OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA or -FCM

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in light of scientific progress, changing regulatory needs, and animal welfare considerations. The first Test Guideline (TG) for the determination of skin sensitization in the mouse, the Local Lymph Node Assay (LLNA; TG 429) was adopted in 2002, and has since then been revised (1). The details of the validation of the LLNA and a review of the associated work have been published (2) (3) (4) (5) (6) (7) (8) (9) (37) (38). In the LLNA, radioisotopic thymidine or iodine is used to measure lymphocyte proliferation and therefore the assay has limited use in regions where the acquisition, use, or disposal of radioactivity is problematic. The LLNA: BrdU-ELISA [Enzyme-Linked Immunosorbent Assay] and the LLNA: BrdU-FCM [Flow Cytometry Method] are non-radioactive modifications to the LLNA test method, which utilise non-radiolabelled 5-bromo-2-deoxyuridine (BrdU) (Chemical Abstracts Service [CAS] No 59-14-3) in an ELISA- or FCM-based test system to measure lymphocyte proliferation. The LLNA: BrdU-ELISA and the LLNA: BrdU-FCM have been validated and recommended by an international independent scientific peer review panel as considered useful for identifying skin sensitizing and non-sensitizing test substances, with certain limitations (10) (11) (12) (37) (38). The validation study for the LLNA: BrdU-FCM was performed in compliance with the performance standards for assessment of proposed similar or modified LLNA test methods for skin sensitization in Annex 1 of the OECD Guideline for the testing of chemicals, Skin sensitization: Local lymph node assay (TG 429). This Test Guideline is designed for assessing skin sensitization potential of chemicals in animals. TG 406 utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (13). The LLNA (TG 429) and the non-radioactive modifications, LLNA: BrdU-ELISA and FCM (TG 442 B) and LLNA: DA (TG 442 A), all provide an advantage over the guinea pig tests in TG 406 (13) in terms of reduction and refinement of animal use.

2. Similar to the LLNA, the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM studies the induction phase of skin sensitization and provides quantitative data suitable for dose-response assessment. Furthermore, an ability to detect skin sensitizers without the necessity for using a radiolabel 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 sensitizers, which could further reduce the use of guinea pigs to test for skin sensitization potential (i.e. TG 406) (13).

DEFINITIONS

3. Definitions used are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. The LLNA: BrdU-ELISA and the LLNA: BrdU-FCM are modified LLNA methods for identifying potential skin sensitizing test substances, with specific limitations. This does not necessarily imply that in all instances the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM should be used in place of the LLNA or guinea pig tests (i.e. TG 406) (13), but rather that the assays are suitable of
equal merit and may be employed as an alternative in which positive and negative results generally no longer require further confirmation (10) (11). The testing laboratory should consider all available information on the test substance prior to conducting the study. Such information will include the identity and chemical structure of the test substance; its physicochemical properties; the results of any other in vitro or in vivo toxicity tests on the test substance; and toxicological data on structurally related test substances. This information should be considered in order to determine whether the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM is are appropriate for the test substance (given the incompatibility of limited types of test substances with the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM [see paragraph 5]) and to aid in dose selection.

