



Original article

Application of BALB/c mouse in the local lymph node assay: BrdU-ELISA for the prediction of the skin sensitizing potential of chemicals

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ABSTRACT

Introduction: Allergic contact dermatitis (ACD) is a skin disease characterized by eczema and itching. A considerable proportion of chemicals induce ACD in humans. More than 10,000 substances should be tested for skin sensitization potential under the Registration, Evaluation, Authorization and Restriction of Chemical substances (REACH) regulation. The Local Lymph Node Assay (LLNA) has been designated as the first-choice *in vivo* assay for sensitization testing by REACH. The LLNA:BrdU-ELISA is a validated non-radioactive modification to the LLNA. For both the LLNA and the LLNA:BrdU-ELISA, CBA/JN mouse is the preferred mouse strain recommended in the regulatory guidelines. However, the availability of CBA/JN mouse in China is only limited to a few animal suppliers, which makes the mouse difficult to obtain. BALB/c mouse, which is widely commercially available, is considered for alternative use but it can only be used in the assay after it has been evaluated by formal validation study. Thus, a validation study was conducted in our laboratory to determine if BALB/c mouse could also be used in the LLNA:BrdU-ELISA.

Methods: Forty-three test substances including 32 LLNA sensitizers and 11 LLNA non-sensitizers, their vehicles and each concentration used were the same as that used in the formal validation study for the LLNA:BrdU-ELISA using CBA/JN mouse. Female BALB/c mice of 8–10 weeks old were randomly allocated to groups (four mice per group). The test substance (25 μ l) or the vehicle alone was applied to the dorsum of both ears daily for 3 consecutive days. A single intraperitoneal injection of 0.5 ml of BrdU (10 mg/ml) solution was given on day 5. On day 6, a pair of auricular lymph nodes from each mouse was excised, weighed and stored at -20°C until BrdU-ELISA was conducted.

Results: This validation study for the LLNA:BrdU-ELISA using BALB/c mouse correctly identified 30 of 31 sensitizers and 8 of 11 non-sensitizers. The accuracy, sensitivity, specificity, false positive rate, false negative rate, positive predictivity values and negative predictivity values in this study, which could indicate the performance of the LLNA:BrdU-ELISA using BALB/c mouse, were not different statistically from that of the validation study for the LLNA:BrdU-ELISA using CBA/JN mouse.

Discussion: This validation study indicates that BALB/c mouse could be used alternatively in the LLNA:BrdU-ELISA for the prediction of the skin sensitizing potential of chemicals.

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1. Introduction

Allergic contact dermatitis (ACD) is a skin disease characterized by eczema and itching. A considerable proportion of chemicals induce ACD in humans and the assessment of skin sensitization potential is an important requirement in the safety assessment process of new chemicals. The Registration, Evaluation, Authorization and Restriction of Chemical substances (REACH) regulation requires that substances produced or imported into the European Union in quantities of 1 ton

or more per year should be tested for skin sensitization potential. It has been estimated that there are more than 10,000 such substances under REACH (Anderson, Siegel, & Meade, 2011). The requirements for the registration of new chemicals under the regulations of Ministry of Environmental Protection of the People's Republic of China are basically identical to that under REACH. The Local Lymph Node Assay (LLNA) (i.e. OECD Test Guideline (TG) 429) (OECD, 2010a) is an alternative to guinea pig models (i.e. OECD TG 406) (OECD, 1992) for the assessment of contact sensitization potential. It has been designated as the first-choice *in vivo* assay for sensitization testing with the REACH regulation (McGarry, 2007). The LLNA:BrdU-ELISA (enzyme-linked immunosorbent assay) (i.e. OECD TG 442B) (OECD, 2010b) is a validated non-radioactive modification to the LLNA, which utilizes non-radiolabelled 5-bromo-2-deoxyuridine (BrdU)

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Table 1
Information on the 43 reference substances tested in this study.

