



Comparison of BALB/c and CBA/J mice for the local lymph node assay using bromodeoxyuridine with flow cytometry (LLNA: BrdU-FCM)



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ARTICLE INFO

Article history:

Received 5 February 2016

Received in revised form

7 July 2016

Accepted 31 October 2016

Available online 2 November 2016

Keywords:

Local lymph node assay

LLNA

BrdU-FCM

Skin sensitization

BALB/c

CBA/J

Flow cytometry

ABSTRACT

The local lymph node assay using 5-bromo-2-deoxyuridine (BrdU) with flow cytometry (LLNA: BrdU-FCM) is a modified LLNA that is used to identify skin sensitizers by counting BrdU-incorporated lymph node cells (LNCs) with flow cytometry. Unlike other LLNA methods (OECD TG 429, 442A and 442B) in which the CBA/J mouse strain is used, LLNA: BrdU-FCM was originally designed to be compatible with BALB/c, a mouse strain that is more widely used in many countries. To justify the substitution of CBA/J for BALB/c, the equivalence of the test results between two strains shall be established prior to the official implementation of LLNA: BrdU-FCM. This study aims to compare the test results of LLNA: BrdU-FCM produced in BALB/c mice with those in CBA/J mice for 18 reference substances, including 13 sensitizers and 5 non-sensitizers, listed in OECD Test Guideline 429. Based on the LLNA: BrdU-FCM test procedure, we selected an appropriate solvent and then performed preliminary tests to determine the non-irritating dose ranges for the main study, which revealed the difference in the irritation responses to 8 of the 18 chemicals between the two strains. In the main study, we measured the changes in the number of total LNCs, which indicated differences in the responses to test chemicals between the two strains. However, the stimulation index obtained with the counts of BrdU-incorporated LNCs with 7-AAD using flow cytometry yielded comparable results and 100% concordance between the BALB/c and CBA/J mouse strains was achieved, suggesting that the performance of LLNA: BrdU-FCM using BALB/c mice was equivalent to that with CBA/J mice.

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1. Introduction

The murine local lymph node assay (LLNA) was introduced as a test method to identify skin sensitizing chemicals that cause allergic contact dermatitis (Kimber et al., 1986). LLNA was adopted by the Organization for Economic Cooperation and Development (OECD) in 2002 (OECD Test Guideline (TG) 429). LLNA provided an alternative to the skin sensitization test method using guinea pigs (OECD TG 406). However, the wide use of LLNA has been hampered due to the employment of radioisotope-labeled ³H-methyl thymidine (thymidine) and resultant difficulty in the disposal of the

radioactive waste in some countries. For this reason, LLNA was modified to replace ³H-methyl thymidine, and the two non-radioisotopic test LLNA methods (LLNA: DA (OECD TG 442A) and LLNA: BrdU-ELISA (OECD TG 442B)) were adopted in 2010. LLNA: DA quantifies the adenosine triphosphate content of lymph nodes and LLNA: BrdU-ELISA measures the incorporation of 5-bromo-2-deoxyuridine (BrdU), an analogue of thymidine, into lymph nodes (OECD TG 442A, 2010b; OECD TG 442B, 2010c) to evaluate LNC proliferation.

LLNA: BrdU-FCM is a newly revised method of LLNA that was originally developed by Jung et al. (2010). LLNA: BrdU-FCM was optimized and validation studies have been completed (Yang et al., 2015; Kim et al., 2016; Ahn et al., 2016). The results of validation studies suggested that the performance of LLNA: BrdU-FCM is

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comparable to those of other LLNAs. Like LLNA: BrdU-ELISA, the LLNA: BrdU-FCM assay replaces radiolabeled thymidine with non-radiolabeled BrdU to quantify LNC proliferation, which occurs in the induction phase of skin sensitization. LLNA: BrdU-ELISA measures the amount of BrdU incorporated into lymph nodes to indirectly quantify lymph node cell proliferation and, therefore, nonspecific addition of BrdU into non-LNCs cannot be excluded. In contrast, LLNA: BrdU-FCM evaluates the proportion of BrdU-incorporated cells, selectively counting live LNCs utilizing 7-aminoactinomycin D (7-AAD) staining for the identification of skin-sensitizing potential.

Furthermore, through employment of multi-color flow cytometry, LLNA: BrdU-FCM could provide additional endpoints that may be instrumental in the characterization of skin sensitizers. For example, the assay may provide information on specific cytokine induction, dendritic cell migration to lymph nodes or T cell differentiation, addressing key events of organ responses presented in adverse outcome pathways (Jung et al., 2012; OECD, 2012; OECD, 2013). In addition, the LLNA: BrdU-FCM assay provides advantages with respect to the 3Rs principle, owing to employment of 2 stages of preliminary tests that can exclude severely irritating doses, minimize pain or distress and, most importantly, reduce the sacrifice of animals by 2–8 animals per chemical (Ahn et al., 2016).

Incidentally, unlike other LLNA methods, the BALB/c strain is employed in LLNA: BrdU-FCM. The original LLNA also used the BALB/c mouse strain but the results of the initial study showed that some substances elicited greater proliferative responses of lymph node cells in CBA mice than in BALB/c mice (Kimber et al., 1986; Kimber and Weisenberger, 1989). Therefore, LLNA was established with CBA mice and it is now recommended as the preferred mouse strain in the OECD test guidelines (Dean et al., 2001; OECD TG 429, 2010a; OECD TG 442A, 2010b; OECD 442B, 2010c; ICCVAM, 2012). According to the LLNA guidelines, however, other mouse strains could be used if there are no remarkable strain-specific differences. Indeed, comparative studies with other strains showed that BALB/c mice exhibit similar level of performance to CBA mice (Woolhiser et al., 2000; Hou et al., 2015). However, in the LLNA: BrdU-FCM assay, the performance of BALB/c and CBA mouse strains has not been compared.

