



## **PRINCIPLE OF THE TEST**

5. The basic principle underlying the LLNA is that sensitisers induce a primary proliferation of lymphocytes in the lymph node draining the site of chemical application. This proliferation is proportional to the dose applied (and to the potency of the allergen) and provides a simple means of obtaining an objective, quantitative measurement of sensitisation. The LLNA assesses this proliferation as a dose-response in which the proliferation in test groups is compared to that in vehicle treated controls. The ratio of the proliferation in treated groups to that in vehicular controls, termed the Stimulation Index, is determined, and must be at least three before a test substance can be further evaluated as a potential skin sensitiser. The methods described here are based on the use of radioactive labelling to measure cell proliferation. However, other endpoints for assessment of proliferation may be employed provided there is justification and appropriate scientific support, including full citations and description of the methodology.

## **DESCRIPTION OF THE ASSAY**

### **Selection of animal species**

6. The mouse is the species of choice for this test. Young adult female mice of CBA/Ca or CBA/J strain, which are nulliparous and non-pregnant, are used. At the start of the study, animals should be between 8-12 weeks old, and the weight variation of the animals should be minimal and not exceed 20% of the mean weight. 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.

## **HOUSING AND FEEDING CONDITIONS**

7. Animals should be individually housed. The temperature of the experimental animal room should be 22°C ( $\pm$  3°C). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

## **PREPARATION OF ANIMALS**

8. The animals are randomly selected, marked to permit individual identification (but not by any form of ear marking), and kept in their cages for at least 5 days prior to the start of dosing to allow for acclimatisation to the laboratory conditions. Prior to the start of treatment all animals are examined to ensure that they have no observable skin lesions.

### **Reliability check**

9. Positive controls are used to demonstrate appropriate performance of the assay and competency of the laboratory to successfully conduct 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 substances are hexyl cinnamic aldehyde (CAS No 101-86-0) and mercaptobenzothiazole (CAS No 149-30-4). There may be circumstances in which, given adequate justification, other control substances, meeting the above criteria, may be used. While ordinarily a positive control group may be required in each assay, there may be situations in which test laboratories will have available historic positive control data to show consistency of a satisfactory response over a six-month or

more extended period. In those situations, less frequent testing with positive controls may be appropriate at intervals of no greater than 6 months. Although the positive control substance should be tested in the vehicle that is known to elicit a consistent response (e.g., acetone:olive oil), there may be certain regulatory situations in which testing in a non-standard vehicle (clinically/chemically relevant formulation) will also be necessary. In such situations the possible interaction of a positive control with this unconventional vehicle should be tested.

## **TEST PROCEDURE**

### **Number of animals and dose levels**

10. A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus a negative control group treated only with the vehicle for the test substance, and a positive control, as appropriate. In those cases in which individual animal data are to be collected, a minimum of five animals per dose group are used. Dose and vehicle selection should be based on the recommendations given in reference (2). Doses are selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5% etc. Existing acute toxicity and dermal irritation data should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximises exposure whilst avoiding systemic toxicity and excessive local skin irritation (2)(11). Except for absence of treatment with the test substance, animals in the control groups should be handled and treated in a manner identical to that of animals in the treatment groups.

11. The vehicle should be selected on the basis of maximising the test concentrations and solubility whilst producing a solution/suspension suitable for application of the test substance. In order of preference, recommended vehicles are acetone/olive oil (4:1 v/v), dimethylformamide, methyl ethyl ketone, propylene glycol and dimethyl sulphoxide (2)(10), but others may be used if sufficient scientific rationale is provided. In certain situations it may be necessary to use a clinically relevant solvent or the commercial formulation in which the test substance is marketed as an additional control. Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system, which wets the skin and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided.

### **Experimental schedule**

12. The experimental schedule of the assay is as follows:

- *Day 1:*

Individually identify and record the weight of each animal. Open application of 25µL of the appropriate dilution of the test substance, the vehicle alone, or the positive control (as appropriate), to the dorsum of each ear.

- *Days 2 and 3:*

Repeat the application procedure carried out on day 1.

- *Days 4 and 5 :*

No treatment.

- *Day 6 :*

Record the weight of each animal. Inject 250µL of phosphate-buffered saline (PBS) containing 20 µCi (7.4e+5 Bq) of <sup>3</sup>H-methyl thymidine into all test and control mice via the tail vein. Alternatively inject 250 µL PBS containing 2 µCi (7.4e + 4 Bq) of <sup>125</sup>I-iododeoxyuridine and 10<sup>-5</sup>M fluorodeoxyuridine into all mice via the tail vein. Five hours (5 h) later, the animals are killed. The draining auricular lymph nodes from each ear are excised and pooled in PBS for each experimental group (pooled treatment group approach);

alternatively pairs of lymph nodes from individual animals may be excised and pooled in PBS for each animal (individual animal approach). Details and diagrams of the node identification and dissection can be found in Annex I of the ICCVAM Immunotoxicology Working Group LLNA Protocol (10).