Substance name	CAS No.	Lot No.	Manufacturer ^a	Vehicle	Concentration (%)
32 LLNA sensitizers					
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	JL140501001	5	DMF	0.5
<i>p</i> -Benzoquinone	106-51-4	80015626	4	AOO	1
2,4-Dinitrochlorobenzene	97-00-7	970318	4	AOO	1
Diphenylcyclopropenone	886-38-4	C175820010	2	AOO	10
Glutaraldehyde	111-30-8	930530	4	AOO	1
4-Phenylenediamine	106-50-3	80100026	4	AOO	10
Formaldehyde	500-00-0	10010094	4	ACE	10
Cobalt chloride	7646-79-9	20015680	4	DMSO	5
4-Methylaminophenol sulfate(MAPS)	55-55-0	TM014501	1	DMF	10
trans-Cinnamaldehyde	14371-10-9	30042962	4	AOO	25
Isoeugenol	97-54-1	TI013201	1	AOO	10
2-Mercaptobenzothiazole	149-30-4	30112714	4	DMF	12.5
Cinnamic aldehyde	104-55-2	ZA12233A	3	AOO	50
<i>m</i> -Aminophenol	591-27-5	80005324	4	AOO	25
Diethyl maleate	141-05-9	JL140422001	5	AOO	25
Trimellitic anhydride	552-30-7	TC004601	1	AOO	10
Nickel sulfate	7786-81-4	10014418	4	DMSO	10
4-Chloroaniline	106-47-8	80032526	4	AOO	25
Sodium lauryl sulfate	151-21-3	30166428	4	DMF	16.7
Citral	5392-40-5	30043463	4	AOO	50
Hexyl cinnamic aldehyde	101-86-0	TH068501	1	AOO	25
Eugenol	97-53-0	TA023201	1	AOO	25
Phenyl benzoate	93-99-2	TB007501	1	AOO	66.7
Cinnamic alcohol	104-54-1	TC036201	1	AOO	50
Cyclamen aldehyde	103-95-7	JL140501001	5	AOO	100
Hydroxycitronellal	107-75-5	JL140319005	5	AOO	100
Imidazolidinyl urea	39236-46-9	TI066501	1	DMF	75
Ethylene glycol imethacrylate	97-90-5	TE010201	1	AOO	100
Linalool	78-70-6	TL004801	1	AOO	100
Ethyl acrylate	140-88-5	80059928	4	AOO	100
Isopropyl myristate	110-27-0	30158628	4	AOO	50
Aniline	62-53-3	10003392	4	AOO	100
11 LLNA non-sensitizers					
Hexane	110-54-3	80068618	4	AOO	100
Lactic acid	50-21-5	30108518	4	DMSO	50
Diethyl phthalate	84-66-2	031117	4	AOO	50
Dimethyl isophthalate	1459-93-4	TI015701	1	AOO	50
2-Hydroxypropyl methacrylate	27813-02-1	XW278130211	4	AOO	50
Glycerol	56-81-5	10010692	4	DMF	50
Isopropanol	67-63-0	40064360	4	AOO	50
Methyl salicylate	119-36-8	30121828	4	AOO	50
Salicylic acid	69-72-7	30163517	4	AOO	25
Sulfanilamide	63-74-1	30172216	4	DMF	50
Propylene glycol	57-55-6	30157018	4	AOO	50

^a , Manufacturers of the test substances: (1) TCI, Tokyo, Japan; (2) Acros, Belgium; (3) Adamas, Belgium; (4) Sinopharm Chemical Reagent Beijing Co., Ltd, Beijing, China; (5) Jianglaishiji, Shanghai, China.

(Chemical Abstracts Service [CAS] No. 59-14-3) in an ELISA-based test system to measure lymphocyte proliferation, thus eliminating the potential occupational exposure to radioactivity and waste disposal issues in the LLNA.