In the present study, we evaluated the performance of CBA/J mice using LLNA: BrdU-FCM to measure the number of lymph node cells, the proportion of BrdU-incorporated cells and stimulation index values with 13 sensitizers and 5 non-sensitizers included in the OECD TG 429 PS (2010a). Also, we assessed the correlation of stimulation indices between BALB/c and CBA/J mice. Two chemicals, 2-mercaptobenzothiazole and methyl methacrylate, which are sensitizers in OECD TG 429 but non-sensitizers in LLNA: BrdU-FCM (Kim et al., 2016; Ahn et al., 2016), were included to identify whether these chemicals are differently classified depending on the mouse strain used.

2. Materials and methods

2.1. Chemicals and materials

Thirteen sensitizers, 5-chloro-2-methyl-4-isothiazolin-3-one (CMI)/2-methyl-4-isothiazolin-3-one (MI), 1-chloro-2,4-dinitrobenzene (DNCB), 4-phenylenediamine (PPD), cobalt chloride (CC), isoeugenol (IE), 2-mercaptobenzothiazole (MBT), citral (CT), hexyl cinnamic aldehyde (HCA), eugenol (EUG), phenyl benzoate (PB), cinnamic alcohol (CA), imidazolidinyl urea (IU) and methyl methacrylate (MMA); five non-sensitizers, chlorobenzene (CB), isopropanol (IP), lactic acid (LA), methyl salicylate (MS) and

salicylic acid (SA); and vehicles used in LLNA were selected (OECD TG 429, 2010a). CMI and MI were mixed, based on Kathon™ CG from Dow chemical company. In brief, the portion of ingredients was 1.15% for CMI, 0.35% for MI, 23.0% for magnesium chloride and water for the rest. Test chemicals, acetone, olive oil, *N,N*-dimethylformamide (DMF), methyl ethyl ketone (MEK), dimethyl sulfoxide (DMSO), magnesium chloride and 5-bromo-2-deoxyuridine (BrdU) were purchased from Sigma-Aldrich (St. Louis, MO, USA). FITC BrdU flow kits and cell strainers (70 µm) were obtained from BD Biosciences (San Jose, CA, USA). Biopsy punches (6 mm) were purchased from Miltex (York, PA, USA). All other reagents were obtained from Sigma-Aldrich and BD Biosciences. Detail information of test chemicals is described in Table 1.

2.2. Animals

Female BALB/c mice (7 weeks old, Specific Pathogen Free) were purchased from Samtako (Osan, Korea) and female CBA/J mice (7 weeks old, Specific Pathogen Free) were obtained from Koatech (Pyeongtaek, Korea). The animals were kept at an animal facility in the Korea Ministry of Food and Drug Safety (Certification Number: 1501MFDS08) in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International Animal Care Policies (Accredited Unit, MFDS: Unit No. 001492). The animals were given access to solid diets (Purina Mills Inc. Seoul, Korea) and sterilized water *ad libitum*. The mice were housed in pathogen-free conditions at 22 ± 1 °C, a relative humidity of $50 \pm 10\%$ and a 12 h of light per day. The mice were acclimated 5 or 7 days prior to the start of the experiment. Mice with a weight that varied more than 20% of the mean weight were not used. Mice with other abnormal observations were also not used. The mice were randomly allocated to the test groups (2 mice per group for preliminary tests and 5 mice per group for the main test), and the average body weight of each group was similar.

2.3. Experimental protocol for preliminary tests

LLNA: BrdU-FCM for the preliminary tests was performed as previously described in Ahn et al. (2016) and based on the LLNA: BrdU-FCM protocol 1.3 provided from the Korean Center for the Validation of Alternative Methods. Test chemicals were dissolved in AOO (acetone: olive oil (4:1, v/v)), DMF, MEK or DMSO to select an appropriate vehicle. A detailed procedure is presented in Fig. 1A. Dissolved test chemicals with maximum solubility were verified no suspensions, layers or precipitation when test chemicals were mixed with appropriate vehicles and leaved 30 min for solid and 5 min for liquid. Doses were determined by performing 2 sets of preliminary tests. The first preliminary test was performed with a single dose at 25% with the negative control. If the maximum solubility of a test chemical was under 25%, a second preliminary test was performed in place of the first preliminary test. Mouse body weights in each group ($N = 2$) were measured on days 1 and 6. Ear thickness was measured at the center of both ears using a thickness gauge on days 1, 3 and 6. The body weight and ear thickness were measured before test solution treatment and sacrifice. Test solutions (25 µl) were topically applied to the back of both ears of each mouse for 3 consecutive days on days 1–3. The mice were sacrificed on day 6 and the central parts of both ears were collected using a 6 mm biopsy punch and weighed. The following observations were regarded as systemic toxicity or severe skin irritation: an erythema score of over 2, a decrease in body weight by more than 5%, an increase in ear weight or thickness by more than 25% on day 6 compared with day 1, or death during the study. The second pre-

Table 1
Test chemical information.

No.	Test chemical	Abb. ^a	CAS No.	Form	Vendor	Purity
1	5-chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one	CMI/MI	26172-55-4/2682-20-4	Liquid	Santa Cruz/Sigma-Aldrich	– ^b
2	1-chloro-2,4-dinitrobenzene	DNCB	97-00-7	Solid	Aldrich	99.4%
3	4-Phenylenediamine	PPD	106-50-3	Solid	Sigma	100.0%
4	Cobalt chloride	CC	7646-79-9	Solid	Sigma-Aldrich	98.4%
5	Isoeugenol	IE	97-54-1	Liquid	Sigma-Aldrich	99.0%
6	2-Mercaptobenzothiazole	MBT	149-30-4	Solid	Aldrich	100.0%
7	Citral	CT	5392-40-5	Liquid	Aldrich	96.5%
8	Hexyl cinnamic aldehyde	HCA	101-86-0	Liquid	Sigma-Aldrich	95.2%
9	Eugenol	EUG	97-53-0	Liquid	Aldrich	99.1%
10	Phenyl benzoate	PB	93-99-2	Solid	Aldrich	99.9%
11	Cinnamic alcohol	CA	104-54-1	Solid	Aldrich	98.3%
12	Imidazolidinyl urea	IU	39236-46-9	Solid	Sigma-Aldrich	91.6% ^c
13	Methyl methacrylate	MMA	80-62-6	Liquid	Sigma-Aldrich	99.9%
14	Chlorobenzene	CB	108-90-7	Liquid	Sigma-Aldrich	99.97%
15	Isopropanol	IP	67-63-0	Liquid	Sigma-Aldrich	99.98%
16	Lactic acid	LA	50-21-5	Liquid	Fluka	90.1%
17	Methyl salicylate	MS	119-36-8	Liquid	Sigma-Aldrich	99.9%
18	Salicylic acid	SA	69-72-7	Solid	Sigma	99.9%

^a Abb., Abbreviations.

