Protocol:

Murine Local Lymph Node Assay (LLNA)

Purpose:

Assessment of Allergic Contact Dermatitis Potential

Recommended by:

ICCVAM Immunotoxicology Working Group – based on an Independent Expert Peer Review Panel Evaluation of the LLNA

ICCVAM Immunotoxicology Working Group Recommended Protocol for the Murine Local Lymph Node Assay (LLNA)[1]: Testing of Chemicals for Contact Sensitizing (Allergic Contact Dermatitis [ACD]) Potential

PREFACE

The murine local lymph node assay (LLNA) is a test method developed to assess whether a chemical has the potential to induce allergic contact dermatitis (ACD) in humans. The LLNA was submitted to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for consideration as an alternative (i.e., stand-alone) test method to the guinea pig (GP) sensitization tests currently accepted by U.S. regulatory agencies. In early 1998, ICCVAM received the submission from Drs. G. Frank Gerberick (Procter & Gamble, U.S.), Ian Kimber (Zeneca, UK), and David A. Basketter (Unilever, UK) (Sponsors). Subsequently, ICCVAM assembled an independent expert Peer Review Panel (PRP) (Table 1.) to evaluate the usefulness of the LLNA for the hazard identification of potential human contact sensitizers. The PRP was asked to evaluate the LLNA submission with emphasis on the performance of the LLNA. They concluded that the LLNA is an acceptable alternative to currently accepted GP test methods for the hazard identification of chemicals with potential to produce ACD. The PRP also concluded that the LLNA offers animal welfare advantages compared to use of the traditional GP methods in that it provides for animal use refinement (i.e., elimination of distress and pain) and reduces the total number of animals required. An ICCVAM Immunotoxicology Working Group (IWG) (Table 2.) then reviewed the PRP report and developed recommendations applicable to the regulatory use of the LLNA. Together, the IWG worked with the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to produce a protocol that would accurately reflect the PRP recommendations.

This protocol, a modification of the Sponsor-supplied protocol, is based on the comments and discussion presented by the PRP and reflects the deliberations and conclusions of the PRP, in addition to specific regulatory considerations. The protocol and related recommendations were approved by ICCVAM and then forwarded with the Panel's report to agencies for their consideration. The purpose of the revised protocol is to provide a flexible guidance document for agencies and companies; it is not the intention of ICCVAM or the IWG that the protocol be considered as mandatory. *Prior to conducting a LLNA test to meet a regulatory requirement, it is recommended that the appropriate regulatory agency be contacted for their current guidance for the conduct and interpretation of this assay.* The revised

[1]A modification of:1) "Draft OECD Guideline for Testing of Chemicals. Skin Sensitisation: Local Lymph Node Assay," [provided by R. J. Fielder, Department of Health (UK), as background information for the ICCVAM peer review] and 2) the Sponsors protocol. Modifications reflect recommendations stated in the ICCVAM Report (NIH Pub. No. 99-4494).

LLNA protocol with the ICCVAM recommendations is provided herein. Additional information on the ICCVAM LLNA review process and deliberations of the PRP can be found at the ICCVAM website (http://iccvam.niehs.nih.gov) or in the PRP report publication (ICCVAM, 1999).

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GENERAL PRINCIPLE OF DETECTION OF SKIN SENSITIZATION USING THE LOCAL LYMPH NODE ASSAY

The basic principle underlying the LLNA is that sensitizers induce proliferation of lymphocytes in the lymph node draining the site of chemical application. Generally, under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization. The test measures cellular proliferation as a function of *in vivo* radioisotope incorporation into the DNA of dividing lymphocytes. The LLNA assesses this proliferation in the draining lymph nodes proximal to the application site (see **Appendix I**). This effect occurs as a dose-response in which the proliferation in test groups is compared to that in concurrent vehicle-treated controls. A positive control is added to each assay to provide an indication of appropriate assay performance.

