Evaluation of Two Nonradiolabeled Murine Local Lymph Node Assays (LLNA) for Potency Categorization of Substances Causing Allergic Contact Dermatitis in Humans

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Abstract

The correct classification of strong skin sensitizers is critical because such substances are considered to have a significant potential for causing allergic contact dermatitis (ACD) in humans. Because the prognosis for ACD is poor, sensitizing substances must be labeled with a description of the potential hazard and the precautions necessary for workers and consumers to avoid development of ACD. A recent ICCVAM evaluation found that the LLNA correctly classified 52% (14/27) of the strong human sensitizers when an effective threshold concentration (EC) ≤ 2% was used as the criterion. Thus, ICCVAM recommends that the LLNA may be used as a screening test to classify substances as strong sensitizers, but that the classification of substances as other than strong sensitizers requires additional information. The OECD recently adopted two test guidelines for nonradiolabeled versions of the LLNA that could be used to classify substances as sensitizers: the LLNA: BrdU-ELISA and the LLNA: DA. Although these LLNA methods use different decision criteria to classify substances for ACD hazard, their accuracy is comparable to that of the LLNA. Of the 136 substances used in the ICCVAM evaluation of the usefulness of the LLNA for potency categorization, LLNA: BrdU-ELISA data were available for 31 substances, and LLNA: DA data were available for 30 substances. An EC ≤ 9% for the LLNA: BrdU-ELISA and an EC ≤ 0.8% for the LLNA: DA classified strong human sensitizers at rates comparable to that of the LLNA. These results suggest that the LLNA: BrdU-ELISA and the LLNA: DA may also be useful for classifying substances as strong human sensitizers.

Introduction

- Allergic contact dermatitis (ACD) is a skin reaction characterized by redness, swelling, and itching that can result from repeated contact with a sensitizing substance.
- Because the prognosis is poor (Hogan et al. 1990), preventing or limiting exposures to ACD hazards is important.

- The U.S. Consumer Product Safety Commission (CPSC), under the Federal Hazardous Substances Act (15 U.S.C. 1261-1278), requires hazard labeling of strong skin sensitizers (16 CFR 1500.3(b)[9]).
  - Although the CPSC uses both human and animal data to determine that a substance is a strong sensitizer, no quantitative criteria are currently applied (**Table 1**).
- In 2007 the CPSC requested that ICCVAM evaluate the usefulness and limitations of the LLNA as a stand-alone test method for potency determinations.
  - Following the CPSC request, the Globally Harmonized System for Classification and Labelling of Chemicals (GHS) was updated to include potency criteria for the LLNA (UN 2009, 2011) (**Table 1**).
  - Based on an evaluation of 136 substances with LLNA and human data, ICCVAM recommended that LLNA with an EC3 ≤ 2% could be used to classify substances as strong sensitizers (GHS 1A) but that LLNA with EC3 > 2% could not be used alone to classify substances as other (than strong) sensitizers (GHS 1B) (ICCVAM 2011).
    - 48% (13/27) of strong human sensitizers were classified as other sensitizers (EC3 > 2%) or as nonsensitizers by the LLNA.
- This poster examines the accuracy of two nonradiolabeled versions of the LLNA, the LLNA: BrdU-ELISA and the LLNA: DA, for classifying substances as strong human skin sensitizers.

Table 1. Classification Systems for Skin Sensitizers

Classification System	Criteria for "Strong" Skin Sensitizer	Criteria for "Other" (Than Strong) Skin Sensitizer
CPSC	Weight-of-evidence approach that considers quantitative or qualitative risk assessment, frequency of occurrence and range of severity of reactions in healthy or susceptible populations, and the results of experimental assays in animals or humans. Human data take precedence over animal data, other data on potency or bioavailability of sensitizers, data on reactions to a cross-reacting substance or to a chemical that metabolizes or degrades to form the same or a cross-reactive substance, the threshold of human sensitivity, epidemiological studies, case histories, occupational studies, and other appropriate *in vivo* and *in vitro* test studies.	Not applicable
GHS	Subcategory 1A High frequency of occurrence in humans, and/or high potency in animals. May consider severity. LLNA EC3 ≤ 2% Positive[a] response in humans at ≤ 500 μg/cm²	Subcategory 1B Low to moderate frequency of occurrence in humans and/or low to moderate potency in animals. LLNA EC3 > 2% Positive[a] response in humans at > 500 μg/cm²

Abbreviations: CPSC = U.S. Consumer Product Safety Commission; EC3 = estimated concentration of a substance needed to produce a stimulation index of 3 in the LLNA; GHS = Globally Harmonized System for Classification and Labelling of Chemicals; LLNA = murine local lymph node assay.