^b CMI/MI mixture contained 1.15% CMI and 0.35% MI.

^c An estimated purity of imidazolidinyl urea calculated using nitrogen content.

liminary test was conducted in the same manner as the first preliminary test and used various doses depending on the results of the first test. The selection process of the second test doses is described below. After the evaluation of skin irritation and systemic toxicity in the second test, 3 doses for which the maximum dose does not cause serious irritation or systemic toxicity were selected for the main study. Test solutions were daily prepared before treatment. Preliminary tests are briefly described in Fig. 1B.

The results of the first preliminary test:

- 1) Systemic toxicity or severe irritation: 10%, 5%, 2.5%, 1%, 0.5% or below
- 2) Non-irritation: 100%, 50%

2.4. Experimental protocol for the main study

LLNA: BrdU-FCM for the main study was performed as previously described in Ahn et al. (2016) and based on the LLNA: BrdU-FCM protocol 1.3 provided from the Korean Center for the Validation of Alternative Methods. Mouse body weights in each group (N = 5) were measured on days 1 and 6. Ear thickness was measured at the center of both ears using a thickness gauge on days 1, 3 and 6. The body weight and ear thickness were measured before test solution treatment and sacrifice. A volume of 25 μ l of the test solutions with various concentrations, a vehicle or a positive control (25% HCA in AOO) were topically treated to the back of both ears of mice for 3 consecutive days on days 1–3. After 2 days (on day 5), mice were intraperitoneally injected with 100 μ l of a freshly prepared BrdU solution (20 mg/ml) in PBS (pH 7.4, 1X) and sacri-

1 ml of cold PBS. The auricular lymph nodes were mashed with a spatula to prepare lymph node cells (LNCs). The LNCs stained with a trypan blue solution were counted using a hemocytometer. Test solutions were daily prepared before treatment. The main study procedure is summarized in Fig. 1C.

2.5. Flow cytometry analysis of incorporated BrdU

Incorporated BrdU was analyzed using a FITC BrdU flow kit following the manufacturer's instructions. Briefly, cells (1.5×10^6 /ml) were washed with $1 \times$ perm/wash buffer and centrifuged ($300 \times g$, 7 min) to remove the supernatant. Cells were suspended for fixation and permeabilization using cytofix/cytoperm buffer. After washing, cells were incubated with cytoperm permeabilization buffer plus and then fixed with cytofix/cytoperm buffer again. After washing again, cells were treated with DNase to expose incorporated BrdU for 1 h at 37 °C in a water bath. Then, cells were washed and stained with a FITC-conjugated anti-BrdU antibody for 20 min at room temperature in the dark. After staining, cells were washed and centrifuged to add staining buffer for transfer to an acquisition tube. Next, a 7-AAD solution was added for DNA labeling. Except DNase exposure, every step was performed on ice. Viable LNCs were gated and a total of 10,000 gated cells were analyzed using BD FACS Calibur™ flow cytometry.

2.6. Stimulation index calculation

Stimulation index (SI) values were calculated using the formula is described below.

$$\text{Stimulation Index (SI)} = \frac{\text{BrdU incorporation rates of the test substance group} \times \text{the number of lymph node cells (cells/mL)}}{\text{BrdU incorporation rates of the negative control group} \times \text{the number of lymph node cells (cells/mL)}}$$

ficed after 24 ± 2 h. After sacrifice (on day 6), the central parts of both ears using a 6 mm biopsy punch were collected and weighed. Additionally, on day 6, auricular lymph nodes were collected, weighed and put into a cell strainer on a 6-well plate filled with

If the stimulation index (SI) was 2.7 or above ($SI \geq 2.7$), a chemical was classified as a sensitizer. Chemicals with a stimulation index (SI) below 2.7 ($SI < 2.7$) were classified as non-sensitizers (Kim et al., 2016).