For both the LLNA and the LLNA:BrdU-ELISA, the mouse is the species of choice and the CBA/JN mouse is the preferred strain recommended in the regulatory guidelines (OECD, 2010a; OECD, 2010b). Meanwhile, the TGs state that "other strains of mouse may be used when sufficient data are generated to demonstrate that significant strain-specific differences in the LLNA:BrdU-ELISA response do not exist" (OECD, 2010b). Adding other strains of mouse in the assay could increase the opportunity of choice for the strains and better meet the actual animal supply conditions in different countries and regions. In China, the availability of CBA/JN mouse is only limited to a few animal suppliers, which makes the mouse difficult to obtain. In contrast, BALB/c mouse is widely commercially available. If BALB/c mouse could be used alternatively in the assay, it would facilitate the LLNA:BrdU-ELISA being used in places where CBA/JN mouse is not available. Further, BALB/c mouse has another virtue for the assay, that is, its fur is white which makes the ear erythema more obvious to observe. In addition, BALB/c mouse have been used in the LLNA or the LLNA:BrdU-ELISA

in some published papers (Auttachot, Germolec, Smith, White, & Guo, 2011; Guo et al., 2013; Guoqun et al., 2011; Haixiong et al., 2008; Takeyoshi, Yamasaki, Yakabe, Takatsuki, & Kimber, 2001; Ulker, Kaymak, & Karakaya, 2014; Xiaoyan et al., 2013.). Based on the above facts, BALB/c mouse is considered to be used in the LLNA:BrdU-ELISA as an alternative to CBA/JN mouse.

According to the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (OECD, 2005), modified test methods which make changes in factors such as numbers of laboratory animals used, feasibility of the proposed revisions, cost and time effectiveness and laboratory capacity, etc., should be evaluated by using the Performance Standards (ICCVAM, 2009) before the adoption of such changes. So, the feasibility of using BALB/c mouse in the LLNA:BrdU-ELISA could only be evaluated by validation studies conforming to these demands (ICCVAM, 2009). At present, the available data (Angers-Loustau, Tosti, & Casati, 2011; Takeyoshi, Noda, & Yamasaki, 2004) could not sufficiently indicate if significant differences in the LLNA:BrdU-ELISA response between using BALB/c mouse and using CBA/JN mouse does not exist. As a result, a formal validation study was conducted in our laboratory to determine if BALB/c mouse could also be used in the LLNA:BrdU-ELISA.

Table 2

The test groups and vehicle groups in the 4 experiments.

Experiment 1	Experiment 2	Experiment 3	Experiment 4
AOO (veh.)	AOO (veh.)	AOO (veh.)	AOO (veh.)
AOO (veh.)	AOO (veh.)	AOO (veh.)	AOO (veh.)
2,4-Dinitrochlorobenzene	2,4-Dinitrochlorobenzene	2,4-Dinitrochlorobenzene	2,4-Dinitrochlorobenzene
2,4-Dinitrochlorobenzene	2,4-Dinitrochlorobenzene	2,4-Dinitrochlorobenzene	2,4-Dinitrochlorobenzene
Hexyl cinnamic aldehyde	Hexyl cinnamic aldehyde	Hexyl cinnamic aldehyde	Hexyl cinnamic aldehyde
Hexyl cinnamic aldehyde	Hexyl cinnamic aldehyde	Hexyl cinnamic aldehyde	Hexyl cinnamic aldehyde
Eugenol	Eugenol	Eugenol	Eugenol
p-Benzoquinone	Citral	Diethyl phthalate	Salicylic acid
Diphenylcyclopropenone	Phenyl benzoate	Dimethyl isophthalate	Propylene glycol
Glutaraldehyde	Cinnamic alcohol	2-Hydroxypropyl methacrylate	DMF (veh.)
4-Phenylenediamine	Cyclamen aldehyde	Isopropanol	DMF (veh.)
trans-Cinnamaldehyde	Hydroxycitronellal	Methyl salicylate	5-Chloro-2-methyl-4-isothiazolin-3-one
Isoeugenol	Ethylene glycol dimethacrylate	DMSO (veh.)	4-Methylaminophenol sulfate
Cinnamic aldehyde	Linalool	Cobalt chloride	2-Mercaptobenzothiazole
m-Aminophenol	Ethyl acrylate	Nickel sulfate	Sodium lauryl sulfate
Diethyl maleate	Isopropyl myristate	Lactic acid	Imidazolidinyl urea
Trimellitic anhydride	Aniline	ACE (veh.)	Glycerol
4-Chloroaniline	Hexane	Formaldehyde	Sulfanilamide

