OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW TEST GUIDELINE

Skin Sensitisation: Local Lymph Node Assay: DA Version

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress, changing regulatory needs, and animal welfare considerations. Toward that end, a modified Local Lymph Node Assay (LLNA) for the determination of skin sensitisation in the mouse, the nonradiolabelled LLNA: DA test method recently underwent validation studies. Based on a formal evaluation and peer review of these studies, the LLNA: DA is useful for identifying skin sensitising and nonsensitising substances, with certain limitations (1)(2)(3)(4). The method is therefore proposed for adoption as an OECD Test Guideline (TG No. to be inserted). This is the third Test Guideline to be promulgated for assessing skin sensitisation potential of chemicals in animals. Test Guideline 429 describes the radiolabelled LLNA and was the first Test Guideline for the determination of skin sensitisation in the mouse (5). The details of the validation of the LLNA and a review of the associated work have been published (6)(7)(8)(9)(10). Test Guideline 406 utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (11).

2. The LLNA: DA was developed as a nonradioactive modification to the LLNA. Similar to the LLNA, the LLNA: DA studies the induction phase of skin sensitisation and provides quantitative data suitable for dose response assessment. Furthermore, an ability to detect skin sensitisers without the necessity for using a radioactive label for DNA eliminates the potential for occupational exposure to radioactivity and waste disposal issues. This in turn may allow for the increased use of mice to detect skin sensitisers, which could further reduce the use of guinea pigs to test for skin sensitisation potential. The LLNA: DA provides certain advantages with regard to animal welfare and a reduced LLNA: DA (rLLNA: DA) for hazard classification of skin sensitising substances can be performed under this Test Guideline (12)(13).

DEFINITIONS

3. Definitions used are provided in Annex 1.

INITIAL CONSIDERATIONS

4. The LLNA: DA is a modified LLNA method for identifying skin sensitising and nonsensitising chemicals, with specific limitations. This does not necessarily imply that in all instances the LLNA: DA should be used in place of the LLNA or guinea pig tests, 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 (1)(2). The LLNA: DA is an *in vivo* method and, as a consequence, will not eliminate the use of animals in the assessment of allergic contact sensitising activity. It has, however, the potential to reduce the number of

animals required for this purpose (e.g., reducing the number of guinea pigs used when the LLNA: DA is used instead of guinea pig assays where the use of radioactivity is prohibited and therefore the LLNA is not used). Moreover, the LLNA: DA offers a substantial refinement of the way in which animals are used for allergic contact sensitisation testing. The LLNA: DA is based upon an evaluation of immunological events stimulated by chemicals during the induction phase of sensitisation. Unlike guinea pig tests (i.e., TG 406) (11), the LLNA: DA does not require that challenge-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA: DA does not require the use of an adjuvant, as is the case for the guinea pig maximisation test, as described in reference (11). Thus, the LLNA: DA reduces animal distress. Despite the advantages of the LLNA: DA over TG 406, there are certain limitations that may necessitate the use of TG 406 (e.g., the testing of certain metals, false positive findings with certain skin irritants) (10), as limitations that have been identified for the LLNA have been recommended to apply also to the LLNA: DA (1).

PRINCIPLE OF THE TEST

5. The basic principle underlying the LLNA: DA is that sensitisers induce proliferation of lymphocytes in the lymph nodes draining the site of chemical application. This proliferation is proportional to the dose and to the potency of the applied allergen and provides a simple means of obtaining a quantitative measurement of sensitisation. The LLNA: DA assesses this proliferation as the proliferation in test groups compared to that in vehicle treated controls. The ratio of the proliferation in treated groups to that in the concurrent vehicle treated control, termed the Stimulation Index (SI), is determined, and should be ≥ 2.5 before a test substance can be considered as a skin sensitiser and should be ≤ 1.7 for the test substance to be considered a nonsensitiser (1). The methods, described here are based on the use of measuring ATP content to indicate an increased number of proliferating cells in the draining auricular lymph nodes. However, other endpoints for assessment of the number of proliferating cells 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. Mice should be group housed (14), unless adequate scientific rationale for housing mice individually is provided. The temperature of the experimental animal room should be 22ºC (± 3ºC). 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.

Preparation of dosing solutions

9. Solid test substances should be dissolved in appropriate solvents/vehicles and diluted, if appropriate, prior to application to an ear of the mice. Liquid test substances may be applied neat or diluted prior to dosing. Insoluble materials, such as those generally seen in medical devices, should be extracted in an appropriate solvent and, if appropriate, further processed prior to application to an ear of the mice. The test substance should be prepared daily unless stability data demonstrate the acceptability of storage.