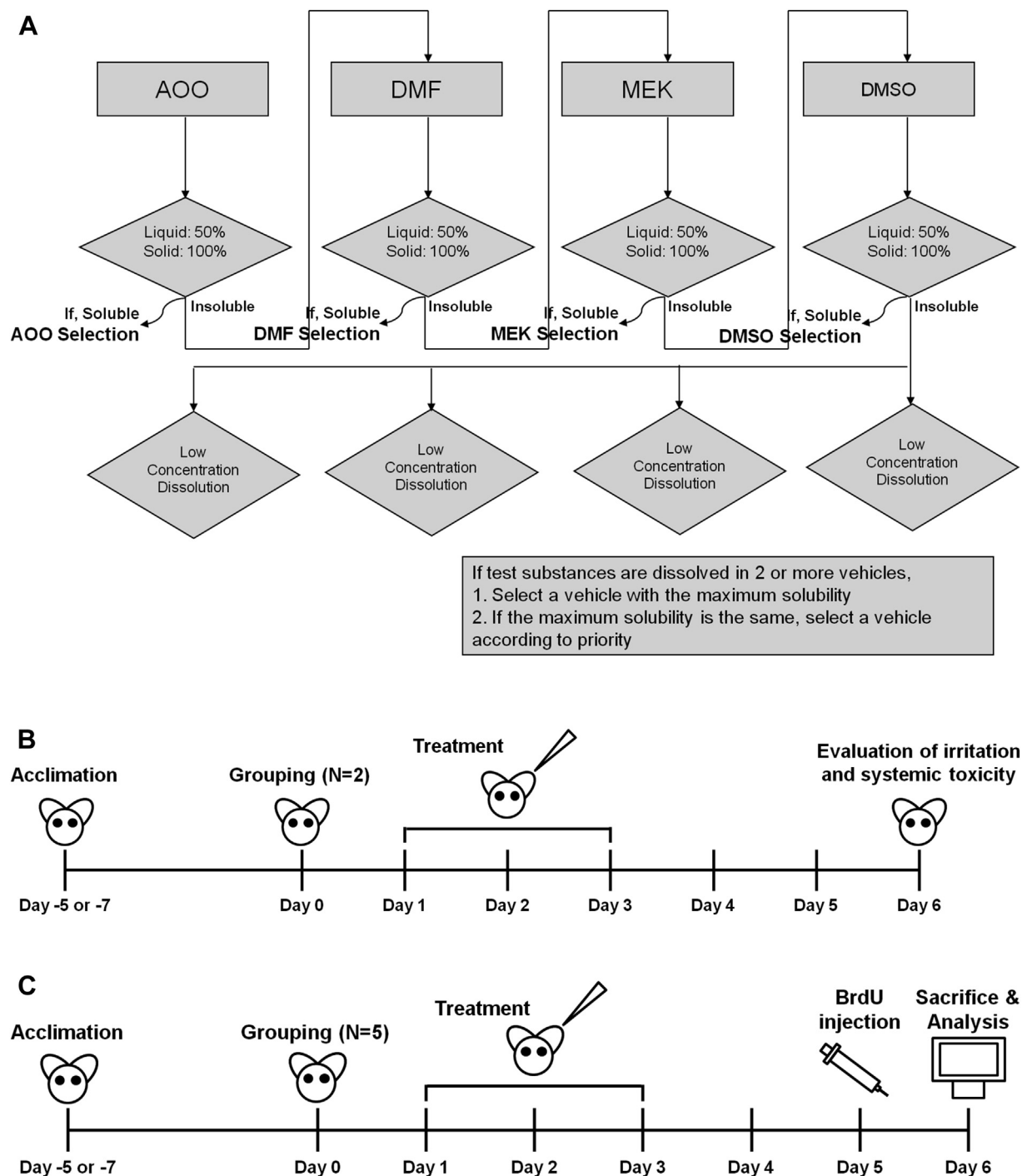


Fig. 1. Schematic diagram for the selection of a vehicle and study designs. (A) A vehicle for each chemical was chosen using a solubility test (AOO→DMF→MEK→DMSO). (B) The first and second preliminary tests were performed following this procedure. (C) The main study proceeded in accordance with this study design.

2.7. Data quotation

The BALB/c mice stimulation index values were from previously published study (Ahn et al., 2016). The number of lymph node cell and percentage of BrdU incorporation, produced from the previous study and unpublished data, were used to compare with CBA/J mice. The previous study was performed with LLNA: BrdU-FCM protocol 1.3. Phenyl benzoate was retested in this study because of different vehicle selection.

2.8. Statistics and others

Statistical analyses of the changes in the number of LNCs, the proportion of BrdU-incorporated LNCs and the correlation of BALB/c with CBA/J mice were performed using Graphpad Prism software (San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant. The EC 2.7 or 3 described an estimated concentration needed to produce a stimulation index of 2.7 or 3. Test chemicals were classified by skin sensitizing potency (ECETOC, 2003).

3. Results

3.1. Results of the preliminary tests for 18 chemicals

To investigate the equivalence between BALB/c and CBA/J in the performance of LLNA: BrdU-FCM, data for 18 reference chemicals with known sensitizing potential were obtained from published studies or produced through conducting experiments in both mouse strains. First, an appropriate solvent and a non-severely irritating dose range for the main study selected through preliminary tests are shown in Table 2. In a vehicle selection test, AOO was chosen as the vehicle for DNCB, IE, CT, HCA, EUG, CA, MMA, CB, IP and MS. DMF was selected as the vehicle for CMI/MI, PPD, CC, MBT, PB, IU, LA and SA. In a dose selection test, the dose ranges of CC, IE, MBT, HCA, EUG, PB, IU, MMA and IP for the two mouse strains were the same. But, the selected doses of CMI/MI, DNCB, PPD, CT, CB, LA, MS and SA for the two mouse strains were different.

3.2. Changes in lymph node cell count

Using the selected dose ranges, the sensitizing potential of 18 chemicals was evaluated with LLNA: BrdU-FCM in BALB/c and CBA/J mice. We weighed both auricular lymph nodes and counted LNCs. Data are shown in Fig. 2. The LNCs significantly increased in both strains treated with 11 sensitizers. When treated with MBT and MMA, LNCs were increased at least one dose in CBA/J mice. For non-sensitizers, chlorobenzene, a significant increase of LNCs showed in CBA/J mice at high dose.

3.3. Analysis of BrdU incorporation in lymph node cells

The percentage of BrdU incorporated LNCs analyzed with flow cytometry are shown in Fig. 3. When 11 chemicals, CMI/MI, DNCB, PPD, CC, IE, CT, HCA, EUG, PB, IU and MS, were treated, the increase in the percentage of BrdU-incorporated LNCs was observed in both mouse strains compared to the vehicle group. After treatment with CB and SA, the BrdU-positive LNC population was increased significantly only in the BALB/c mice as compared to the vehicle

control. In contrast, exposure of CA and LA increased the proportion of BrdU-incorporated LNCs in CBA/J mice. MBT, MMA and IP were not affected BrdU incorporation in the LNCs of both mouse strains.