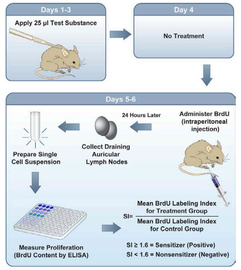
[a] Human evidence can also include (1) diagnostic patch test data with a relatively high incidence of reactions in a defined population in relation to relatively low exposure or (2) other epidemiology evidence with a relatively high incidence of allergic contact dermatitis in relation to relatively low exposure.

[b] Human evidence can also include (1) diagnostic patch test data with a relatively low but substantial incidence of reactions in a defined population in relation to relatively high exposure or (2) other epidemiology evidence with a relatively low but substantial incidence of allergic contact dermatitis in relation to relatively high exposure.

Methods

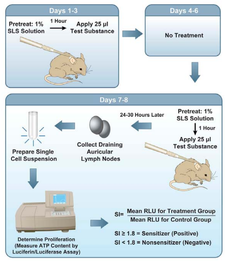
- **LLNA: BrdU-ELISA Test Method**
  - The assay measures the nucleotide analog bromodeoxyuridine (BrdU) using an enzyme-linked immunosorbent assay (ELISA) to assess lymph node cell proliferation (Takeyoshi et al. 2001) (**Figure 1**).
  - ICCVAM recommended use of the LLNA: BrdU-ELISA to classify substances as potential sensitizers (stimulation index [SI] ≥ 1.6) or nonsensitizers (ICCVAM 2010a).
  - OECD Test Guideline 442B Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA, which includes the SI ≥ 1.6 criterion to classify substances as skin sensitizers, was adopted on July 22, 2010 (OECD 2010a).
- **LLNA: DA Test Method (developed by Daicel Chemical Industries, Ltd.)**
  - The assay measures adenosine triphosphate content in draining auricular lymph nodes as an estimate of cell number to assess lymph node cell proliferation (Yamashita et al. 2005; Idehara et al. 2008) (**Figure 2**).
  - ICCVAM recommended use of the LLNA: DA to classify substances as potential sensitizers (SI ≥ 1.8) or nonsensitizers (ICCVAM 2010b).
  - OECD Test Guideline 442A Skin Sensitization: Local Lymph Node Assay: DA, which includes the SI ≥ 1.8 criterion to classify substances as skin sensitizers, was adopted on July 22, 2010 (OECD 2010b).

Figure 1. LLNA: BrdU-ELISA Test Method Protocol



Abbreviations: SI = stimulation index.

Figure 2. LLNA: DA Test Method Protocol



Abbreviations: RLU = relative luminescence unit; SI = stimulation index; SLS = sodium lauryl sulfate.

Human Data

- These data were obtained from the literature as reported by ICCVAM (2011).
- The human repeat insult patch test (HRIPT) and the human maximization test (HMT) involve the administration of occluded patches loaded with test substance to the skin for 9 (HRIPT) or 5 (HMT) on-and-off periods of 24 or 48 hours, respectively, in order to attempt to induce an allergic reaction (Kligman and Epstein 1975; Politano and Api 2008).
- Following a rest period of 10 to 14 days, volunteers are again exposed to the test substance in an occluded patch on naive skin for 24 (HRIPT) or 48 (HMT) hours.
- Erythema and edema (including papules, vesicles, or bullae) observed after patch removal indicate ACD and are recorded as positive reactions.
- For substances that produce no skin irritation, the HMT includes a patch pretreatment of the skin with 5% sodium lauryl sulfate for the 24-hour period before the induction patch treatments in order to break the stratum corneum barrier, producting a "brisk dermatitis" (Kligman and Epstein 1975).
- Induction thresholds (the minimum concentrations that produce positive reactions) are reported as microgram weight of applied substance per square centimeter of skin (μg/cm²).