5. The LLNA: BrdU-ELISA and the LLNA: BrdU-FCM is are an in vivo methods and, as a consequence, will not eliminate the use of animals in the assessment of allergic contact sensitizing activity. They have, however, the potential to reduce the animal use for this purpose when compared to the guinea pig tests (TG 406) (13). Moreover, the LLNA: BrdU-ELISA ELISA and the LLNA: BrdU-FCM offers a substantial refinement of the way in which animals are used for allergic contact sensitization testing, since unlike the TG 406, the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM does not require that challenge-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM does not require the use of an adjuvant, as is the case for the guinea pig maximisation test (13). Thus, the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM reduces animal distress. Despite the advantages of the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM over TG 406 (13), 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 [such as some surfactant-type substances] (6) (1), solubility of the test substance). In addition, test substance classes or substances containing functional groups shown to act as potential confounders (15) may necessitate the use of guinea pig tests (i.e. TG 406 (13)). Limitations that have been identified for the LLNA (1) have been recommended to apply also to the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM (10). Other than such identified limitations, the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM should be applicable for testing any test substances unless there are properties associated with these substances that may interfere with the accuracy of the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM. In addition, consideration should be given to the possibility of borderline positive results when Stimulation Index (SI) values between 1.6 and 1.9 are obtained (see paragraphs 31-32) in the LLNA: BrdU-ELISA. This is based on the validation database of 43 substances using an SI ≥ 1.6 (see paragraph 6) for which the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers, but incorrectly identified two of 11 LLNA non-sensitizers with SI values between 1.6 and 1.9 (i.e. borderline positive) (10). However, as the same dataset was used for setting the SI-values and calculating the predictive properties of the test, the stated results may be an over-estimation of the real predictive properties. For FCM, consideration should be given to the possibility of false negative results. According to the validation study on the LLNA: BrdU-FCM using 18 essential reference chemicals listed in the TG 429 PS, the LLNA: BrdU-FCM has a limitation since one moderate skin sensitizer, 2-mercaptobenzothiazole, and one borderline skin sensitizer, methyl methacrylate, were misclassified in the LLNA: BrdU-FCM (36) (37).

PRINCIPLE OF THE TEST

6. The basic principle underlying the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM is that sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of test substance 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 sensitization. Proliferation is
measured by comparing the mean proliferation in each test group to the mean proliferation in the vehicle treated control group (VC). The ratio of the mean proliferation in each treated group to that in the concurrent VC group, termed the SI, is determined, and should be ≥1.6 (LLNA: BrdU-ELISA) or ≥2.7 (LLNA: BrdU-FCM) before further evaluation of the test substance as a potential skin sensitiser is warranted. The methods described here are based on the use of measuring BrdU content to indicate an increased number of proliferating cells in the draining auricular lymph nodes. BrdU is an analogue of thymidine and is similarly incorporated into the DNA of proliferating cells. The incorporation of BrdU is measured by ELISA or FCM, which utilises an antibody specific for BrdU that is also labelled with peroxidase or fluorescein isothiocyanate (FITC). As for ELISA, when the substrate is added, the peroxidase reacts with the substrate to produce a coloured product that is quantified at a specific absorbance using a microtiter plate reader. The FCM quantifies the number of BrdU-incorporating viable cells using a flow cytometer.

DESCRIPTION OF THE ASSAY

Selection of animal species

7. The mouse is the species of choice for this test. Validation studies for the LLNA: BrdU-ELISA were conducted exclusively with the CBA/J strain, which is therefore considered the preferred strain (10) (12). In the LLNA: BrdU-FCM, both BALB/c and CBA/J mice are considered the preferred strain based on the results of the validation study and other references (36) (37). Young adult female mice, 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. Alternatively, 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: BrdU-ELISA or the LLNA: BrdU-FCM response do not exist.

Housing and feeding conditions

8. Mice should be group-housed (16), unless adequate scientific rationale for housing mice individually is provided. The temperature of the experimental animal room should be 22 ± 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

9. 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 five 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

10. Solid test substances should be dissolved or suspended in 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 substances, such as those generally seen in medical devices, should be subjected to an exaggerated extraction in an appropriate solvent to reveal all extractable constituents for testing prior to application to an ear of the mice. Test substances should be prepared daily unless stability data demonstrate the acceptability of storage.

