

B.42. SKIN SENSITISATION: LOCAL LYMPH NODE ASSAY

1. METHOD

This test method is equivalent to the OECD TG 429 (2002)

1.1 INTRODUCTION

The Local Lymph Node Assay (LLNA) has been sufficiently validated and accepted to justify its adoption as a new Method (1)(2)(3). This is the second method for assessing skin sensitisation potential of chemicals in animals. The other method (B.6) utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (4).

The LLNA provides an alternative method for identifying skin sensitising chemicals and for confirming that chemicals lack a significant potential to cause skin sensitisation. This does not necessarily imply that in all instances the LLNA should be used in place of guinea pig test, but rather that the assay is of equal merit and may be employed as an alternative in which positive and negative results generally no longer require further confirmation.

The LLNA provides certain advantages with regard to both scientific progress and animal welfare. It studies the induction phase of skin sensitisation and provides quantitative data suitable for dose response assessment. The details of the validation of the LLNA and a review of the associated work have been published (5)(6)(7)(8). In addition, it should be noted that the mild/moderate sensitisers, which are recommended as suitable positive control substances for guinea pig test methods, are also appropriate for use with the LLNA (6)(8)(9).

The LLNA is an *in vivo* method and, as a consequence, will not eliminate the use of animals in the assessment of contact sensitising activity. It has, however, the potential to reduce the number of animals required for this purpose. Moreover, the LLNA offers a substantial refinement of the way in which animals are used for contact sensitisation testing. The LLNA is based upon consideration of immunological events stimulated by chemicals during the induction phase of sensitisation. Unlike guinea pig tests the LLNA does not require that challenged-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA does not require the use of an adjuvant, as is the case for the guinea pig maximisation test. Thus, the LLNA reduces animal distress. Despite the advantages of the LLNA over traditional guinea pig tests, it should be recognised that there are certain limitations that may necessitate the use of traditional guinea pigs tests (e.g., false negative findings in the LLNA with certain metals, false positive findings with certain skin irritants)(10).

See also Introduction part B.

1.2 PRINCIPLE OF THE TEST METHOD

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 relationship 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.

1.3 DESCRIPTION OF THE TEST METHOD

1.3.1 Preparations

1.3.1.1 *Housing and feeding conditions*

Animals should be individually housed. The temperature of the experimental animals room should be 22°C ($\pm 3^\circ\text{C}$). 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.

1.3.1.2 *Preparation of animals*

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.

1.3.2 Test Conditions

1.3.2.1 *Experimental animals*

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.

1.3.2.2 *Reliability check*

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, EINECS No 202-983-3) and mercaptobenzothiazole (CAS No 149-30-4, EINECS No 205-736-8). 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 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 non-standard vehicle (clinically/chemically relevant formulation) will also be necessary. In such situation the possible interaction of a positive control with this unconventional vehicle should be tested.

1.3.2.3 *Number of animals, dose levels and vehicle selection.*

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, as appropriate, a positive control. In those cases in which individual animal data are to be collected, a minimum of five animals per dose group are used. 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.

Dose and vehicle selection should be based on the recommendations given in reference (1). 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).

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.

1.3.3 **Test procedure**

1.3.3.1 *Experimental schedule*

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 + 8 Bq) of ³H-methyl thymidine into all test and control mice via the tail vein. Alternatively inject 250 µL PBS containing 2 µCi (7.4e + 7 Bq) of ¹²⁵I-iododeoxyuridine and 10⁻⁵ M fluorodeoxyuridine into all mice via the tail vein.

Five hours 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 reference 10.

1.3.3.2 *Preparation of cell suspensions*

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 10 ml of scintillation fluid for ³H-counting, or transferred directly to gamma counting tubes for ¹²⁵I-counting.

1.3.3.3 *Determination of cell proliferation (incorporated radioactivity)*

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

1.3.3.4 *Observations*

1.3.3.4.1 *Clinical observations*

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.

1.3.3.4.2 *Body Weights*

As stated in section 1.3.3.1, individual animal body weights should be measured at the start of the test and at the scheduled kill of the animals.

1.3.4 **Calculation of results**

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/animal within each test substance group and the positive control group by the mean DPM/animal for the solvent/vehicle control group. The average SI for vehicle treated controls is then 1.

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 (8)(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.

The decision process with regard to a positive response includes a stimulation index ≥ 3 together with consideration of dose-response and, where appropriate, statistical significance (3)(6)(8)(12)(14).

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 sensitizers, whether it causes excessive skin irritation and the nature of the dose response seen. These and other considerations are discussed in detail elsewhere (7).

2 **DATA**

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

3 **REPORTING**

TEST REPORT

The test report should contain the following information:

Test substance:

- identification data (e.g., CAS number, if available; source; purity; known impurities; lotnumber);
- 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 the total amount of substance applied
- details of food and water quality (including diet type/source, water source).

Reliability check:

- a summary of the results of the 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 the start of dosing and at scheduled kill.
- a table of mean (pooled approach) and individual (individual approach) DPM values as well as the range of values for both approaches and the 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.

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