Reliability check

10. Positive controls are used to demonstrate appropriate performance of the assay by responding with adequate and reproducible sensitivity to a sensitising substance for which the magnitude of the response is well characterised. Inclusion of a concurrent positive control is recommended because it demonstrates competency of the laboratory to successfully conduct each assay and allows for an assessment of intra- and inter-laboratory reproducibility and comparability. The positive control should produce a positive LLNA: DA response at an exposure level expected to give an increase in the SI $\geq$ 2.5 over the negative control group. The positive control dose should be chosen such that the induction is reproducible but not excessive. Preferred positive control substances are hexyl cinnamic aldehyde (Chemical Abstracts Service [CAS] No 101-86-0) and eugenol (CAS No 97-53-0). There may be circumstances in which, given adequate justification, other positive control substances, meeting the above criteria, may be used.

11. While inclusion of a concurrent positive control group is recommended, there may be situations in which periodic testing (i.e., at intervals $\leq$6 months) of the positive control substance may be adequate for laboratories that conduct the LLNA: DA regularly (i.e., conduct the LLNA: DA at a frequency of no less than once per month) and have an established historical positive control database that demonstrates the laboratory's ability to obtain reproducible and accurate results with positive controls. Adequate proficiency with the LLNA: DA can be successfully demonstrated by generating consistent results with the positive control in at least 10 independent tests conducted within a reasonable period of time (i.e., less than one year).

12. A concurrent positive control group should always be included when there is a procedural change to the LLNA: DA (e.g., change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test

animals), and such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of the previously established historical database in determining the necessity for establishing a new historical database to document consistency in the positive control results.

13. Investigators should be aware that the decision to conduct a positive control on a periodic basis instead of concurrently has ramifications on the adequacy and acceptability of negative study results generated without a concurrent positive control during the interval between each periodic positive control study. For example, if a false negative result is obtained in the periodic positive control study, all negative test substance results obtained in the interval between the last acceptable periodic positive control study and the unacceptable periodic positive control study will be questioned. Any study reports associated with these negative test substance results should immediately be amended to report the failed positive control test. In order to demonstrate that the prior negative test substance study results are acceptable, a laboratory would be expected to repeat all negative studies, which would require additional expense and increased animal use. Simply repeating a failed periodic positive control study is not scientifically valid. These implications should be carefully considered when determining whether to include concurrent positive controls or to only conduct periodic positive controls. Consideration should also be given to using fewer animals in the concurrent positive control group when this is scientifically justified and if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used without substantially increasing the frequency with which studies will need to be repeated.

14. 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. If the concurrent positive control substance is tested in a different vehicle than the test substance, then a separate vehicle control for the concurrent positive control should be included.

15. In instances where substances of a specific chemical class or range of responses are being evaluated, benchmark controls may be useful to demonstrate that the test method is functioning properly for detecting the skin sensitisation potential of a test substance. Appropriate benchmark controls should have the following properties:

- structural and functional similarity to the class of the substance being tested;
- known physical/chemical characteristics;
- supporting data on known effects in animal models;
- known potency for sensitisation response.

TEST PROCEDURE

Number of animals and dose levels

16. A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus a concurrent negative control group treated only with the vehicle for the test substance, and a concurrent positive control (see paragraphs 10-14). 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.

17. Dose and vehicle selection should be based on the recommendations given in references (6) and (16). 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 (15)(16). In the absence of such information, an initial prescreen test may be necessary (see paragraphs 20-23).

18. The vehicle should not interfere with or bias the test result and should be selected on the basis of maximising the solubility in order to obtain the highest concentration achievable 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), *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulphoxide (9)(16), 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.

19. The processing of lymph nodes from individual mice allows for the assessment of interanimal variability and a statistical comparison of the difference between test substance and vehicle control group measurements. In addition, evaluating the possibility of reducing the number of mice in the positive control group is only feasible when individual animal data are collected.

Prescreen test

20. The purpose of the prescreen test is to provide guidance for selecting the maximum dose level to use in the main LLNA: DA study. The maximum dose level tested should be a concentration of 100% (i.e., neat substance) for liquid substances or the maximum soluble concentration (for solids), unless available information suggests that this concentration induces systemic toxicity or excessive local irritation after topical application in the mouse.

21. In the absence of such information, a prescreen test should be performed using three dose levels of the test substance, in order to define the appropriate dose level to test in the LLNA: DA. Six mice (two per concentration) are used, and the prescreen test is conducted under identical conditions as the main LLNA: DA study, except there is no assessment of lymph node proliferation. All mice will be observed daily for any clinical signs of systemic toxicity or local irritation at the application site. Body weights are recorded pre-test and prior to termination (Day 8). Both ears of each mouse are observed for erythema and scored using

198 Table 1. Ear thickness measurements are taken using a thickness gauge (e.g., digital
199 micrometer or Peacock Dial thickness gauge) on Day 1 (pre-dose), Day 3 (approximately 48
200 hours after the first dose), Day 7 (24 hours prior to termination) and Day 8. Excessive local
201 irritation is indicated by an erythema score ≥3 and/or ear swelling of ≥25% (17)(18).