Reliability check

11. Positive controls (PC) are used to demonstrate appropriate performance of the assay by responding with adequate and reproducible sensitivity to a sensitizing test substance for which the magnitude of the response is well characterised. Inclusion of a concurrent PC 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. Some regulatory authorities also require a PC for each study and therefore users are encouraged to consult the relevant authorities prior to conducting the LLNA: BrdU-ELISA or the LLNA: BrdU-FCM. Accordingly, the routine use of a concurrent PC is encouraged to avoid the need for additional animal testing to meet such requirements that might arise from the use of a periodic PC (see paragraph 12). The PC should produce a positive LLNA: BrdU-ELISA or the LLNA: BrdU-FCM response at an exposure level expected to give an increase in the SI ≥ 1.6 (LLNA: BrdU-ELISA) or SI ≥ 2.7 (LLNA: BrdU-FCM) over the negative control (NC) group. The PC dose should be chosen such that it does not cause excessive skin irritation or systemic toxicity and the induction is reproducible but not excessive (e.g. SI > 14 or > 27 would be considered excessive for the LLNA: BrdU-ELISA, or the LLNA: BrdU-FCM, respectively). Preferred PC test substances are 25% hexyl cinnamic aldehyde (CAS No 101-86-0) and 25% eugenol (CAS No 97-53-0) in acetone: olive oil (4:1, v/v). There may be circumstances in which, given adequate justification, other PC test substances, meeting the above criteria, may be used.

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

13. A concurrent PC group should always be included when there is a procedural change to the LLNA: BrdU-ELISA or the LLNA: BrdU-FCM (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 PC results.

14. Investigators should be aware that the decision to conduct a PC study on a periodic basis instead of concurrently has ramifications on the adequacy and acceptability of negative study results generated without a concurrent PC during the interval between each periodic PC study. For example, if a false negative result is obtained in the periodic PC study, negative test substance results obtained in the interval between the last acceptable periodic PC study and the unacceptable periodic PC study may be questioned. Implications of these outcomes should be carefully considered when determining
whether to include concurrent PCs or to only conduct periodic PCs. Consideration should also be given to using fewer animals in the concurrent PC group when this is scientifically justified and if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used (17).

15. Although the PC test substance should be tested in the vehicle that is known to elicit a consistent response (e.g. acetone: olive oil; 4:1, v/v), there may be certain regulatory situations in which testing in a non-standard vehicle (clinically/chemically relevant formulation) will also be necessary (18). If the concurrent PC test substance is tested in a different vehicle than the test substance, then a separate VC for the concurrent PC should be included.

16. In instances where test substances of a specific chemical class or range of responses are being evaluated, benchmark test substances may also be useful to demonstrate that the test methods are functioning properly for detecting the skin sensitization potential of these types of test substances. Appropriate benchmark test substances should have the following properties:

- structural and functional similarity to the class of the test substance being tested;
- known physical/chemical characteristics;
- supporting data from the LLNA: BrdU-ELISA or the LLNA: BrdU-FCM;
- supporting data from other animal models and/or from humans.

TEST PROCEDURE

Number of animals and dose levels

17. A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus a concurrent NC group treated only with the vehicle for the test substance, and a PC group (concurrent or recent, based on laboratory policy in considering paragraphs 11-15). Testing multiple doses of the PC should be considered especially when testing the PC on an intermittent basis. 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.

18. Dose and vehicle selection should be based on the recommendations given in the references 2 and 19. Consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. Adequate scientific rationale should accompany the selection of the concentration series used. All existing toxicological information (e.g. acute toxicity and dermal irritation) and structural and physicochemical information on the test substance of interest (and/or structurally related test substances) should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximises exposure while avoiding systemic toxicity and/or excessive local skin irritation (19)(20). In the absence of such information, an initial pre-screen test may be necessary (see paragraphs 21-24).

19. Alternatively, the pre-screen test could be conducted in two phases initiating with and start at 25%, thus minimizing pain and distress on laboratory animals by avoiding their exposure to extremely high concentrations of a skin irritant (35) (37). If no toxicity or irritation is found at 25%, only 50% and 100% are needed in the second phase, not requiring additional test with lower concentrations (less than 50%). If toxicity or irritation, however, is found at 25%, concentrations will be lowered, resulting in the selection of consecutive concentration sequence lower than 25% (10%, 5%, 2.5%, 1%, 0.5%, etc).
4920. 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 while producing a solution/suspension suitable for application of the test substance. Recommended vehicles are acetone: olive oil (4:1 v/v), N,N-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulphoxide (6) 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 substances are incorporated into a vehicle system, which wets the skin and does not immediately run off, by incorporation of appropriate solubilisers (e.g. 1% Pluronic® L92). Thus, wholly aqueous vehicles are to be avoided.