3.4. Comparison of stimulation index values and predictive capacity in BALB/c and CBA/J

The stimulation index based on the number of LNCs and BrdU incorporation ratio from two strains was shown in Table 3. CMI/MI, DNCB, PPD, CC, IE, CT, HCA, EUG, PB, CA and IU were classified as skin sensitizers, while MBT, MMA, CB, IP, LA, MS and SA were evaluated as non-sensitizers in both mouse strains. EC2.7 values of 18 chemicals between two mouse strains were largely similar (Table 4) although some chemicals, PPD, CT and HCA, showed differences in EC2.7 values between two mouse strains and the sensitization potency was mostly higher in CBA/J than in BALB/c. The test results (sensitizer or non-sensitizer decision) for 18 chemicals indicated that the performance of LLNA: BrdU-FCM was equivalent in BALB/c and CBA/J. The concordance between tLLNA and LLNA: BrdU-FCM using both mouse strains was 16/18 (88.89%) (Table 4). Additionally, the scatter plot indicated that the stimulation index values of BALB/c was significantly and highly correlated with those of CBA/J (r value = 0.7868) (Fig. 4).

4. Discussion

Similar to other LLNA methods including traditional LLNA, LLNA: DA and LLNA: BrdU-ELISA, LLNA: BrdU-FCM was developed to evaluate the potential of skin sensitization by measuring the proliferation of LNCs. LLNA: BrdU-FCM reflects the “Reduce” and “Refine” concepts among the 3Rs principle over guinea pig tests, like other LLNA methods. In particular, the 2-step preliminary tests employed in LLNA: BrdU-FCM could reduce the pain inflicted on animals and minimize the number of animals needed, which is an ethical advantage over the other LLNAs. The performance of LLNA: BrdU-FCM was confirmed through the within- and between-laboratory reproducibility using 2 OECD TG 429 reference sensitizers, DNCB and HCA (Yang et al., 2015), and predictive capacity using 22 OECD TG 429 chemicals (Kim et al., 2016). The reproducibility of LLNA: BrdU-FCM was evaluated using the EC threshold calculation and stimulation index. The results met the acceptance criteria stated in the OECD TG 429 performance standards, and the predictive capacity of the test method demonstrated that 14 of 16 sensitizers and 6 of 6 non-sensitizers were classified correctly (Kim et al., 2016). Recently, new predictive capacity using LLNA: BrdU-FCM protocol 1.3 indicated that 11 of 13 sensitizers and 5 of 5 non-sensitizers were correctly classified (Ahn et al., 2016). Two chemicals, MBT and MMA, which are reported to be skin sensitizers by LLNA (OECD TG 429, 2010a), were classified wrongly as non-skin sensitizers in the LLNA: BrdU-FCM using both mouse strains. However, conflicting results are being produced for these two sensitizers by LLNA methods as reported in several papers, suggesting that MBT and MMA have low skin sensitizing potential and accordingly may be often misclassified as non-sensitizers (ICCVAM, 2010a; ICCVAM, 2010b; Basketter et al., 2012; Kolle et al., 2013).

LLNA: BrdU-FCM is designed to use BALB/c mice instead of CBA/J mice, which is the recommended mouse strain in the other LLNA methods. For this reason, we performed LLNA: BrdU-FCM using BALB/c and CBA/J strains to evaluate the potential differences in the classification of test chemicals depending on mouse strain used. An appropriate vehicle was selected for each chemical through the vehicle selection test (Table 2). The vehicle selection is an important factor which substantially contributes to the variability of LLNA (Hoffmann, 2015) since testing the same chemical with different

Table 2
Preliminary tests results.

No.	Test chemical	Vehicle ^a	Main study dose selection (%)	
			BALB/c ^{b,c}	CBA/J
1	5-chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one	DMF	2.5, 5, 10	1, 2.5, 5
2	1-chloro-2,4-dinitrobenzene	AOO	0.1, 0.25, 0.5	0.05, 0.1, 0.25
3	4-Phenylenediamine	DMF	0.5, 1, 2.5	5, 10, 25
4	Cobalt chloride	DMF	0.25, 0.5, 1	0.25, 0.5, 1
5	Isoeugenol	AOO	5, 10, 25	5, 10, 25
6	2-Mercaptobenzothiazole	DMF	5, 10, 25	5, 10, 25
7	Citral	AOO	10, 25, 50	2.5, 5, 10
8	Hexyl cinnamic aldehyde	AOO	5, 10, 25	5, 10, 25
9	Eugenol	AOO	5, 10, 25	5, 10, 25
10	Phenyl benzoate	DMF	10, 25, 50	10, 25, 50
11	Cinnamic alcohol	AOO	10, 25, 50	10, 25, 50
12	Imidazolidinyl urea	DMF	10, 25, 50	10, 25, 50
13	Methyl methacrylate	AOO	25, 50, 100	25, 50, 100
14	Chlorobenzene	AOO	10, 25, 50	25, 50, 100
15	Isopropanol	AOO	25, 50, 100	25, 50, 100
16	Lactic acid	DMF	10, 25, 50	1, 2.5, 5
17	Methyl salicylate	AOO	10, 25, 50	25, 50, 100
18	Salicylic acid	DMF	1, 2.5, 5	0.1, 0.25, 0.5

^a DMF, N,N-dimethylformamide; AOO, acetone: olive oil (4:1, v/v).

^b Data taken from Ahn et al., 2016.

^c Phenyl benzoate was retested.