2. Materials and methods

2.1. Chemicals

Forty-three reference substances including 32 LLNA sensitizers and 11 LLNA non-sensitizers were tested in this validation study (Table 1). The 43 substances were the same as that used in the ICCVAM (Inter-agency Coordinating Committee on the Validation of Alternative Methods) formal validation study for the LLNA:BrdU-ELISA using CBA/JN mouse (ICCVAM, 2010a). Each of the 43 test substances was tested at only one concentration (conc.), which was the highest concentration tested in the ICCVAM formal validation study for the LLNA:BrdU-ELISA except that 2,4-dinitrochlorobenzene (1%), glutaraldehyde (1%), hexyl cinnamic aldehyde (25%) and eugenol (25%) were tested at a lower concentration. Each of the substances was dissolved in the same vehicles (veh.) as in the ICCVAM formal validation study for the LLNA:BrdU-ELISA (ICCVAM, 2010b) including AOO (acetone: olive oil (4:1)), *N,N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and acetone (ACE). BrdU (C-22859, Arcos, Belgium) was dissolved in physiological saline at a concentration of 10 mg/ml. BrdU ELISA kit (Catalogue Number 11 647 229 001) was supplied by Roche Applied Science, Mannheim, Germany.

2.2. Animals

Special pathogen-free female BALB/c mice of 8–10 weeks old were obtained from Beijing HFK Bio-Technology Co., Ltd. (Beijing China, Permit No. SCXK(Jing)2009-0007). The mice were housed in animal rooms maintained at a temperature of 20–23 °C and a relative humidity of 40–55%. The rooms were ventilated at a frequency of 10–15 cycles/h and lighted artificially for 10 h daily. The animals were allowed free access to a laboratory diet (Beijing Keao Xieli Feed Ltd., Beijing, China. Permit No. SCXK(Jing)2009-0012) and tap water. All procedures regarding the use and handling of animals were reviewed and approved by the Animal Care and Use Committee of the Chinese Center for Disease Control and Prevention.

2.3. Test designs and procedures

In this validation study, 4 experiments were conducted sequentially. Each experiment includes vehicle control (VC) groups, two 2,4-dinitrochlorobenzene groups, two hexyl cinnamic aldehyde groups, one eugenol group and other test substance groups (Table 2). For each experiment, mice were randomly allocated to groups (four mice per group). The test procedures conform completely to the procedures and demands in OECD TG 442B (OECD, 2010a) and ICCVAM

Recommended Test Method Protocol (ICCVAM, 2010c). The test procedures were performed mainly as the following: 25 µl of the appropriate dilution of the test substance or the vehicle alone was applied to the dorsum of both ears daily for 3 consecutive days; a single intraperitoneal injection 0.5 ml (5 mg/mouse) of BrdU (10 mg/ml) solution was given on day 5. On day 6, a pair of auricular lymph nodes from each mouse was excised, weighed and stored at –20 °C until BrdU ELISA analysis to measure the level of BrdU incorporation (ICCVAM, 2010c).

2.4. ELISA for BrdU incorporation

The incorporation of BrdU into lymph node cells (LNC) was measured using a commercial cell proliferation assay kit (Roche Applied Science, Mannheim, Germany, Catalogue Number 11 647 229 001). The test procedures were done mainly as the following (ICCVAM, 2010c). First, the bilateral auricular lymph nodes of each animal were crushed, passed through a #70 nylon mesh, and LNC were suspended in 20 ml of physiological saline. Then, 100 µl of the cell suspension was added to the wells of a flat-bottomed microplate (Lot 112640; Thermo Scientific, Roskilde, Denmark) in triplicate. Simultaneously, 100 µl of physiological saline was added to the blank wells. After centrifugation (300g, 10 min), 75 µl supernatants were removed from each well by suction and then the cells were dried at 60 °C for about 1 h. Then, 200 µl of FixDenat solution was added to each well, and the plate was allowed to stand for 30 min at room temperature. After removing the FixDenat solution thoroughly by flicking off and tapping, 100 µl of diluted anti-BrdU antibody solution was added to each well and incubated at room temperature for 90 min. Antibody conjugates were removed by flicking off and rinsing the wells three times with washing buffer. After removing the washing buffer by tapping, 100 µl of the substrate solution was added. Absorbance (ABS) at 370 nm with a reference wavelength of 492 nm was determined with an EPOCH microplate reader (BioTek Instruments Inc., Highland Park, Winooski, VT, USA. Serial #130325B) at 10, 15, and 20 min later. The optimal result achieving a mean absorbance of the VC group within 0.1–0.2 was used.