Chemical Database for Analysis

- Data for analysis were identified from the corresponding ICCVAM evaluations of the LLNA: BrdU-ELISA (ICCVAM 2010a), the LLNA: DA (ICCVAM 2010b), and the traditional LLNA for potency categorization (ICCVAM 2011) (see **Table 2**) and included:
  - 30 substances with traditional LLNA, human, and LLNA: BrdU-ELISA data
  - 31 substances with traditional LLNA, human, and LLNA: DA data
- Human studies were used as the reference data.
  - Induction threshold concentrations from the HRIPT and HMT were expressed as the dose per unit area that produced a 5% response in the population tested (DSA_{05}).
  - "Strong" sensitizers (GHS 1A) are defined as having $DSA_{05} \leq 500$ μg/cm². "Other" sensitizers (GHS 1B) are defined as having $DSA_{05} > 500$ μg/cm².

Analyses

- The correct, underprediction, and overprediction rates for strong (GHS 1A) human sensitizers, other (GHS 1B) human sensitizers, and nonsensitizers were calculated for multiple threshold induction concentrations for the LLNA: BrdU-ELISA EC1.6[a] and the LLNA: DA EC1.8[b]
  - These results were compared to the prediction rates of the traditional LLNA EC3 for the same substances.

[a] EC1.6 is an estimated concentration of a substance needed to produce a stimulation index of 1.6 in LLNA: BrdU-ELISA.

[b] EC1.8 is an estimated concentration of a substance needed to produce a stimulation index of 1.8 in LLNA: DA.

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Table 2. Database for Potency Analysis of Nonradiolabeled LLNA Methods

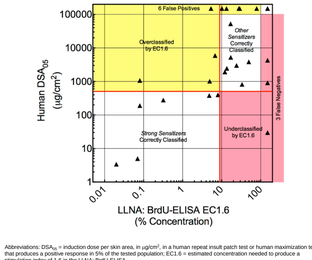
Substance[a]	Human DSA_{05} (μg/cm²)[b]	LLNA EC3[b]	LLNA: BrdU-ELISA EC1.6[b]	LLNA: DA EC1.8[b]
2,4-Dinitrochlorobenzene	3.4	0.04	0.02	0.02
5-Chloro-2-methyl-4-isothiazolin-3-one	5.0	0.01	0.07	0.01
4-Phenylenediamine	30	0.12	NC	0.04
Potassium dichromate	106	0.12	NT	0.06
Formaldehyde	191	1.40	0.08	0.47
Cobalt chloride	279	0.57	0.32	0.38
Cinnamic aldehyde	382	1.00	4.81	0.63
Diethyl maleate	400	3.27	8.05	0.89
Butyl glycidyl ether	437	30.9	NT	17.5
Ethyl acrylate	818	32.0	33.3	6.79
Citral	915	5.00	NC	4.11
Isoeugenol	1016	1.40	4.93	0.82
Glutaraldehyde	1073	0.16	0.08	0.1
2-Mercaptobenzothiazole	1930	2.60	12.1	7.99
Aniline	2463	33.0	13.6	NT
Cinnamic alcohol	3002	20.0	24	5.23
Imidazolidinyl urea	3846	24.0	49.6	6.28
Sulfanilamide	4310	NC	NC	NC
Hydroxycitronellal	5237	23.0	17.1	8.67
Eugenol	5926	11.0	6.8	2.63
Benzocaine	10140	7.80	NT	3.11
Phenyl benzoate	52500	9.50	16.9	0.65
Benzalkonium chloride	Negative	0.07	NT	0.4
Cyclamen aldehyde	Negative	22.0	28.9	NT
Diethyl phthalate	Negative	NC	NC	NC
Glycerol	Negative	NC	NC	NT
Hexane	Negative	NC	78.9	82.2
Hexyl cinnamic aldehyde	Negative	8.90	10.2	6.16
Isopropanol	Negative	NC	5.33	NC
Isopropyl myristate	Negative	44.0	NC	NT
Linalool	Negative	55.0	27.6	NT
Methyl salicylate	Negative	17.0	NC	NC
Propylene glycol	Negative	NC	NC	NT
Resorcinol	Negative	5.9	NT	3.9
Salicylic acid	Negative	12.0	NC	17.7
Sodium lauryl sulfate	Negative	4.0	13.3	1.64

Abbreviations: DSA_{05} = induction dose per skin area, in μg/cm²; in a human repeat insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC1.6 = estimated concentration of a substance needed to produce a stimulation index of 1.6 in LLNA: BrdU-ELISA; EC1.8 = estimated concentration of a substance needed to produce a stimulation index of 1.8 in the LLNA: DA; EC3 = estimated concentration of a substance needed to produce a stimulation index of 3 in the traditional LLNA; NC = not calculated because the substance was negative; NT = not tested.