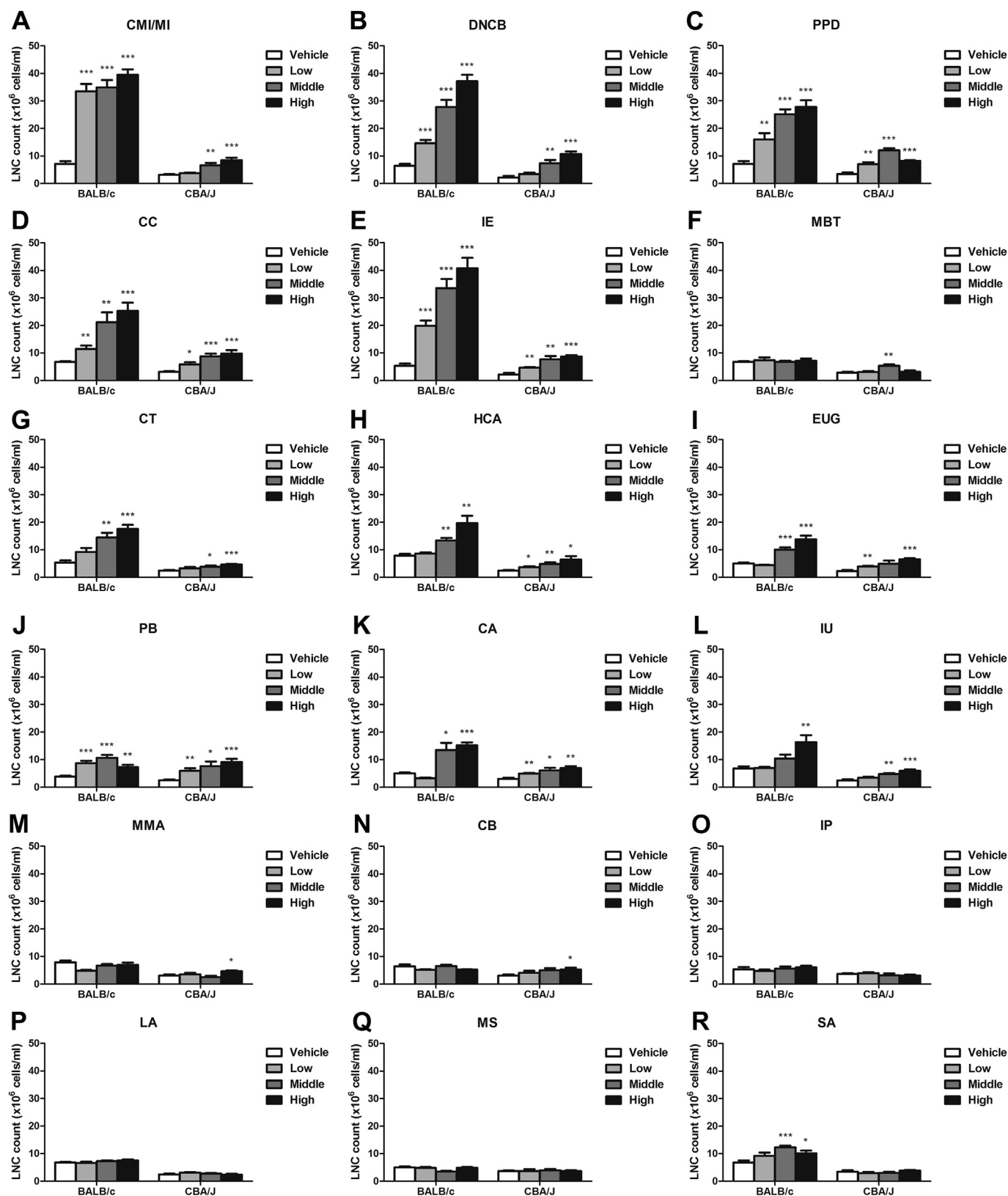


Fig. 2. Changes in the number of LNCs for 18 test chemicals. As shown in (A) to (R), each chemical was topically applied to the dorsum of the ears of the mice on days 1–3. After treatment, auricular lymph nodes were collected on day 6. The auricular lymph nodes were meshed and prepared LNCs. LNCs were stained with a trypan blue solution and counted by hemocytometer. BALB/c LNC count data previously produced but unpublished in [Ahn et al. \(2016\)](#) were used to compare with CBA/J mice. Values are the mean $\pm$ S.D from five mice per group (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus vehicle control; Student's *t*-test). S.D, standard deviation.