2021. The processing of lymph nodes from individual mice allows for the assessment of inter-animal variability and a statistical comparison of the difference between test substance and VC group measurements (see paragraph 33). In addition, evaluating the possibility of reducing the number of mice in the PC group is only feasible when individual animal data are collected (17). Further, some national regulatory authorities require the collection of individual animal data. Regular collection of individual animal data provides an animal welfare advantage by avoiding duplicate testing that would be necessary if the test substance results originally collected in one manner (e.g. via pooled animal data) were to be considered later by regulatory authorities with other requirements (e.g. individual animal data).

Pre-screen test

2122. In the absence of information to determine the highest dose to be tested (see paragraph 18), a pre-screen test should be performed in order to define the appropriate dose level to test in the LLNA: BrdU-ELISA or the LLNA: BrdU-FCM. The purpose of the pre-screen test is to provide guidance for selecting the maximum dose level to use in the main LLNA: BrdU-ELISA ELISA or the LLNA: BrdU-FCM study, where information on the concentration that induces systemic toxicity (see paragraph 24) and/or excessive local skin irritation (see paragraph 23) is not available. The maximum dose level tested should be a concentration of 100% of the test substance for liquids or the maximum possible concentration for solids or suspensions. Alternatively, the pre-screen test could be conducted in two phases initiating with 25% (see paragraph 19).

2223. The pre-screen test is conducted under conditions identical to the main LLNA: BrdU-ELISA or LLNA: BrdU-FCM study, except there is no assessment of lymph node proliferation and fewer animals per dose group can be used. One or two animals per dose group are suggested. 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 6). Both ears of each mouse are observed for erythema and scored using Table 1 (20). Ear thickness measurements are taken using a thickness gauge (e.g. digital micrometer or Peacock Dial thickness gauge) on Day 1 (pre-dose), Day 3 (approximately 48 hours after the first dose), and Day 6. Additionally, on Day 6, ear thickness could be determined by ear punch weight determinations, which should be performed after the animals are humanely killed. Excessive local irritation is indicated by an erythema score ≥3 and/or ear thickness of ≥25% on any day of measurement (21) (22). The highest dose selected for the main LLNA: BrdU-ELISA or LLNA: BrdU-FCM study will be the next lower dose in the pre-screen concentration series (see paragraph 18) that does not induce systemic toxicity and/or excessive local skin irritation.
### Table 1. Erythema Scores

<table>
<thead>
<tr>
<th>Observation</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No erythema</td>
<td>0</td>
</tr>
<tr>
<td>Very slight erythema (barely perceptible)</td>
<td>1</td>
</tr>
<tr>
<td>Well-defined erythema</td>
<td>2</td>
</tr>
<tr>
<td>Moderate to severe erythema</td>
<td>3</td>
</tr>
<tr>
<td>Severe erythema (beet redness) to eschar formation preventing grading of erythema</td>
<td>4</td>
</tr>
</tbody>
</table>

In addition to a 25% increase in ear thickness (21) (22), a statistically significant increase in ear thickness in the treated mice compared to control mice has also been used to identify irritants in the LLNA (22) (23) (24) (25) (26) (27) (28). However, while statistically significant increases can occur when ear thickness is less than 25%, they have not been associated specifically with excessive irritation (25) (26) (27) (28) (29).

The following clinical observations may indicate systemic toxicity (30) when used as part of an integrated assessment and therefore may indicate the maximum dose level to use in the main LLNA: BrdU-ELISA or LLNA: BrdU-FCM: changes in nervous system function (e.g. pilo-erection, ataxia, tremors, and convulsions); changes in behaviour (e.g. aggressiveness, change in grooming activity, marked change in activity level); changes in respiratory patterns (i.e. changes in frequency and intensity of breathing such as dyspnea, gasping, and rales), and changes in food and water consumption. In addition, signs of lethargy and/or unresponsiveness and any clinical signs of more than slight or momentary pain and distress, or a >5% reduction in body weight from Day 1 to Day 6 and mortality should be considered in the evaluation. Moribund animals or animals showing signs of severe pain and distress should be humanely killed (31).