[a] Listed in order of decreasing human potency.

[b] If more than one value per substance was identified, values represent a geometric mean.

Figure 3. LLNA: BrdU-ELISA and Human Potency



Abbreviations: DSA_{05} = induction dose per skin area, in μg/cm², in a human repeat insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC1.6 = estimated concentration needed to produce a stimulation index of 1.6 in the LLNA: BrdU-ELISA.

Notes: A red vertical line indicates a classification cutoff of 9% for the LLNA: BrdU-ELISA EC1.6. A red horizontal line shows a cutoff of 500 μg/cm² for the human DSA_{05} — a cutoff applied by GHS to differentiate "strong" sensitizers from "other" sensitizers. Negative results were assigned a single value >100% for the LLNA: BrdU-ELISA and >100,000 μg/cm² for the human DSA_{05}. Six true negatives are overplotted in the unshaded area in the upper right corner of the graph, and the number of false positives and false negatives (relative to human data) are indicated on the graph.

Results

Figures 3 and **4** show scatter plots for the human and LLNA: BrdU-ELISA or LLNA: DA data in **Table 2**. **Table 3** shows classification rates for the nonradiolabeled LLNA assays to predict human potency based on the GHS cutoff of 500 μg/cm² for the human DSA_{05}. The two figures and table are color coded to indicate substances that are correctly classified (unshaded), overclassified (yellow), or underclassified (pink) using selected cutoffs for the two LLNA tests.

Table 3. Classification Rates for Prediction of Human Potency

Table 3a. LLNA EC3 and LLNA: BrdU-ELISA EC1.6 Cutoffs[a] for 31 Substances

Classification Cutoff for "Strong" versus "Other" Sensitizers	"Strong" Human Sensitizers (DSA_{05} ≤ 500 μg/cm²)		"Other" Human Sensitizers (DSA_{05} > 500 μg/cm²)		Human Nonsensitizers		Overall Correct Potency Classification[b]
	Correct	Under	Under	Correct	Over	Over	Correct
LLNA EC3 ≤ 2%	86% (6/7)	14% (1/7)	8% (1/12)	75% (9/12)	17% (2/12)	58% (7/12)	42% (5/12)	65% (20/31)
LLNA: BrdU-ELISA EC1.6 ≤ 9%	86% (6/7)	14% (1/7)	58% (7/12)	25% (3/12)	25% (3/12)	50% (6/12)	50% (6/12)	61% (19/31)
LLNA: BrdU-ELISA EC1.6 ≤ 6%	71% (5/7)	29% (2/7)	17% (2/12)	67% (8/12)	17% (2/12)	50% (6/12)	50% (6/12)	61% (19/31)
LLNA: BrdU-ELISA EC1.6 ≤ 2%	57% (4/7)	43% (3/7)	75% (9/12)	8% (1/12)	50% (6/12)	50% (6/12)	50% (6/12)	61% (19/31)

Abbreviations: DSA_{05} = induction dose per skin area, in μg/cm²; in a human repeat insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC1.6 = estimated concentration of a substance needed to produce a stimulation index of 1.6 in the LLNA: BrdU-ELISA; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8 in the LLNA: DA; EC3 = estimated concentration of a substance needed to produce a stimulation index of 3, which is the threshold value for a substance to be considered a sensitizer in the LLNA; GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2011); LLNA = murine local lymph node assay.

Note: Column headings are defined as follows: correct = correct classification; under = underclassification; over = overclassification.

[a] Potency classification used cutoff values of ≤2%, 6%, or 9% for strong sensitizers and >2%, 6%, or 9% for other sensitizers. "Strong" or "other" sensitizers were identified using the respective EC values listed in the table for the traditional LLNA and nonradiolabeled LLNA assay. The comparable cutoff for the LLNA: BrdU-ELISA is indicated in the table (see row with the bold border for EC1.6 ≤ 9%).

[b] The overall correct classification rate is based on human data classifications for "strong" sensitizers, "other" sensitizers, and nonsensitizers (UN 2011).