202 **Table 1 Erythema Scores**

Observation	Value
No visual effect	0
Slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema (beet redness)	3
Eschar (i.e., piece of dead tissue that is cast off from the surface of the skin)	4

203 22. In addition to a 25% increase in ear swelling (17)(18), a statistically significant
204 increase in ear swelling in the treated mice compared to control mice has also been used to
205 identify irritants in the LLNA (19)(20)(21)(22)(23)(24)(25). While statistically significant
206 increases can occur when ear swelling is less than 25%, they have not been associated
207 specifically with excessive irritation (21)(22)(23)(24)(25). Additionally, an adequately robust
208 statistical comparison would require that a vehicle control group be included and that more
209 than two mice per group be tested. Both of these requirements would substantially increase
210 the number of mice used in a prescreen test. For this reason, a threshold increase in ear
211 swelling above pre-dosing levels is recommended for this prescreen test.

212 23. Test guidelines for assessing acute dermal toxicity recommend a number of clinical
213 observations for assessing systemic toxicity (26)(27). The following clinical observations,
214 which are based on test guidelines and current practices (28), may indicate systemic toxicity
215 when used as part of an integrated assessment and therefore may indicate the maximum dose
216 level to use in the main LLNA: DA:

217 • Changes in nervous system function (e.g., piloerection, ataxia, tremors, and
218 convulsions)

219 • Changes in behavior (e.g., aggressiveness, change in grooming activity,
220 marked change in activity level)

221 • Changes in respiratory patterns (i.e., changes in frequency and intensity of
222 breathing such as dyspnea, gasping, and rales)

223 • Changes in food and water consumption

224 • Lethargy and/or unresponsiveness

225 • Any clinical signs of more than slight or momentary pain and distress

226 • Reduction in body weight >10% from Day 1 to Day 8

- Mortality

Reduced LLNA

24. Use of a rLLNA: DA protocol has the potential to reduce the number of animals used in the LLNA: DA by omitting the middle and low dose groups (12)(13). This is the only difference between the LLNA: DA and the rLLNA: DA and thus, the test substance concentration evaluated in the rLLNA: DA should be the maximum concentration that does not induce overt systemic toxicity and/or excessive local irritation in the mouse. The rLLNA: DA should be used for the hazard classification of skin sensitising substances if dose-response information is not needed, provided there is adherence to all other LLNA: DA protocol specifications, as described in this Test Guideline. To further reduce animal use, the rLLNA: DA should be used routinely as an initial test to determine allergic contact dermatitis potential of chemicals and products before conducting the LLNA: DA. Negative substances can be classified as nonsensitisers and positive substances can be classified as sensitisers.

Main study experimental schedule

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

- *Day 1:*
  Individually identify and record the weight of each animal and any clinical observations. Apply 1% sodium lauryl sulfate (SLS) solution to the dorsum of each ear by using a brush dipped in the SLS solution to cover the entire dorsum of each ear with four to five strokes. One hour after the SLS treatment, apply 25 µL of the appropriate dilution of the test substance, the vehicle alone, or the concurrent positive control (see paragraphs 10-14), to the dorsum of each ear.
- *Days 2, 3 and 7:*
  Repeat the 1% SLS pretreatment and test substance application procedure carried out on Day 1.
- *Days 4, 5, and 6:*
  No treatment.
- *Day 8:*
  Record the weight of each animal and any clinical observations. Approximately 24 to 30 hours after the start of application on Day 7, humanely kill the animals. Excise the draining auricular lymph nodes from each mouse ear and process separately in PBS for each animal. Details and diagrams of the node identification and dissection can be found in reference (29).

Preparation of cell suspensions

26. A single cell suspension of lymph node cells bilaterally from individual animals is prepared by gentle mechanical disaggregation through 200 micron-mesh stainless steel gauze or another acceptable technique for generating a single-cell suspension.

Determination of cellular proliferation (measurement of ATP content of lymphocytes)

27. ATP is measured by the luciferin/luciferase method using an ATP measurement kit which measures bioluminescence in relative luminescence units (RLU). The assay time from time of animal sacrifice to measurement of ATP content for each individual animal should be uniform, within approximately 30 minutes, because the ATP content is considered to gradually decrease with time after animal sacrifice. Thus, the series of procedures from excision of auricular lymph nodes to ATP measurement should be performed without delay.