2.5. Calculation of results

BrdU labelling index is defined as

$$\text{BrdU labelling index} = (\text{ABS}_{370} - \text{ABS blank}_{370}) - (\text{ABS}_{492} - \text{ABS blank}_{492})$$

Results for each treatment group are expressed as the mean stimulation index (SI). The SI is derived from dividing the mean BrdU labelling

index/mouse within each test substance group by the mean BrdU labeling index for the solvent/VC group. The average SI for the VCs is one. A positive response is defined as the mean SI of the test group ≥ 1.6 .

Based on the positive and negative responses results, the accuracy, sensitivity, specificity, false positive rate, false negative rate, positive predictivity value and negative predictivity value of this study, which could indicate the performance of the LLNA:BrdU-ELISA using BALB/c mouse, were calculated and compared with that of the LLNA:BrdU-ELISA using CBA/JN mouse in the ICCVAM formal validation study.

2.6. Statistical analyses

The accuracy, sensitivity, specificity, false positive rate, false negative rate, positive predictivity values and negative predictivity values in this validation study were compared with that in the ICCVAM formal validation study for the LLNA:BrdU-ELISA using CBA/JN mouse (ICCVAM, 2010d). Chi-square test was used with $P < 0.05$ as the level of significant difference. Statistical Product and Service Solutions (SPSS) 19.0 was used for the statistical test.

Table 3

The results of LLNA:BrdU-ELISA using BALB/c mice in this study and that of LLNA:BrdU-ELISA using CBA/JN mice in the ICCVAM formal validation study.

Substance name	ICCVAM results: ^a LLNA:BrdU-ELISA in CBA/JN mice		This study results: LLNA:BrdU-ELISA in BALB/c mice	
	Mean SI	Identified	Mean SI	Identified
32 LLNA sensitizers				
0.5% 5-Chloro-2-methyl-4-isothiazolin-3-one	4.83	+	12.28	+
1% <i>p</i> -benzoquinone	6.90	+	4.37	+
1% 2,4-Dinitrochlorobenzene	4.83/5.50/8.37/4.30/ 16.94/12.98 mean: 8.82	+	2.11/8.77/3.23/4.67/3.92/4.00/2.19/4.33 mean: 4.15	+
10% Diphenylcyclopropanone	11.61/9.34	+	5.42	+
1% Glutaraldehyde	2.25/3.72/28.60	+	2.78	+
10% 4-Phenylenediamine	14.70	+	5.63	+
10% Formaldehyde	16.59/4.4/1.97	+	3.0	+
5% Cobalt chloride	3.68	+	3.29	+
10% 4-Methylaminophenol sulfate	3.98	+	3.43	+
10% trans-Cinnamaldehyde	4.11/3.50/3.37/5.86 mean: 4.21	+	2.73	+
10% Isoeugenol	2.36/2.43/7.20/8.36 mean: 5.09	+	3.24	+
12.5% 2-Mercaptobenzothiazole	1.62	+	2.14	+
50% Cinnamic aldehyde	3.97	+	3.00	+
25% <i>m</i> -Aminophenol	3.06	+	2.40	+
25% Diethyl maleate	6.27	+	2.46	+
10% Trimellitic anhydride	7.85	+	4.23	+
30% Nickel sulfate	4.53/2.58/2.66 mean: 3.26	+	3.06	+
25% 4-Chloroaniline	2.53	+	Not available (NA)	NA
16.7% Sodium lauryl sulfate	2.64	+	1.78	+
50% Citral	16.4	+	5.15	+
25% Hexyl cinnamic aldehyde	2.44/2.41/2.08/2.06/2.59/8.58/2.87/3.05/2.61/1.57 mean: 2.93	+	2.28/3.48/2.62/3.33/2.83/2.75/2.44/2.43 mean: 2.74	+
25% Eugenol	15.15/2.45/2.73/4.18 mean: 6.13	+	2.10/2.28/2.67/2.43 mean: 2.37	+
66.7% Phenyl benzoate	3.37	+	2.31	+
50% Cinnamic alcohol	2.74	+	3.27	+
100% Cyclamen aldehyde	1.97/5.72	+	2.42	+
100% Hydroxycitronellal	1.34/4.78	+	2.83	+
75% Imidazolidinyl urea	2.27	+	2.14	+
100% Ethylene glycol imethacrylate	3.11	+	2.62	+
100% Linalool	1.18/4.7	+	1.83	+
100% Ethyl acrylate	4.95	+	2.67	+
50% Isopropyl myristate	4.19	+	6.67	+
100% Aniline	2.1	+	1.33	—
11 LLNA Non-sensitizers				
100% Hexane	1.76	+	2	+
50% Lactic acid	2.53/1.89/1.80 mean: 2.07	+	1.34	—
50% Diethyl phthalate	0.88	—	0.88	—
50% Dimethyl isophthalate	1.26	—	1.31	—
50% 2-Hydroxypropyl methacrylate	1.13	—	0.75	—
50% Glycerol	1.29	—	1.21	—
50% Isopropanol	2.22/0.98/0.94/1.01/ 1.57/2.04 mean: 1.46	—	0.94	—
50% Methyl salicylate	1.44/1.40/1.43	—	1.50	—
25% Salicylic acid	1.26	—	2.42	+
50% Sulfanilamide	1.26	—	0.71	—
50% Propylene glycol	1.57/0.7	—	2.17	+