DESCRIPTION OF THE LOCAL LYMPH NODE ASSAY

Sex and strain of animals

1. Young adult female mice (nulliparous and non-pregnant) of the CBA/Ca or CBA/J strain should be used at age 8-12 weeks. All animals should be age-matched (preferably within a one-week time frame). Females are used because the existing database is predominantly based on this gender. Other strains and males should not be used until it is sufficiently demonstrated that significant strain- and/or gender-specific differences in the LLNA response do not exist.

Preparation of animal

2. The temperature of the experimental animal room should be 21^{o}C (± 3^{o}C) and the relative humidity 30-70%. When artificial lighting is used, the light cycle should be 12 hours light:12 hours dark. For feeding, standard laboratory mouse diets should be used with an unlimited supply of drinking water. The mice should be acclimatized for at least 5 days prior to the start of the test. Animals may be housed individually, or caged in small groups of the same sex. Healthy animals are randomly assigned to the control and treatment groups. The animals are uniquely identified prior to being placed on study. Although a variety of techniques exist to uniquely mark mice, any method that involves identification via ear marking (e.g., ear tags) should not be used.

Preparation of doses

3. Solid test substances should be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be prepared daily unless stability data demonstrate the acceptability of storage.

Test conditions

Solvent/vehicle

4. The solvent/vehicle should be selected on the basis of maximizing the test concentrations while producing a solution/suspension suitable for application of the test substance. In order of preference, recommended solvents/vehicles are acetone/olive oil (4:1 v/v), *N*,*N*-dimethylformamide (DMF), methyl ethyl ketone (MEK), propylene glycol (PG), and dimethyl sulfoxide (DMSO), but others may be used (Kimber and Basketter, 1992). Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided. It may be necessary for regulatory purposes to test the chemical in the clinically relevant solvent or product formulation.

Controls

5. Concurrent negative (solvent/vehicle) and positive controls should be included in each test. In some circumstances, it may be useful to include a naïve control. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals of the treatment groups.

6. Positive controls are used to ensure the appropriate performance of the assay. The positive control should produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) >3 over the negative control group. The positive control dose should be chosen such that the induction is clear but not excessive. Preferred positive control substances are hexyl cinnamic aldehyde (HCA) and mercaptobenzothiazole. There may be circumstances where, given adequate justification, other positive control substances may be used.

   Although the positive control substance should be tested in the vehicle that is known to elicit a consistent response (i.e., acetone:olive oil), there may be certain regulatory situations where a non-standard vehicle (clinically/ chemically relevant formulation) is necessary to test the effect (interaction) of a positive control with this unconventional vehicle.

Methodology

7. A minimum of five successfully treated animals is used per dose group, with a minimum of three consecutive concentrations of the test substance plus a solvent/vehicle control and a positive control group. Test substance treatment doses should be based on the recommendations given in Kimber and Basketter (1992) and in the ICCVAM Peer Review Panel (Panel) Report (ICCVAM, 1999). Doses are selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. The maximum concentration tested should be the highest achievable level while avoiding overt systemic toxicity and excessive local irritation. To identify the appropriate maximum test substance dose, an initial toxicity test, conducted under identical experimental conditions except for an assessment of lymph node proliferative activity, may be necessary. To support an ability to identify a dose-response relationship, data must be collected on at least three test substance treatment doses, in addition to the concurrent solvent/ vehicle control group. For negative LLNA studies, the concurrent positive control must induce a SI >3 relative to its vehicle-treated control (see Section 6).

8. The LLNA experimental procedure is performed as follows:

   Day 1 – Individually identify and record the weight of each mouse prior to dermal applications. Apply 25 µL/ear of the appropriate dilution of the test substance, or the positive control, or the vehicle alone to the dorsum of both ears.

   Days 2 and 3 – Repeat the application procedure as carried out on day 1.

   Days 4 and 5 - No treatment.