### **Preparation of cell suspensions**

13. A single cell suspension of lymph node cells (LNC) either from pooled treatment groups or bilaterally from individual animals is prepared by gentle mechanical disaggregation through 200 µm-mesh stainless steel gauze. Lymph node cells are washed twice with an excess of PBS and precipitated with 5% trichloroacetic acid (TCA) at 4°C for 18h(2). Pellets are either re-suspended in 1 mL TCA and transferred to scintillation vials containing 1.0 mL of scintillation fluid for <sup>3</sup>H-counting, or transferred directly to gamma counting tubes for <sup>125</sup>I-counting.

### **Determination of cellular proliferation (incorporated radioactivity)**

14. Incorporation of <sup>3</sup>H-methyl thymidine is measured by β-scintillation counting as disintegrations per minute (DPM). Incorporation of <sup>125</sup>I-iododeoxyuridine is measured by <sup>125</sup>I-counting and also is expressed as DPM. Depending on the approach used, the incorporation will be expressed as DPM/treatment group (pooled approach) or DPM/animal (individual approach).

## **OBSERVATIONS**

### **Clinical observations**

15. Animals should be carefully observed once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. All observations are systematically recorded with individual records being maintained for each animal.

### **Body weights**

16. As stated in paragraph 12, individual animal body weights should be measured at the start of the test and at the scheduled kill of the animals.

## **CALCULATION OF RESULTS**

17. Results are expressed as the Stimulation Index (SI). When using the pooled approach, the SI is obtained by dividing the pooled radioactive incorporation for each treatment group by the incorporation of the pooled vehicle control group; this yields a mean SI. When using the individual approach, the SI is derived by dividing the mean DPM /mouse within each test substance group and the positive control group by the mean DPM/mouse for the solvent/vehicle control group. The average SI for vehicle treated controls is then 1.

18. Use of the individual approach to calculate the SI will enable the performance of a statistical analysis of the data. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequalities of variances and other related problems that may necessitate a data transformation or a non-parametric statistical analysis. An adequate approach for interpreting the

data is to evaluate all individual data of treated and vehicle controls, and derive from these the best fitting dose response curve, taking confidence limits into account (10)(12)(13). 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.

19. The decision process with regard to a positive response includes a stimulation index  $\geq 3$ , together with consideration of dose-response and, where appropriate, statistical significance (3)(6)(10)(13)(14).

20. If it is necessary to clarify the results obtained, consideration should be given to various properties of the test substance, including whether it has a structural relationship to known skin sensitisers, whether it causes excessive skin irritation, and the nature of the dose response seen. These and other considerations are discussed in detail elsewhere (7).

## **DATA AND REPORTING**

### **Data**

21. Data should be summarised in tabular form showing the mean and individual DPM values and stimulation indexes for each dose (including vehicle control) group.

### **Test report**

22. The test report should contain the following information:

#### Test substance:

- identification data (e.g. CAS number, if available; source; purity; known impurities; lot number);
- physical nature and physicochemical properties (e.g. volatility, stability, solubility);
- if mixture, composition and relative percentages of components.

#### Vehicle:

- identification data (purity; concentration, where appropriate; volume used);
- justification for choice of vehicle.

#### Test animals:

- strain of mice used;
- microbiological status of the animals, when known;
- number, age and sex of animals;
- source of animals, housing conditions, diet, etc.

#### Test conditions:

- details of test substance preparation and application;
- justification for dose selection (including results from range finding study, if conducted);- vehicle and test substance concentrations used, and total amount of substance applied;
- details of food and water quality (including diet type/source, water source).

## Reliability check:

- a summary of results of latest reliability check, including information on substance, concentration and vehicle used;
- concurrent and/or historical positive and negative control data for testing laboratory.

## Results:

- individual weights of animals at start of dosing and at scheduled kill;
- a table of mean/median (pooled approach) and individual (individual approach) DPM values, as well as the range of values for both approaches, and stimulation indices for each dose (including vehicle control) group;
- statistical analysis, where appropriate;
- time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal.

## Discussion of results:

- A brief commentary on the results, the dose-response analysis, and statistical analyses, where appropriate, with a conclusion as to whether the test substance should be considered a skin sensitiser.

**LITERATURE**

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