#### Main study experimental schedule

The experimental schedule of the assays is as follows:

- **Day 1:**
  - Individually identify and record the weight of each animal and any clinical observation. Apply 25 μL of the appropriate dilution of the test substance, the vehicle alone, or the PC (concurrent or recent, based on laboratory policy in considering paragraphs 11-15), to the dorsum of each ear.

- **Days 2 and 3:**
  - Repeat the application procedure carried out on Day 1.

- **Day 4:**
  - No treatment.

- **Days 5:**
Inject intra-peritoneally 0.5 mL (5 mg/mouse) of BrdU (10 mg/mL) solution in the LLNA: BrdU-ELISA, or 0.1 mL (2 mg/mouse) of BrdU (20 mg/mL) solution in the LLNA: BrdU-FCM.

- **Day 6:**

  Record the weight of each animal and any clinical observation. Approximately 24 hours (24 h) after BrdU injection, humanely kill the animals. Excise the draining auricular lymph nodes from each mouse ear and process separately in phosphate buffered saline (PBS) for each animal. Details and diagrams of the lymph node identification and dissection can be found in reference (17). To further monitor the local skin response in the main study, additional parameters such as scoring of ear erythema or ear thickness measurements (obtained either by using a thickness gauge, or ear punch weight determinations at necropsy) may be included into the study protocol.

**Preparation of cell suspensions**

2627. From each mouse, a single-cell suspension of lymph node cells (LNC) excised bilaterally is prepared by gentle mechanical disaggregation through 200 micron-mesh stainless steel gauze or another acceptable technique for generating a single-cell suspension (e.g. use of a disposable plastic pestle to crush the lymph nodes followed by passage through a #70 nylon mesh). The procedure for preparing the LNC suspension is critical in these assays and therefore every operator should establish the skill in advance. Further, the lymph nodes in NC animals are small, so careful operation is important to avoid any artificial effects on SI values. In each case, the target volume of the LNC suspension should be adjusted to a determined optimised volume (approximately 15 mL) for the LLNA: BrdU-ELISA (The optimised volume is based on achieving a mean absorbance of the NC group within 0.1-0.2). For the LLNA: BrdU-FCM, total lymph node cells should be calculated and then $1.5 \times 10^6$ lymph node cells are needed in the next step.

**Determination of cellular proliferation (measurement of BrdU content in DNA of lymphocytes or BrdU-incorporating lymphocytes)**

2728. BrdU is measured by ELISA using a commercial kit (e.g. Roche Applied Science, Mannheim, Germany, Catalogue Number 11 647 229 001). Briefly, 100 μL of the LNC suspension is added to the wells of a flat-bottom microplate in triplicate. After fixation and denaturation of the LNC, anti-BrdU antibody is added to each well and allowed to react. Subsequently, the anti-BrdU antibody is removed by washing and the substrate solution is then added and allowed to produce chromogen. Absorbance at 370 nm with a reference wavelength of 492 nm is then measured. In all cases, assay test conditions should be optimised (see paragraph 26).

29. BrdU is measured by the FCM using a commercially available kit (e.g. BD Pharmingen, Franklin Lakes, NJ, USA, Catalogue Number 559619). Briefly, the LNC suspension ($1.5 \times 10^6$) is washed once with PBS by centrifugation and then re-suspended. Cells are permeabilised with the buffer supplied with the kit and then treated with DNase. After washing, FITC-conjugated anti-BrdU antibodies are added followed by adding 7-aminoactinomycin D (7-AAD) solution after one more wash. The number of BrdU-incorporated cells within the viable 7-AAD-expressing cell population ($10^4$ cells) is counted with a flow cytometer.
**OBSERVATIONS**

**Clinical observations**

2830. Each mouse should be carefully observed at least 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 local skin irritation, or corrosion of skin for euthanasia (31).