OBSERVATIONS

Clinical observations

28. Each mouse 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 records being maintained for each mouse. Monitoring plans should include criteria to promptly identify those mice exhibiting systemic toxicity, excessive irritation, or corrosion of skin for euthanasia.

Body weights

29. As stated in paragraph 25, individual animal body weights should be measured at the start of the test and at the scheduled kill.

CALCULATION OF RESULTS

30. Results for each treatment group are expressed as the mean SI. The SI is derived by dividing the mean RLU/mouse within each test substance group and the concurrent positive control group by the mean RLU/mouse for the solvent/vehicle control group. The average SI for vehicle treated controls is then one.

31. Collecting data at the level of the individual mouse will enable a statistical analysis for presence and degree of dose response in the data. Any statistical assessment should include an evaluation of the dose response relationship as well as suitably adjusted comparisons of test groups (e.g., pair-wise dosed group versus concurrent solvent/vehicle control comparisons). Statistical analyses may include, for instance, linear regression or William's test to assess dose-response trends, and Dunnett's test for pairwise comparisons. 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. In any case, the investigator should be alert to possible "outlier" responses for individual mice within a group that may necessitate analysis both with and without outliers.

32. The decision process with regard to a positive response includes a SI $\geq$ 2.5, and the decision process with regard to a negative response includes a SI $\leq$ 1.7 (1). Dose response, chemical toxicity, solubility, and, where appropriate, statistical significance should be considered together with SI values to arrive at a final decision (7)(10)(27)(30).

33. If an SI value that falls into the range 2.5 > SI > 1.7 is obtained, an integrated assessment of the SI value should be considered in conjunction with all other available and

relevant information (e.g., dose response information, statistical analyses of treated vs. control animals, peptide-binding activity, molecular weight, results from related chemicals, other testing data), to determine if there is sufficient information on which to base an accurate determination of sensitisation potential, or if additional testing is necessary (1). Consideration should also 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 in the mouse, and the nature of the dose response seen. These and other considerations are discussed in detail elsewhere (8).

DATA AND REPORTING

Data

34. Data should be summarised in tabular form showing the individual animal RLU values, the group mean RLU/animal, its associated error term, and the mean SI for each dose group compared against the concurrent solvent/vehicle control group.

Test report

35. The test report should contain the following information:

Test substance and control substances:

- 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.

Solvent/vehicle:

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

Test animals:

- source of CBA mice;
- microbiological status of the animals, when known;
- number and age 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);
- details of treatment and sampling schedules;
- methods for measurement of toxicity;
- criteria for considering studies as positive or negative;
- details of any protocol deviations and an explanation on how the deviation affects the study design and results.

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 (solvent/vehicle) control data for testing laboratory;
- If a concurrent positive control was not included, the date and laboratory report for the most recent periodic positive control and a report detailing the historical positive control data for the laboratory justifying the basis for not conducting a concurrent positive control.

Results:

- individual weights of mice at start of dosing and at scheduled kill; as well as mean and associated error term for each treatment group.
- time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal;
- a table of individual mouse RLU values and SIs for each dose treatment group;
- mean and associated error term for RLU/mouse for each treatment group and the results of outlier analysis for each treatment group;
- calculated SI and an appropriate measure of variability that takes into account the interanimal variability in both the test substance and control groups;
- dose response relationship;
- statistical analysis, where appropriate.

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.

Quality assurance statement for Good Laboratory Practice compliant studies:

- statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. The statement should also confirm that the final report reflects the raw data.

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ANNEX 1

False negative: A substance incorrectly identified as negative or non-active by a test method, when in fact it is positive or active.

False positive: A substance incorrectly identified as positive or active by a test, when in fact it is negative or non-active.

Hazard: The potential for an adverse health or ecological effect. The adverse effect is manifested only if there is an exposure of sufficient level.

Inter-laboratory reproducibility: A measure of the extent to which different qualified laboratories, using the same protocol and testing the same substances, can produce qualitatively and quantitatively similar results. Inter-laboratory reproducibility is determined during the prevalidation and validation processes, and indicates the extent to which a test can be successfully transferred between laboratories, also referred to as between-laboratory reproducibility.

Intra-laboratory reproducibility: A determination of the extent that qualified people within the same laboratory can successfully replicate results using a specific protocol at different times. Also referred to as within-laboratory reproducibility.

Quality assurance: A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures, and the accuracy of data transfer, are assessed by individuals who are independent from those performing the testing.

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility.

Skin sensitisation: An immunological process that results when a susceptible individual is exposed topically to an inducing chemical allergen, which provokes a cutaneous immune response that can lead to the development of contact sensitisation.

Stimulation Index (SI): A value calculated to assess the skin sensitisation potential of a test substance that is the ratio of the proliferation in treated groups to that in the concurrent vehicle control group.