Office of Pesticide Programs:

Expansion of the Traditional Local Lymph Node Assay for the Assessment of Dermal Sensitization Potential of End Use Pesticide Products; and

Adoption of a "Reduced" Protocol for the Traditional LLNA (Limit Dose)

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Office of Pesticide Programs: Expansion of the Traditional Local Lymph Node Assay for the Assessment of Dermal Sensitization Potential of End Use Pesticide Products; and Adoption of a "Reduced" Protocol for the Traditional LLNA (Limit Dose)

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Executive Summary

The Office of Pesticide Programs (OPP) is expanding the existing OPPTS 870.2600 dermal sensitization guideline from assessing only the technical grade active ingredients to include end use pesticide products using the radiolabeled (or traditional) Local Lymph Node Assay (LLNA). None of the non-radiolabeled LLNA test methods are included in this expansion. Separately, OPP is adopting the "reduced or limit dose" LLNA (rLLNA) radiolabeled assay, which will further reduce the number of animals used in laboratory tests by 40% when compared to the multi-dose LLNA.

Objective

The objective of this policy is to expand the usefulness the traditional (or radiolabeled) LLNA protocol for assessment of dermal sensitization potential to single component pesticide products (i.e., technical grade active ingredients) and to pesticides with multiple components (i.e., end use products). In most cases, the traditional LLNA continues to be the preferred assay because it incorporates replacement, refining, and reducing animal testing (also referred to as "the 3 R's" or the three main goals animal testing) while also providing a more quantitative assessment of dermal sensitization in a way that is less subjective than similar guinea pig assays.

Background

At the ICCVAM independent scientific public peer review panel meeting held on April 28-29, 2009 at the National Institutes of Health (NIH), Bethesda, MD, the applicability domain of the radiolabeled LLNA method was examined. The peer panel performed a retrospective review of data derived from over 600 substances (single component compounds) which included 104 end use pesticide products tested in the radiolabeled LLNA. The first ICCVAM evaluation in 1999 had 209 substances (single component) evaluated in the mouse LLNA. This radiolabeled LLNA guideline was formally adopted by OECD in 2001. OPP presented the LLNA to the Science Advisory Panel (SAP) in 2002. The revised dermal sensitization OPPTS guideline 870.2600 was published in 2003. The OPPTS guideline lists the LLNA which uses the mouse as the preferred assay over the 2 other traditional guinea pigs

assays used for dermal sensitization. The other acceptable assays are the Guinea-Pig Maximization Test (GPMT) or Buehler Test (BT).

The 2009 ICCVAM peer review panel re-evaluated new information:

> The second review of the ICCVAM peer panel contained an additional database with 22 end use pesticide products which contained both guinea pig and mouse LLNA data. The 22 end use products represented 9 active ingredients (i.e., one insecticide, six herbicides and two fungicides). There was concordance of the radiolabeled LLNA mouse and guinea pig data test results for 54% of the end use products (12/22), of which 3/22 were positive and 9/22 were negative in both sets of results. The LLNA radiolabeled test method gave positive results for the 10 remaining end use products, all of which were negative by guinea pig testing. Therefore, the LLNA radiolabeled procedure gave positive results for 13/22 (59%) of these products; while the guinea pig test (either BT or GPMT) gave positive results for 3/22 (14%). The false negative (i.e., under prediction) rate for the LLNA in this sample was 0% (0/3). No comparative human data on these 22 pesticide end use products were available to confirm these results.

LLNA does not appear to under predict results from the guinea pig testing**.** The limited database indicates a bias towards a positive or sensitizing result in LLNA (13/22; 59%) over the guinea pig test (3/22; 14%) but this conclusion is based on a very small sample size of 3. The Agency believes the bias would be alleviated if the dataset were larger. Regardless, these data indicate the LLNA has utility for hazard classification of end use pesticide products, since it is as protective as the other assays. The panel could not make a prediction if the guinea pig assay may have missed classifying an end use product as a sensitizer or not, since no comparable human data exist. A quick review of the National Pesticide Information Center's incident data base during 2008 for "hives or contact dermatitis" showed no significant failure in the guinea pig assay in classifying an end use product as a sensitizer. Only 3 human incidents out of a total of 1,823 were attributed to hives or contact dermatitis.

The retrospective analysis by ICCVAM also showed the LLNA under predicts for single components (i.e., Active Ingredients) when compared with the BT and GMPT; but the LLNA end use product data over predicts both the BT and the GPMT. Again no comparable human data exist to compare to this results.

OPP Expansion:

OPP Evaluation: No antimicrobial, biopesticide or microbial end use pesticide products were found in the ICCVAM subset of LLNA available data. But it is expected that these pesticide products would react in a similar manner. OPP considers this expansion of the guidelines to be public health protective. Effective immediately, OPP will now evaluate both negative and positive LLNA radiolabeled assays on end use pesticide products. Listed below are the specifics:

1. This expansion of the OPPTS guidelines will <u>not</u> allow the registrant to "test out" of a positive sensitizing result by submitting another assay on the same end use pesticide product. This guiding principle is consistent with a longstanding OPP practice when

conflicting results are submitted; i.e., protect human health. If the end use pesticide product (i.e., composition) is significantly amended a new assay might be considered, however, please consult with the Agency in this case. The radiolabeled LLNA assay will continue as the preferred test method for dermal sensitization for OPP and all of the published LLNA protocol principles apply.

2. Also, it should be emphasized that dermal sensitization testing may be waived, if the end use pesticide product is a known skin sensitizer. The registrant may request the most restrictive labeling in this scenario.

3. Non-radio labeled assays for dermal sensitization is not part of the expansion. The non radio labeled assays will not be accepted for review until their full adoption by the OECD and new guidelines are published by OECD and OPPTS.

