available in the literature (10–12). These experiments have demonstrated that HCA is identified routinely as a contact sensitizer in the LLNA, with comparable EC3 values obtained between laboratories and for repeat assays within the same laboratory. Suggested doses for HCA (formulated in AOO) for determination of EC3 values are: 50%, 25%, 10%, 5% and 2.5%. The EC3 value for this material is approximately 10%.

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