Table 3b. LLNA EC3 and LLNA: DA EC1.8 at Various Cutoffs[a] for 30 Substances

Classification Cutoff for "Strong" versus "Other" Sensitizers	"Strong" Human Sensitizers (DSA_{05} ≤ 500 μg/cm²)		"Other" Human Sensitizers (DSA_{05} > 500 μg/cm²)		Human Nonsensitizers		Overall Correct Potency Classification[b]
	Correct	Under	Correct	Over	Over	Correct	
LLNA: EC3 ≤ 2%	78% (7/9)	22% (2/9)	8% (1/12)	75% (9/12)	17% (2/12)	67% (8/12)	33% (4/12)	63% (19/30)
LLNA: DA EC1.8 ≤ 2%	89% (8/9)	11% (1/9)	8% (1/12)	67% (8/12)	25% (3/12)	67% (8/12)	33% (4/12)	63% (19/30)
LLNA: DA EC1.8 ≤ 0.8%	78% (7/9)	22% (2/9)	8% (1/12)	75% (9/12)	17% (2/12)	67% (8/12)	33% (4/12)	63% (19/30)
LLNA: DA EC1.8 ≤ 0.5%	67% (6/9)	33% (3/9)	8% (1/12)	58% (7/12)	33% (4/12)	67% (8/12)	33% (4/12)	53% (16/30)

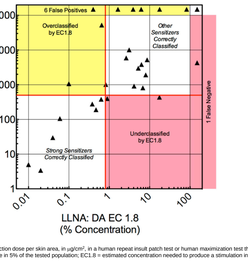
Abbreviations: See **Table 3a**.

Note: Column headings are defined as follows: correct = correct classification; under = underclassification; over = overclassification.

[a] Potency classification used cutoff values of ≤0.5%, 0.8%, or 2% for "strong" sensitizers and >0.5%, 0.8%, or 2% for "other" sensitizers. Sensitizers were identified using the respective EC values listed in the table for the traditional LLNA and the two nonradiolabeled LLNA assays. The cutoff for the LLNA: DA is indicated in the table (see row with bold border for EC1.8 ≤ 0.8%.)

[b] The overall correct classification rate is based on human data classifications for "strong" sensitizers, "other" sensitizers, and nonsensitizers (UN 2011).

Figure 4. LLNA: DA and Human Potency

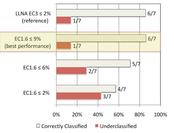


Abbreviations: DSA_{05} = induction dose per skin area, in μg/cm²; in a human repeat insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8 in the LLNA: DA.

Notes: A red vertical line indicates a classification cutoff of 0.8% for the LLNA: DA EC1.8. A red horizontal line shows a cutoff of 500 μg/cm² for the human DSA_{05} — a cutoff applied by GHS to differentiate "strong" sensitizers from "other" sensitizers. Three true negatives are overplotted in the unshaded area in the upper right corner of the graph, and the number of false positives and false negatives (relative to human data) are indicated on the graph.

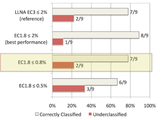
Figure 5. Classification of "Strong" Sensitizers

Figure 5a. Cutoffs for the LLNA: BrdU-ELISA EC1.6



Abbreviations: EC1.6 = estimated concentration of a substance needed to produce a stimulation index of 1.6 in the LLNA: BrdU-ELISA; EC3 = estimated concentration of a substance needed to produce a stimulation index of 3 in the LLNA.
Note: Cutoffs are compared using values from **Table 3a** and **Figure 3**.

Figure 5b. Cutoffs for the LLNA: DA EC1.8



Abbreviations: EC1.8 = estimated concentration of a substance needed to produce a stimulation index of 1.8 in the LLNA: DA; EC3 = estimated concentration of a substance needed to produce a stimulation index of 3 in the LLNA.
Note: Cutoffs are compared using values from **Table 3b** and **Figure 4**.

Conclusions

- EC1.6 ≤ 9% for the LLNA: BrdU-ELISA and EC1.8 ≤ 0.8% for the LLNA: DA classified strong sensitizers at rates comparable to that of the LLNA EC3 ≤ 2%.
- These results suggest that the LLNA: BrdU-ELISA and LLNA: DA may also be useful for classifying substances as strong sensitizers.
- When relative potency information is required, these nonradiolabeled tests should
  - Further reduce and refine animal use for ACD hazard assessments in comparison to guinea pig test methods, while ensuring human safety
  - Reduce costs and environmental hazards associated with the use of radioactive substances

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