APPENDIX A

Description of the LLNA: DA Protocol

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NICEATM has requested a detailed protocol from Daicel Chemical Industries, Ltd. but it has not yet been provided (when that protocol is received, this appendix will be updated accordingly). Manuscripts detailing the results of the LLNA: DA validation studies and specifics of the methodology are reported to be currently undergoing peer review for publication. Thus, the description of the LLNA: DA methodology provided here represents information taken from poster and platform presentations made at the 2007 6th World Congress (Daicel Chemical Industries, Ltd. 2007, Omori et al. 2007, Yoshimura et al. 2007) and from Yamashita et al. 2005 where the development of the LLNA: DA was first described.

Introduction

The LLNA: DA is similar to the traditional LLNA in that it determines the sensitization potential of a test substance by measuring the proliferation of lymphocytes in the auricular lymph nodes draining the site of exposure (ears). One main difference is that the LLNA: DA employs non-radioactive methods to assess lymphocyte proliferation by measuring adenosine triphosphate (ATP) content in the lymph nodes and thus foregoing the use of tritiated thymidine or iodine-125 based measurements used by the traditional LLNA. In addition, there are major differences between the two protocols that relate to test substance application and timing for the collection of the lymph nodes. In the traditional LLNA, the test substance is administered on three consecutive days (days 1, 2, and 3). On day 6, tritiated thymidine or iodine-125 is administered via the tail vein and the lymph nodes are excised five hours later. A lymph node cell suspension is then prepared and tritiated thymidine or iodine-125 incorporation is determined by β-scintillation or γ-scintillation counting. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and 7. During the initial development of the LLNA: DA, the study group (Yamashita et al. 2005) determined the optimal dosing schedule by evaluating whether the addition of a fourth application (day 7) was useful for increasing lymph node proliferation. Based on a statistically significant increase in lymph node weight-based Stimulation Indexes (SIs) for mice that received a fourth application of the test substance, this protocol was decided upon. Furthermore, one hour prior to each application of the test substance, 1% sodium lauryl sulfate (SLS) is applied to the dorsum of the treated ears to increase absorption of the test substance across the skin (van Och et al. 2000). Lastly, twenty-four to 30 hours after the last test substance application, the auricular lymph nodes

are excised and a lymph node cell suspension is prepared, and the ATP content is measured by luciferin-luciferase assay.

LLNA: DA Test Method Protocol:

Animal Selection and Preparation

Sex and strain of animals

Female CBA/JN mice were 8 to 12 weeks old.

Preparation of animals

The temperature and humidity inside the animal holding room was maintained at 23 ± 2°C and 55% ± 15%, respectively, and each room was ventilated at a frequency of 8-10 cycles per hour.

Preparation of Substance Doses

Suitable vehicles and concentrations for testing were determined based on previous reports (Hariya et al. 1999, Van Och et al. 2000).

SLS was dissolved in water and was used to pretreat the test substance application site (ears) prior to application of the agents (or vehicle). Application of 1% SLS prior to ear challenge is known to help improve the detection sensitivity of the test (van Och et al. 2000). Various researchers have shown that 1% SDS does not elicit a positive response in the traditional LLNA but when applied prior to test substance administration there is generally an increased response compared to the test substance alone (van Och et al. 2000; De Jong et al. 2002).

The moderate sensitizer alpha-hexyl cinnamic aldehyde (HCA) was used as the positive control.

Test Procedure Methodology

Four mice per group:

Day 1 – The dorsum of both ears was pretreated with a 1% SLS solution. One hour later, 25 μL of three concentrations of test substance or the relevant vehicle alone was applied to the dorsum of both ears.

Days 2 and 3 – Repeat of the application procedure as carried out on day 1.

Days 4, 5, and 6 – No treatment

Day 7 – Repeat of the application procedure as carried out on day 1.

Day 8 (24-30 hours after last application)– For each group, draining auricular lymph nodes were excised, weighed, and pooled.

Assessment of Lymphocyte Proliferation

Lymph node cells (LNCs) were crushed and spread between two glass slides and then scraped and suspended in 1 ml phosphate buffered saline (PBS). The cell suspension was then diluted 1:100 in PBS and a 100 μL aliquot was used to measure ATP content by luciferin-luciferase assay (several measurement kits are available; Kikkoman Co., Tokyo, Japan).

$$ATP + Luciferin + O_2 \xrightarrow{Luciferase} Oxyluciferin + AMP + PPi + CO_2 + Light$$

The intensity of light emitted was measured with a luminometer (Lumitester™ C-100) and was an indicator of ATP content and thus an index of cell number.

Measurement was performed immediately after lymph node excision

Statistical Analysis

The mean relative light unit (RLU) value for each experimental group was calculated, and the SI was derived. The SI was calculated as the mean ATP content in the LNC suspension obtained from the test group to that in the LNC suspension obtained from the control group according to **Equation 1** below:

$$SI = \frac{mean\ ATP\ content\ of\ \ auricular\ lymph\ nodes\ in\ test\ treatment\ group\ (RLU)}{mean\ ATP\ content\ of\ \ auricular\ lymph\ nodes\ in\ vehicle\ treatment\ group\ (RLU)}$$

The cut-off point for a positive result was SI ≥ 3 and any result with an SI < 3 was considered negative.

The confidence intervals (CIs) for the SI values were calculated using the following formula:

$$\exp\left(\ln(SI) \pm 1.96\sqrt{(Var(\ln SI))}\right) \text{ where, } Var(\ln SI) \cong \frac{SE(Y)^2}{Mean(Y)^2} + \frac{SE(X)^2}{Mean(X)^2}$$

Good laboratory Practices (GLP)

The studies were not conducted in compliance of GLP guidelines. However, all of the participating laboratories were GLP compliant laboratories. In addition, all of the laboratories that participated in the interlaboratory validation studies used the same experimental protocol and took part in a one-day seminar that explained the protocol and execution of the test method. Also, the same luminometer and the same dose of each coded substance were used in all the laboratories. Furthermore, a formatted file for the entry of the experimental data and information was prepared using Microsoft EXCEL. The file was then distributed to the participating laboratories prior to the experiment. A preliminary test was performed with only a positive control substance to confirm that the experimental protocol was being adequately documented among the laboratories. After filling in the data, files from all the experimental laboratories were collected and the data were analyzed (Omori et al. 2007).