   Day 6 – Record the weight of each mouse. Inject 250 µL of sterile phosphate-buffered saline (PBS) containing 20 µCi of ^{3}H-methyl thymidine (^{3}H–TdR) or 250 µL PBS containing 2 µCi of ^{125}I-iododeoxy-uridine (^{125}IU) and 10^{-5} M fluorodeoxy-uridine into each experimental mouse via the tail vein (Loveless et al., 1996; Kimber et al., 1995). Five hours later, the draining ("Auricular") lymph node of each ear is excised and pooled in PBS for each animal. Both bilateral draining lymph nodes must be collected (see diagram and description of dissection in **Appendix I**). A single cell suspension of lymph node cells (LNC) is prepared for each mouse. The single cell suspension is prepared in PBS by either gentle mechanical separation through 200-mesh stainless steel gauze or another acceptable technique for generating a single cell suspension. LNC are washed twice with an excess of PBS and the DNA precipitated with 5% trichloroacetic acid (TCA) at 4^{o}C for approximately 18h.

   For ^{3}H – TdR method, pellets are resuspended in 1 mL TCA and transferred to 10 mL of scintillation fluid. Incorporation of tritiated thymidine is measured by β-scintillation-counting as disintegrations per minute (dpm) for each mouse and expressed as dpm/mouse. For the ^{125}IU method, the 1 mL TCA pellet is transferred directly into gamma counting tubes. Incorporation of ^{125}IU is determined by gamma counting and also expressed as dpm/mouse.

   Observations: Mice should be carefully observed for any clinical signs, either of local irritation at the application site or of systemic toxicity. Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded, with records being maintained for each individual mouse.

9. Results for each treatment group are expressed as the mean SI. The SI is the ratio of the mean dpm/mouse within each test substance treatment group and the positive control treated group against the mean dpm/mouse for the solvent/vehicle treated control group. However, the investigator should be alert to possible "outlier" responses for individual animals within a group that may necessitate the use of an alternative measure of response (e.g., median rather than mean) or elimination of the outlier. Each SI should include an appropriate measure of variability that takes into account the inter-animal variability in both the dosed and control groups (ICCVAM, 1999).

   In addition to an assessment of the magnitude of the SI, a statistical analysis should be conducted. This assessment should include an assessment of the dose-response relationship as well as pair-wise dosed group versus concurrent solvent/vehicle concurrent control comparisons (e.g., linear regression analysis to assess dose-response trends; Dunnett's test to make pairwise comparisons). In choosing an appropriate method of statistical

analysis, the investigator should be aware of possible inequality of variances and other related problems that may necessitate a data transformation or a non-parametric statistical analysis.

Data and reporting

10. Individual mouse dpm data should be presented in tabular form, along with the group mean dpm/mouse, it's associated error term, the SI (and associated error term) for each dose group compared against the concurrent solvent/vehicle control group.

Evaluation and interpretation of results

11. In general, when the SI for any single treatment dose group is ≥3, the test substance is regarded as a skin sensitizer (Basketter et al., 1996; ICCVAM, 1999; Kimber et al., 1994). However, the magnitude of the SI should not be the sole factor used in determining the biological significance of a skin sensitization response. A quantitative assessment may be performed by statistical analysis of individual animal data and may provide a more complete evaluation of the test agents (see Section 9). Factors that should be considered include the results of the SI, statistical analyses, the strength of the dose-response relationship, chemical toxicity, solubility, and the consistency of the vehicle and positive control responses. Equivocal results should be clarified by considering statistical analysis, structural relationships, available toxicity information, and dose selection.

12. A test substance not meeting the above criteria is considered a non-sensitizer in this test.

13. The test report should contain the following information:

Test substance, controls, and solvent/ vehicles

- identification data and CAS no., if known;
- physical nature and purity;
- physiochemical properties relevant to the conduct of the study;
- stability of the test substance, if known; and
- lot number of the test substance.

Solvent/vehicle:

- use of the regulatory relevant vehicle;
- justification for choice of solvent/vehicle; and
- solubility and stability of the test substance in the solvent/vehicle.