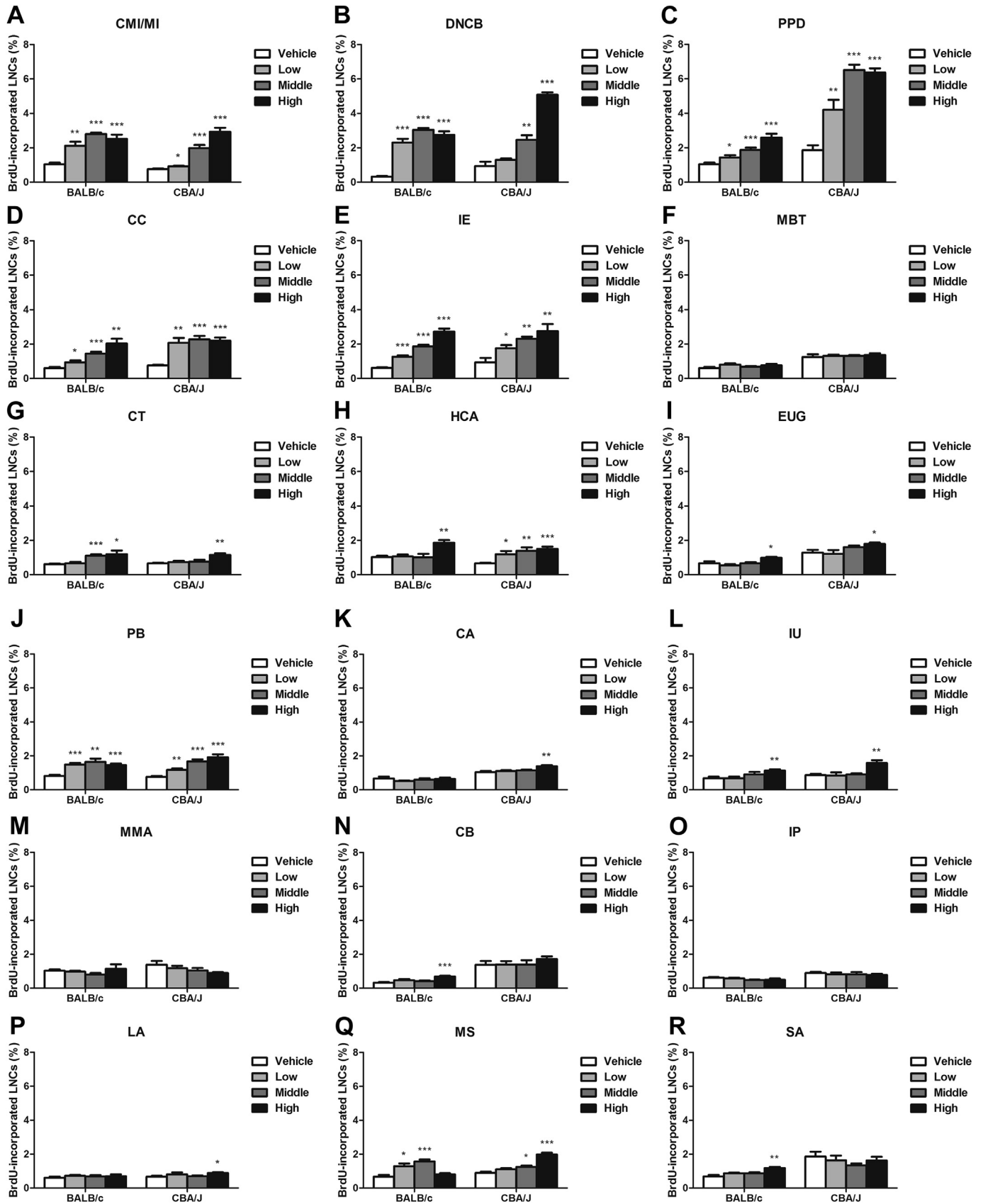


Fig. 3. Analysis of BrdU-incorporated lymph node cells using flow cytometry. As shown in (A) to (R), each chemical was topically applied to the dorsum of the ears of the mice on days 1–3. Mice were intraperitoneally injected with BrdU solution on day 5. After injection, auricular lymph nodes were collected on day 6. The LNCs derived from the auricular lymph nodes were analyzed by flow cytometry using FITC-conjugated anti-BrdU antibodies and 7-AAD. BALB/c BrdU incorporation data previously produced in [Ahn et al. \(2016\)](#) but unpublished were used to compare with CBA/J mice. Values are the mean $\pm$ S.D from five mice per group (* p < 0.05, ** p < 0.01 and *** p < 0.001 versus vehicle control; Student's t -test). S.D, standard deviation.

Table 3
Comparison of stimulation index values in BALB/c and CBA/J.

No.	Test chemical	BALB/c ^{a,b}				CBA/J			
		Dose (%)		SI (mean ± standard deviation)		Dose (%)		SI (mean ± standard deviation)	
		V ^c	L ^c	M ^c	H ^c	PC ^c	Max SI	N/S ^d	Max SI
1	CMI/MI	2.5, 5, 10	1.00 ± 0.32	9.99 ± 3.45	13.42 ± 1.85	13.55 ± 1.77	6.24 ± 1.44	13.55	S
2	DNCB	0.1, 0.25, 0.5	1.00 ± 0.39	15.57 ± 4.25	38.97 ± 6.12	47.29 ± 9.45	9.01 ± 1.75	47.29	S
3	PPD	0.5, 1, 2.5	1.00 ± 0.32	3.24 ± 1.34	6.48 ± 1.48	10.02 ± 3.26	6.24 ± 1.44	10.02	S
4	CC	0.25, 0.5, 1	1.00 ± 0.25	2.60 ± 1.00	7.80 ± 4.06	12.83 ± 6.03	11.86 ± 3.15	12.83	S
5	IE	10, 25	1.00 ± 0.32	7.66 ± 1.33	19.30 ± 4.37	34.91 ± 11.73	5.42 ± 1.48	34.91	S
6	MBT	5, 10, 25	1.00 ± 0.25	1.44 ± 0.58	1.13 ± 0.27	1.30 ± 0.50	11.86 ± 3.15	1.44	N
7	CT	10, 25, 50	1.00 ± 0.32	1.98 ± 1.02	5.05 ± 1.85	8.88 ± 4.43	5.42 ± 1.48	8.88	S
8	HCA	5, 10, 25	1.00 ± 0.27	1.71 ± 0.40	1.75 ± 0.95	4.34 ± 0.74	6.50 ± 3.78	4.34	S
9	EUG	5, 10, 25	1.00 ± 0.38	0.71 ± 0.19	2.01 ± 0.75	3.94 ± 0.74	3.89 ± 3.28	3.94	S
10	PB	10, 25, 50	1.00 ± 0.29	4.06 ± 1.39	5.64 ± 2.28	3.19 ± 0.50	3.06 ± 0.79	5.64	S
11	CA	10, 25, 50	1.00 ± 0.38	0.49 ± 0.15	2.29 ± 0.89	2.78 ± 0.65	3.89 ± 3.28	2.78	S
12	IU	10, 25, 50	1.00 ± 0.35	1.05 ± 0.37	2.11 ± 1.04	4.10 ± 1.52	12.27 ± 4.27	4.10	S
13	MMA	25, 50, 100	1.00 ± 0.27	0.57 ± 0.10	0.65 ± 0.19	0.87 ± 0.23	6.50 ± 3.78	0.87	N
14	CB	10, 25, 50	1.00 ± 0.39	1.12 ± 0.49	1.25 ± 0.36	1.67 ± 0.23	9.01 ± 1.75	1.67	N
15	IP	25, 50, 100	1.00 ± 0.32	0.86 ± 0.29	0.83 ± 0.24	0.89 ± 0.27	5.42 ± 1.48	0.89	N
16	LA	10, 25, 50	1.00 ± 0.25	1.15 ± 0.32	1.18 ± 0.16	1.28 ± 0.50	11.86 ± 3.15	1.28	N
17	MS	10, 25, 50	1.00 ± 0.38	1.77 ± 0.33	1.61 ± 0.52	1.14 ± 0.27	3.89 ± 3.28	1.77	N
18	SA	1, 2.5, 5	1.00 ± 0.35	1.76 ± 0.49	2.26 ± 0.33	2.57 ± 0.22	12.27 ± 4.27	2.57	N

^a Data taken from Ahn et al., 2016.