4. Effective dates: Effective immediately. The expansion will be in place until the OECD guideline 429 is revised and formally adopted by the OECD member countries and then published as an OPPTS guideline.

5. EPA concurs with the ICCVAM recommendation that the LLNA should be used to test any a.i. or product. The LLNA should not be used if the properties of the chemical or product interfere with the LLNA in detecting sensitizing substances.

Reduced LLNA radiolabeled assay

Within the expansion of the guidelines on use of the traditional LLNA for dermal sensitization testing of end use pesticide products, OPP is also adopting for use the "reduced" or "limit dose" LLNA radiolabeled assay (rLLNA). The same limitations that apply to the LLNA apply to the rLLNA. The rLLNA radiolabeled (limit dose) assay uses a negative control group and a high dose group. The rLLNA omits the middle and low dose groups, and instead uses a test concentration that is the maximum concentration that does not induce excessive local irritation or overt systemic toxicity in the mouse. The rLLNA assay must use the same protocol provisions published in the present OPPTS guidelines with 5 animals per group, and presentation of individual (not pooled) data, historical controls and mouse strains. The rLLNA can be used in cases where dose-response information is not needed. All other protocol provisions of the current OPPTS guideline should be applied to the rLLNA.

OPP LABELING STATEMENTS

Typical Statements for Dermal Sensitization:

Label Statements:
Product is a sensitizer or is positive for sensitization. Prolonged or frequently repeated skin contact may cause allergic reactions in some individuals.
Or
Product is not a sensitizer or is negative for sensitization. No labeling is required for this result.

Note: USEPA does not make a distinction between weak, moderate or strong sensitizers.

Local Lymph Node Assay (LLNA) - OPPTS Protocol

INTRODUCTION

OPPTS 870.2600 Skin Sensitization

- Purpose is to identify substances (end use and manufacturing use products) with skin sensitization potential. Testing is not required if the product is a known skin sensitizer.
- The LLNA is the preferred alternative method, where applicable, to the traditional guinea pig test because it demonstrates an equivalent prediction of human allergic contact dermatitis as compared to the other sensitization tests, and provides quantitative data and an assessment of dose-response, gives consideration to animal welfare concerns, and is suitable for testing colored substances.
- Other Acceptable methods: a) Guinea-Pig Maximization Test (GPMT); or b) Buehler Test.
- LLNA may not be appropriate for all types of test materials, such as certain metallic (i.e., nickel) compounds, high molecular weight proteins, strong dermal irritants and test materials that do not sufficiently adhere to the ear for an acceptable period of time during treatment.
- It should be recognized that there are certain testing situations that may necessitate the use of traditional sensitivity tests.
- When using the LLNA, particular care should be taken to ensure that aqueous and or hydrophilic materials are incorporated into a vehicle system that wets the ear and does not immediately run off. The guideline has a list of applicable vehicles.

LLNA LIMITATIONS

Materials that are wholly aqueous, strong irritants or sensitizing metals (nickel) appear to be problematic for the LLNA. These types of pesticide end use products should be tested using other acceptable dermal sensitization assays.

PRINCIPLES OF DETECTION OF THE LOCAL LYMPH NODE ASSAY (LLNA)

- Sensitizers induce proliferation of lymphocytes
- Proliferation is proportional to the dose applied
- Quantitative (objective) test
- Dose response built into guideline (3 doses)
- Simple, yet requires skills and practice

PRINCIPLE OF THE LOCAL LYMPH NODE ASSAY (LLNA)

- Sensitizers 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 sensitization.
- The ratio of the proliferation in treated groups to that in vehicular controls, termed the Stimulation Index, is determined. Stimulation index ≥ 3 are indicative of the test substance is a skin sensitizer.

- Radioactive labeling is used to measure cell proliferation.

PROTOCOL HIGHLIGHTS

- 3HTdR is used for detection as this incorporates with lymph node DNA
- Radio activity from excised lymph tissue is measured
- β-scintillation counter is used for monitoring
- 5-6 CBA/ca female mice are used per group
- The highest concentration should not produce systemic toxicity or excessive local dermal irritation
- Pretest screening test may be necessary using a few mice starting with 100% concentration of the test substance- then dose in a linear manner
- 3-5 consecutive concentrations are used (e.g., 100, 50, 25, 10, 5, 2.5, or 1%)
- Each animal receives 25 ul of the test substance to the dorsum of each ear to groups of 5 mice
- Animals are treated on days 1 , 2 and 3
- No treatment on days 4 and 5
- Day 6 tail vein injection with tritiated methyl-thymidine
- Animals sacrificed 5 hours later
- Auricular lymph nodes removed and prepared for cell suspension and scintillation counting
- 3 concentrations of positive control added to the test with 5 mice in each concentration 100%, 50% and 25% all (preferably) in acetone and olive oil 4:1
- Day 6 all test and controls are injected in tail vein with 250 ml of 0.01 M phosphate buffer saline pH 7.2 containing 20 m Ci of Thymidine
- Then the pellets are suspended in 1 ml of Trichloroacetic acid (TCA) and transferred to 10 ml of scintillation fluid
- Produce a stimulation index i.e., test/control ratio
- Single cell suspension is prepared by mechanical disintegration through 200 mesh stainless steel gauze. Cells washed twice with PBS and precipitated with 5% TCA at 4 degrees C for 18 hours
- Incorporation of Thymidine is measured by liquid scintillation counter as DPM (disintegrations/minute) for each mouse. The stimulation index for one of the 3 concentrations should be ≥ 3 for a test to be labeled as a sensitizer or a positive result

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