Test animals:

- strain of mice used;
- number, age, and sex of mice;
- source, housing conditions, diet, etc.;
- individual weight of the animals at the start and end of the test, including body weight range, mean and associated error term for each group; and
- microbiological status of the mouse

Test conditions:

- positive and negative (vehicle/ solvent) control data;
- data from range-finding study, if conducted;
- rationale for dose level selection;
- details of test substance preparation;
- details of the administration of the test substance;

- details of food and water quality;
- detailed description of treatment and sampling schedules;
- methods for measurement of toxicity;
- criteria for considering studies as positive, negative, or equivocal.

Results:

- signs of toxicity;
- dpm/mouse values for each mouse within each treatment group;
- mean and associated error term for dpm/mouse for each treatment group;
- calculated SI and associated error term for each test substance treatment dose group and concurrent positive control group;
- dose-response relationship;
- statistical analyses and method applied;
- concurrent and historical negative control data as established in the testers laboratory;
- concurrent positive control data

Discussion of the results

Conclusion

REFERENCES

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). (1999). The murine local lymph node assay: A test method for assessing the ACD potential of chemicals/compounds. NIH Publication No. 99-4494, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina. Available on the Internet at http://iccvam.niehs.nih.gov.

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Appendix I: An Approach to Dissection and Identification of the Draining ("Auricular") Lymph Nodes

BACKGROUND

Although minimal technical training of the LLNA is required, extreme care must be taken to obtain appropriate and consistent dissection of the lymph nodes. It is recommended that technical proficiency be achieved by the dissection and identification of the lymph nodes draining the ear by: a) practice dissection on mice that have been injected with a colored agent (dye); and/or b) practice dissection with mice sensitized with a strong positive sensitizer. Brief descriptions of these practice dissections are provided below. Recognizing that nodes from vehicle treated and naïve mice are smaller, laboratories performing the LLNA must also gain proficiency in the dissection of these nodes. It may be helpful for laboratories inexperienced in this procedure to request guidance from laboratories that have successfully performed the LLNA.

TRAINING AND PREPARATION FOR NODE IDENTIFICATION

Identification of the draining node – colored treatment

There are several methods that can be used to provide color identification of the draining nodes. These techniques may be helpful for initial identification and should be performed to ensure proper isolation of the appropriate node. Examples of such treatments are listed below. It should be noted, that other such protocols may be used effectively.

A. ***Evan's Blue Dye treatment:***

Inject approximately 0.1 ml of 2% Evan's Blue Dye (prepared in sterile saline) intradermally into the pinnae of an ear. Euthanize the mouse after several minutes and continue with the dissection as noted below.

B. ***Colloidal carbon and other dye treatments:***

Colloidal carbon and India ink are examples of other dye treatments that may be used (Tilney, 1971).

Identification of the draining node – application of strong sensitizers

For the purpose of node identification and training, a strong sensitizer is recommended. This agent should be applied in the standard acetone:olive oil vehicle (4:1). Suggested sensitizers used for this training exercise include 0.1% oxazolone, 0.1% (w/v) 2,4-dinitrochlorobenzene, and 0.1% (v/v) dinitrofluorobenzene. After treating the ear with a strong sensitizer, the draining node will dramatically increase in size, thus aiding in the identification and location of the node.

Using a procedure similar to that listed in the protocol, the agent is applied to the dorsum of both ears (25 µL/ear) for three consecutive days. On the fourth day, the mouse is euthanized. Identification and dissection (listed below) of the node should be performed in these animals prior to practice in non-sensitized or vehicle-treated mice, where the node is significantly smaller.

Please note: Due to the exacerbated response, the suggested sensitizers are not recommended as controls for the assay performance. They should only be used for training and node identification purposes.

