APPENDIX A

BrdU-ELISA Local Lymph Node Assay Protocol

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This appendix describes the protocol used to obtain the BrdU-ELISA LLNA results (Takeyoshi et al. 2003; 2004a; 2004b; 2005; 2006; 2007a) evaluated in this BRD on the validation status of the BrdU-ELISA LLNA. The protocol used by Takeyoshi et al. is contrasted with the ICCVAM recommended LLNA protocol (ICCVAM 1999; Dean et al. 2001), which was used to develop OECD Test Guideline 429 (OECD 2002). NICEATM has requested a detailed BrdU-ELISA protocol but it has not yet been provided. When that protocol is received, this appendix will be updated accordingly.

Animal Selection and Preparation

Animal Species Selection

- Female CBA/JN mice were used.
- Age or weight of mice was not specified. ICCVAM (1999) and Dean et al. (2001) recommend mice be 8-12 weeks old and that weight variations between the mice should not exceed 20% of the mean weight.

Housing and Feeding Conditions

- Experimental animal room temperature was 23 ± 2 °C. ICCVAM (1999) and Dean et al. (2001) recommend 21 ± 3 °C.
- Experimental animal room humidity was 55% ± 15%. ICCVAM (1999) and Dean et al. (2001) recommend 30 - 70%.
- Experimental animal rooms were ventilated at 10-15 cycles per hour. ICCVAM (1999) and Dean et al. (2001) make no recommendation on ventilation.
- Experimental animal rooms were lighted artificially for 12 hours per day.
- Mice had free access to laboratory diet and drinking water.

Animal Preparation

- Not reported. ICCVAM (1999) and Dean et al. (2001) recommend that mice be acclimated for at least 5 days prior to the start of the test.

Control Substances

Solvent/Vehicle Control

- The solvent and vehicle control for all tests was acetone:olive oil (4:1 v/v)

Positive Control

- 50% Hexyl cinnamic aldehyde was used as a concurrent positive control.

Test Procedure

Number of Animals per Dose

- Used four mice per dose group and four mice for the vehicle control group. ICCVAM (1999) and Dean et al. (2001) recommend at least five mice per group.

Selection of Doses

- At least three doses were tested (e.g., 100%, 50%, 25%).
- Dose selection was based on previous sensitization information such as dose range finding tests, guinea pig test results, or human potency. ICCVAM (1999) and Dean et al. (2001) indicate that the highest dose tested should not induce systemic toxicity and/or excessive skin irritation.

Dosing Schedule and Collection of Lymph Node Cells

- Days 1 through 3
  - Test substance or vehicle (25 μL) was applied to the dorsum of each ear.
- Day 5
  - BrdU was intraperitoneally injected (5 mg/mouse).
- Day 6
  - The draining auricular lymph nodes were removed, weighed, and stored at -20°C until ELISA analysis.

Observations

- None reported. ICCVAM (1999) and Dean et al. (2001) indicate that mice should be observed for clinical signs of local, excessive irritation or corrosion, or systemic toxicity.

Assessment of Lymphocyte Proliferation and Interpretation of Results

- A commercial cell proliferation assay kit was used to quantify the incorporation of BrdU into lymph node cells (Boehringer Mannheim Corp., Indianapolis, IN, USA; Cat. No. 1647229).
- Lymph nodes were crushed, passed through a #70 nylon mesh and cells were suspended individually in 15 mL physiological saline. ICCVAM (1999) and Dean et al. (2001) recommend using #200 stainless steel mesh.
- 100 μL cell suspension was added, in triplicate, to the wells of a flat bottom microplate.
- After centrifugation at 3000g for 10 minutes, the supernatants were removed and 200 μL Fix-Denat was added to each well.
- The plate was allowed to stand at room temperature for 30 minutes, Fix-Denat was removed and the 100 μL diluted anti-BrdU antibody was added to each well.
- Cells were rinsed three times with phosphate buffered saline and then 100 μL substrate solution containing tetramethylbenzidine was added and allowed to react for 15 minutes at room temperature.
- Absorbance was read at 370 nm with a microplate reader with a reference wavelength of 492 nm. The absorbance was defined as the BrdU labeling index.

- The means and standard errors for the labeling index were calculated from the individual animal data for each treatment group. The mean absorbances for the treatment groups were divided by the mean absorbance of the control group to determine the stimulation index (SI) for each treatment group. If any concentration of test substance produced SI $\geq$ 3, the substance was identified as a sensitizer.
- For some tests, the concentration of a test substance required to produce an EC3 was derived by linear interpolation between the two concentration points above and below SI =3.

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