**Body weights**

2931. As stated in paragraph 2526, individual animal body weights should be measured at the start of the test and at the scheduled humane kill.

**CALCULATION OF RESULTS**

3032. Results for each treatment group are expressed as the mean SI. The SI for the LLNA: BrdU-ELISA is derived by dividing the mean BrdU labelling index/mouse within each test substance group and the PC group by the mean BrdU labelling index for the solvent/VC group. The average SI for the VCs is then one.

The BrdU labelling index is defined as:

\[
\text{BrdU labelling index} = (\text{ABS}_{em} - \text{ABS}_{blankem}) - (\text{ABS}_{ref} - \text{ABS}_{blankref})
\]

Where; em = emission wavelength; and ref = reference wavelength.

33. The SI for the LLNA: BrdU-FCM is derived by dividing the total number of BrdU-incorporating LNCs from each individual mouse applied with each test substance group and the PC group by the mean number of BrdU-incorporating LNCs in the solvent/VC group. The average SI for the VCs is then one.

The total number of BrdU-incorporating LNCs is defined as (See Annex 2 paragraph 7):

\[
\text{Total number of BrdU-incorporating LNCs} = \frac{\text{percentage of BrdU incorporation (}\%\text{ of Q2)} \times \text{the total number of LNCs (cells/mL)}}{}
\]

3434. The decision process regards a result as positive when SI ≥ 1.6 in the LLNA: BrdU-ELISA (10) or SI ≥ 2.7 in the LLNA: BrdU-FCM (36) (37). However, the strength of the dose-response relationship, the statistical significance and the consistency of the solvent/vehicle and PC responses may also be used when determining whether a borderline result (i.e. SI value between 1.6 and 1.9 in the LLNA: BrdU-ELISA) is declared positive (3) (6) (32).

3235. For a borderline positive response between an SI of 1.6 and 1.9, users may want to consider additional information such as dose-response relationship, evidence of systemic toxicity or excessive irritation, and where appropriate, statistical significance together with SI values to confirm that such
results are positives (10). Consideration should also 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 in the mouse, and the nature of the dose-response observed. These and other considerations are discussed in detail elsewhere (4).

3336. Collecting data at the level of the individual mouse will enable a statistical analysis for presence and degree of dose-response relationship in the data. Any statistical assessment could 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, e.g. linear regression or Williams’s test to assess dose-response trends, and Dunnett’s test for pair-wise 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 may need to carry out SI calculations and statistical analyses with and without certain data points (sometimes called “outliers”).

DATA AND REPORTING

Data

3437. Data should be summarised in tabular form showing the individual animal BrdU labelling index values, the group mean BrdU labelling index/animal for the LLNA: BrdU-ELISA, or the individual animal BrdU-incorporating LNCs, the group mean BrdU-incorporating LNCs/animal for the LLNA: BrdU-FCM, or, its associated error term (e.g. SD, SEM), and the mean SI for each dose group compared against the concurrent solvent/vehicle control group.

Test report

3538. The test report should contain the following information:

Test substance and control 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 formulation, 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 or BALB/c mice;
- microbiological status of the animals, when known;
- number and age of animals;
- source of animals, housing conditions, diet, etc.;
Test conditions:

- source, lot number, and manufacturer’s quality assurance/quality control data (antibody sensitivity and specificity and the limit of detection) for the ELISA or the FCM kit;
- details of test substance preparation and application;
- justification for dose selection (including results from pre-screen test, if conducted);
- vehicle and test substance concentrations used, and total amount of test 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 test substance, concentration and vehicle used;
- concurrent and/or historical PC and concurrent negative (solvent/vehicle) control data for testing laboratory;
- if a concurrent PC was not included, the date and laboratory report for the most recent periodic PC and a report detailing the historical PC data for the laboratory justifying the basis for not conducting a concurrent PC;