DISSECTION APPROACH

Lateral Dissection (Figure 1)

Although lateral dissection is not the conventional approach used to obtain the nodes draining the ear, it may be helpful as a training procedure when used in combination with the ventral dissection. This approach is performed bilaterally (on both sides of the mouse). After the mouse is euthanized, it is placed in a lateral position. The facial and neck area is wetted with 70% ethanol. Using scissors and forceps, an initial cut is made from the neck area slightly below the ear. This incision is carefully extended toward the mouth and nose. During this procedure, the tip of the scissors should be angled slightly upward to prevent the damage of deeper tissue. The glandular tissue in the area is gently retracted using the forceps. Using the masseter muscle, facial nerves, blood vessels, and the bifurcation of the jugular vein as landmarks, the draining node is isolated and removed (***Figure 1***). The draining node ("Auricular") will be positioned adjacent to the masseter muscle and proximal to and slightly above the jugular bifurcation.

Ventral Dissection (Figure 2)

The most commonly used dissection approach is from the ventral surface of the mouse. This approach allows both right and left draining nodes to be obtained without repositioning the mouse. With the mouse ventrally exposed, the neck and abdomen area is wetted with 70% ethanol. Using scissors and forceps, carefully make the first incision across the chest and between the arms. Make a second incision up the mid-line, perpendicular to the initial cut, and then cut up to the chin area. Reflect the skin to expose the external jugular veins in the neck area. Care should be used to avoid salivary tissue at the midline and nodes associated with this tissue. The nodes draining the ear ("Auricular") are located distal to the masseter muscle, away from the midline, and near the bifurcation of the jugular veins.

ACCURACY IN IDENTIFICATION

The nodes can be distinguished from glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. The application of sensitizing agents (especially the strong sensitizers used in training) will cause an enlargement of the node size. If a dye is injected for training purposes, the node will take on the tint of the dye.

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Figure 1: Lateral Dissection

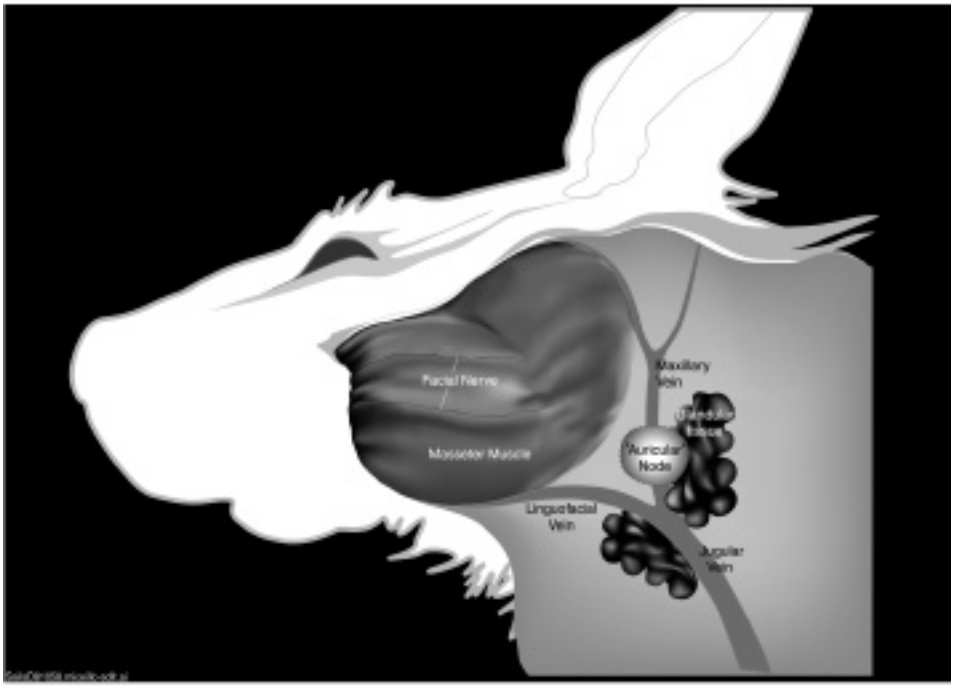

Figure 2: Ventral Dissection