Each group has 4 mice. The mean SI of each group was the average SI of the 4 animals. A positive response is defined as the mean SI of the test group ≥ 1.6 . “+” indicates positive response, “—” indicates negative response.

^a , Data from ICCVAM LLNA:BrdU-ELISA Evaluation Report (ICCVAM, 2010b; ICCVAM, 2010e).

Table 4

Comparison of the performance of LLNA:BrdU-ELISA using BALB/c mice with that of using CBA/JN mice.

Comparison	n	Accuracy		Sensitivity		Specificity		False positive rate		False negative rate		Positive predictivity value		Negative predictivity value	
		%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
LLNA:BrdU-ELISA in BALB/c mice	42	90.5	38/42	96.8	30/31	72.7	8/11	27.3	3/11	3.2	1/31	90.9	30/33	88.9	8/9
LLNA:BrdU-ELISA in CBA/JN mice ^a	43	95.3	41/43	100	32/32	82	9/11	18	2/11	0	0/32	94	32/34	100	9/9

Chi-square test, $P > 0.05$. No statistically significant difference exists between LLNA:BrdU-ELISA using BALB/c mouse and LLNA:BrdU-ELISA using CBA/JN mouse regarding the accuracy, sensitivity, specificity, false positive rate, false negative rate, positive predictivity values and negative predictivity values.

^a , Data from ICCVAM LLNA:BrdU-ELISA Evaluation Report (ICCVAM, 2010d).

3. Results

The mean SI of each test group is shown in Table 3. In this validation study for the LLNA:BrdU-ELISA using BALB/c mouse, the ICCVAM recommended decision criterion of $SI \geq 1.6$ to identify substances as potential sensitizers (i.e. a response is identified positive if the mean SI of the test group ≥ 1.6) was used according to OECD TG 442B. The results showed that the data of one sensitizer (i.e. hydroxycitronellal) was not gained because the animals in this group died of toxicity, 30 of the 31 other LLNA sensitizers were identified correctly as positive. Eight of the 11 LLNA non-sensitizers were identified correctly as negative. Generally, the SI values gained in this study using BALB/c mouse were smaller than the SI values gained in the LLNA:BrdU-ELISA using CBA/JN mouse.