Results:

- individual weights of mice at start of dosing and at scheduled humane kill; as well as mean and associated error term (e.g. SD, SEM) 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 BrdU labelling indices for the LLNA: BrdU-ELISA or BrdU-incorporating LNCs for the LLNA: BrdU-FCM, and SI values for each treatment group;
- mean and associated error term (e.g. SD, SEM) for BrdU labelling index or BrdU-incorporating LNCs /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 inter-animal variability in both the test substance and control groups;
- dose-response relationship;
- statistical analyses, 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 sensitizer.

**LITERATURE**


OECD/OCDE


(36) Submitted manuscript: Lee YS, Yi JS, Kim JH, Jung MS, Seo IK, Ahn IY, Ko KY, Kim TS, Lim KM, Sohn SJ. Comparison of BALB/c and CBA/J mice for the local lymph node assay using flow cytometry.


ANNEX I

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with “concordance” to mean the proportion of correct outcomes of a test method (33).

Benchmark test substance: A sensitizing or non-sensitizing substance used as a standard for comparison to a test substance. A benchmark substance should have the following properties: (i) a consistent and reliable source(s); (ii) structural and functional similarity to the class of substances being tested; (iii) known physical/chemical characteristics; (iv) supporting data on known effects; and (v) known potency in the range of the desired response.

False negative: A test substance incorrectly identified as negative or non-active by a test method, when in fact it is positive or active (33).

False positive: A test substance incorrectly identified as positive or active by a test, when in fact it is negative or non-active (33).

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 test substance, can produce qualitatively and quantitatively similar results. Inter-laboratory reproducibility is determined during the pre-validation and validation processes, and indicates the extent to which a test can be successfully transferred between laboratories, also referred to as between-laboratory reproducibility (33).

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 (33).

Outlier: An outlier is an observation that is markedly different from other values in a random sample from a population.

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 (33).

Skin sensitization: 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 sensitization.

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

**Test substance:** Any material tested using this TG, whether it is a single compound or consists of multiple components (*e.g.* final products, formulations). When testing formulations, consideration should be given to the fact that certain regulatory authorities only require testing of the final product formulation. However, there may also be testing requirements for the active ingredient(s) of a product formulation.
ANNEX 2

SUMMARY OF THE LLNA: BrdU-FCM PROTOCOL

I. PROTOCOL

1. The LLNA: BrdU-FCM is a me-too test, and it complied with the essential test method components described in paragraph 5 of OECD TG 429 Annex 1 (34) (35) (37). The final version of the protocol can be found in (DB-ALM of EURL-ECVAM) or in the validation study report for the LLNA: BrdU-FCM (37).

Observation of general symptoms and erythema

2. General symptoms are observed and recorded on a daily basis. Erythema on ears is scored each day prior to the application of test substances in accordance with the Draize test method. The mice are weighed on day 1 and 6, and the average thickness of both ears is calculated on day 1 (before treatment), 3 (before treatment) and 6 (before autopsy).

Application of test substances

3. Doses of 100%, 50%, 25%, 10%, 5%, 2.5%, 1% and 0.5% are chosen according to OECD TG 429. In the pre-screen test, the solubility of each chemical is evaluated, and the maximum concentrations without serious irritation or systemic toxicity are determined. The test substance or negative control substance in 25 µL vehicle is applied to the dorsum of each ear. The test substances are applied carefully in a circle using the side of a micropipette tip.

Injection of BrdU solution

4. A single intraperitoneal injection of 100 µL BrdU solution (20 mg/mL) is given to each mouse at 24 ± 2 hours before sacrifice.

Collection of lymph nodes

5. The method of euthanasia, including CO₂ gas-asphyxiation, which minimizes pain and distress on animals, is chosen. After sacrifice, the weights of both auricular lymph nodes are measured.

BrdU staining

6. The FITC BrdU Flow Kit (e.g. Cat. No. 559619, BD Pharmingen™) is used for BrdU staining. The fluorescence staining kit is commercially available.