No statistically significant differences existed between the LLNA:BrdU-ELISA using BALB/c mouse and the LLNA:BrdU-ELISA using CBA/JN mouse regarding the accuracy, sensitivity, specificity, false positive rate, false negative rate, positive predictivity value and negative predictivity value (Table 4).

4. Discussion

In this study, BALB/c mouse is considered to be used alternatively in the LLNA:BrdU-ELISA based on the reasons stated in the Introduction. The feasibility of using BALB/c mouse in the LLNA:BrdU-ELISA should be evaluated by validation studies conforming to the related demands (ICCVAM, 2009). At present, no such data have been generated. As a result, this validation study was conducted. This study could provide evidence about the performance of the LLNA:BrdU-ELISA using BALB/c mouse based on the following aspects: (1) This study conforms completely to the ICCVAM-recommended performance standards (ICCVAM, 2009) and OECD TG 442B (OECD, 2010a). (2) The same 43 reference substances in the formal validation study for the LLNA:BrdU-ELISA (ICCVAM, 2010a) using CBA/JN mouse were tested in this validation study using BALB/c mouse. (3) The 22 reference substances demanded by the ICCVAM-recommended performance standards (ICCVAM, 2009) for evaluating modified LLNA were among the 43 reference substances. (4) The performance of the LLNA:BrdU-ELISA using BALB/c mouse in this study was compared with that of the LLNA:BrdU-ELISA using CBA/JN mouse in the ICCVAM formal validation study.

The results that the SI values gained in the LLNA:BrdU-ELISA using BALB/c mouse were generally smaller than that using CBA/JN mouse (Table 3), accordant with that of Takeyoshi et al.'s study (Takeyoshi et al., 2004). Mouse strain difference might be the reason but the SI variations might also come from many other factors, of which the differences in preparing the LNC suspension and the many steps of ELISA procedures are the main source of variations in different laboratories. Thus, the smaller SI values gained in this study may relate to inter-laboratory variations. In the ICCVAM formal validation study for the LLNA:BrdU-ELISA using CBA/JN mouse, the coefficient of variation for the EC 1.6 values of seven sensitizers ranged from 31% to 93% (ICCVAM, 2010f).

Although smaller SI values were obtained, the LLNA:BrdU-ELISA using BALB/c mouse in this study has correctly identified most of the sensitizers and non-sensitizers (Table 3). The results in Table 4 show that no significant differences exist between the performance of the LLNA:BrdU-ELISA using BALB/c mouse and that using CBA/JN mouse could support BALB/c mouse be used alternatively in the LLNA:BrdU-ELISA. Furthermore, the eight repeated results for groups receiving 25% hexyl cinnamic aldehyde in this study have shown that the mean SI values were almost the same and were consistent with the mean SI values obtained in CBA/JN mouse groups receiving 25% hexyl cinnamic aldehyde (Table 3). These results have further verified that the test system in BALB/c mouse have gained adequate proficiency. Finally, some LLNA:BrdU-ELISA using BALB/c mouse have been reported, in which the tested sensitizers and non-sensitizers were correctly identified (Auttachot et al., 2011; Guo et al., 2013; Guoqun et al., 2011; Haixiong et al., 2008; Takeyoshi et al., 2001; Ulker et al., 2014; Xiaoyan et al., 2013).

In conclusion, there was no statistical evidence of any differences between the performance of the LLNA:BrdU-ELISA using BALB/c mouse and that of using CBA/JN mouse that could be detected in this validation study. The results indicate that BALB/c mouse could be used alternatively in the LLNA:BrdU-ELISA for the prediction of the skin sensitizing potential of chemicals. It should be pointed out that the lack of statistically significant differences between the performance of the LLNA:BrdU-ELISA using BALB/c mouse and that of using CBA/JN mouse does not necessarily prove that the two models are equivalent. That is because the sample size of this study is small relative to the large number of chemicals to be identified for skin sensitizing potential. Therefore, the results need be confirmed with more test substances in the future. Finally, the inter-laboratory validation studies for the reproducibility of the LLNA:BrdU-ELISA using BALB/c mouse need be conducted to further validate that BALB/c mouse could be used alternatively in the assay.

Conflict of interest

The authors declare that there are no conflict of interest.

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