Measurement of BrdU with flow cytometry

7. For the measurement of BrdU incorporated LNCs with a flow cytometer (e.g. BD FACSCalibur™, Beckman Coulter Cytomics FC 500), blank, non-treated, negative control substance-treated and test substance-treated samples are prepared before the first measurement. To analyze test results, the FSC-SSC dot plot and the 7-AAD-BrdU dot plot are set up. An upper-right quadrant (Q2) was set up to exhibit no LNCs from a mouse in which neither BrdU nor test substance was applied.
Then, the Q2 quadrant was adjusted to display 1% events, using LNCs from the mouse in which BrdU was injected in the absence of test substance application. After completion of these gating procedures (see Figure 3), LNCs from the vehicle control or the test substance application group were analyzed to obtain the percentage of BrdU-incorporating cells within the viable 7-AAD-expressing cell population. Then the total number of BrdU-incorporating LNCs was calculated by multiplication of the percentage with total number of LNCs.

II. SELECTION OF THE VEHICLE AND HIGHEST CONCENTRATIONS

8. A vehicle with highest solubility is selected for the test substance (see Figure 1).

9. The three concentrations that do not induce systemic toxicity or serious irritation are chosen in the 1\textsuperscript{st} and 2\textsuperscript{nd} toxicity/irritation tests (see Figure 2). If a test substance is applied firstly at the concentration of 100\%, extreme toxicity could be induced. For this reason, the 1\textsuperscript{st} pre-screen test is performed at 25\%, and in the 2\textsuperscript{nd} test, the highest concentration is determined based on the results of 1\textsuperscript{st} pre-screen. If no toxicity or irritation is found at 25\%, only 50\% and 100\% concentrations are needed in the 2\textsuperscript{nd} test, not requiring to test lower concentrations (less than 25\%). If toxicity or irritation is found at 25\%, concentrations will be lowered since severe

**Figure 1. Procedure for determining a vehicle**

AOO, acetone:olive oil (4:1); DMF, dimethylformamide; MEK, methyl ethyl ketone; DMSO, dimethylsulfoxide
toxicity will be induced at 50% or 100%, thereby relieving more pain and distress on laboratory animals and reducing animal testing.

III. EVALUATION OF SKIN SENSITIZATION POTENCY

10. Murine auricular lymph nodes are taken from the four different groups as below to measure the proportion of BrdU-incorporated lymphocytes using flow cytometry.
   - Blank group (n=1): Neither injection of BrdU nor treatment of test substances
   - Non-treatment group (n=1): Injection of BrdU but no treatment of test substances
   - Negative control treatment group (n≥4): Injection of BrdU and treatment of a vehicle
   - Test substance treatment group (n≥4): Injection of BrdU and treatment of test substances

11. To calculate the stimulation index, flow cytometry is set up as below (see Figure 3):
   - A: Set up the Q2 area using the blank samples so that no cell can be found in it.
   - B: Set up the Q2 area again using non-treatment samples such the BrdU-incorporated LNCs is about 1% in it.
   - C, D: Analyze the negative control-treated samples and the test substance-treated samples, and calculate the gated % in the Q2 area that is set up in A and B.

12. The SI is calculated as below:

   i) A gated % in the Q2 area, which is set up for each mouse in the negative control group, is calculated. The gated % is then multiplied by the number of LNCs (cells/mL) to work out a proliferated LNCs for each mouse. The mean of LNC proliferation rates of all mice in the negative control group is calculated.

Figure 2. Procedure for selecting the highest concentration to be tested
ii) A gated % in the Q2 area, which is set up for each mouse in the test substance treatment group, is calculated. The gated % is then multiplied by the number of LNCs (cells/mL) to work out a proliferated LNCs.

iii) The SI for each mouse in the test substance treatment group is calculated by dividing i) by ii).

**Figure 3. Flow cytometry configuration for the calculation of % of BrdU incorporation (% of Q2)**