^b Phenyl benzoate was retested.

^c V, vehicle control; L, low dose; M, middle dose; H, high dose; PC, positive control.

^d N, Non-sensitizer; S, Sensitizer.

vehicles often under- or overestimates the sensitization potency. Unlike the traditional LLNA, DMF was selected as a vehicle for PPD, PB and SA instead of AOO and CC and LA instead of DMSO (Table 4). However, the different vehicle selection in our study did not affect the results demonstrating that our vehicle selection procedure may be appropriate. We performed 2-sets of preliminary tests to find the range of maximum non-irritating doses to be used in the main test. Selected doses of CC, IE, MBT, HCA, EUG, PB, CA, IU, MMA, IP and MS in BALB/c and CBA/J mice were the same. However, the doses of CMI/MI, DNCB, PPD, CT, CB, LA, MS and SA were different between the two mouse strains, reflecting that there may be strain-difference in the reactivity of irritancy towards these chemicals (Table 2). Interestingly, although the non-irritating doses for main study were different for the two mouse strains, the decision results were not affected.

In the main test, in compliance with the LLNA: BrdU-FCM protocol 1.3 (Ahn et al., 2016), the number of lymph node cells were counted, BrdU incorporation analyzed and stimulation index values were calculated. There were no meaningful differences in the number of LNCs in two mouse strains (Fig. 2). There were differences in the responses to some test chemicals, but in regard to the final classification of the sensitizers, these were insignificant. BALB/c and CBA/J mice presented comparable levels of BrdU incorporation at each dose (Fig. 3). In our opinion, there was no remarkable difference in two mouse strains, which could significantly affect the final classification results. Supporting this, although SI values of each chemical were different in both mouse strains, agreement in the classification for 18 test chemicals between those strains was 100% (18/18) (Tables 3 and 4). Moreover, SI values from two mouse strains were significantly correlated (r value = 0.7868 and $p < 0.0001$) (Fig. 4) and the agreement with tLLNA of LLNA: BrdU-FCM was 88.89% for both mouse strains (16/18). Collectively, we could not observe meaningful strain-differences in the skin sensitization-related changes, including the number of LNCs and the LNC proliferation. Overall, we can conclude that the performance of LLNA: BrdU-FCM with BALB/c is comparable to that with CBA/J.

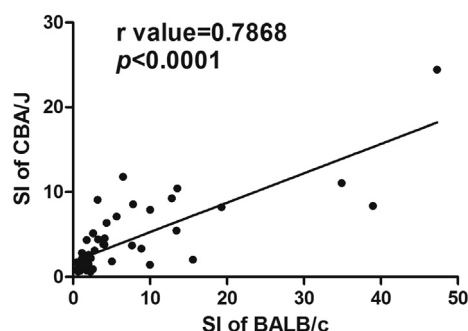
There are several studies that corroborate our findings. Woolhiser et al. (2000) compared the skin sensitizing potential of six mouse strains, including C57BL/6, SJL/J, BALB/c, CBA, DBA/2 and B6C3F1, with three chemicals employing the original radioisotopic LLNA method. The study results showed that BALB/c, B6C3F1 and DBA/2 mice had performance equal or similar to CBA/J mice in regard to the stimulation index and proliferation response, supporting that BALB/c, B6C3F1 and DBA/2 could also be used in LLNA. Hou et al. (2015) also analyzed the SI with 43 substances in LLNA: BrdU-ELISA using BALB/c mice, compared with ICCVAM results. Their analysis demonstrated that the BALB/c mouse strain represented a potential alternative mouse strain because there was no significant difference in the results compared with CBA/JN mice. Additionally, it has been reported in several studies that the skin sensitization potential of test chemicals was correctly determined in LLNA: BrdU-ELISA using BALB/c mice (Takeyoshi et al., 2001; Auttachoat et al., 2011; Guo et al., 2013; Ulker et al., 2014; Arancioglu et al., 2015).

In conclusion, we examined and compared the performance of LLNA: BrdU-FCM in BALB/c and CBA/J strains with 13 sensitizers and 5 non-sensitizers. Similar level of performances was observed for both strains, and there were no strain-specific results. Furthermore, all of the test chemicals were identically classified in both strains. These findings demonstrated that the results of LLNA: BrdU-FCM using CBA/J mice are comparable to those using BALB/c mice. Additionally, our results suggest that CBA/J mice could also be used as another recommended mouse strain for LLNA: BrdU-FCM.

Table 4

Performance of LLNA: BrdU-FCM using BALB/c and CBA/J mice with traditional LLNA.

No.	Test chemical	Traditional LLNA (OECD TG 429)			LLNA: BrdU-FCM BALB/c ^{a,b}			LLNA: BrdU-FCM CBA/J		
		Vehicle ^c	EC3	Category ^d	Vehicle ^c	EC2.7	Category ^d	Vehicle ^c	EC2.7	Category ^d
1	CMI/MI	DMF	0.009	Extreme	DMF	1.062 ^e	Moderate	DMF	1.45	Moderate
2	DNCB	AOO	0.049	Extreme	AOO	0.016 ^e	Extreme	AOO	0.05	Extreme
3	PPD	AOO	0.11	Strong	DMF	0.101	Strong	DMF	0.04 ^e	Extreme
4	CC	DMSO	0.6	Strong	DMF	0.199	Strong	DMF	0.17 ^e	Strong
5	IE	AOO	1.5	Moderate	AOO	1.198 ^e	Moderate	AOO	3.78 ^e	Moderate
6	MBT	DMF	1.7	Moderate	DMF	—	Negative	DMF	—	Negative
7	CT	AOO	9.2	Moderate	AOO	13.08	Weak	AOO	7.71	Moderate
8	HCA	AOO	9.7	Moderate	AOO	15.11	Weak	AOO	2.39	Moderate
9	EUG	AOO	10.1	Weak	AOO	16.46	Weak	AOO	13.28	Weak
10	PB	AOO	13.6	Weak	DMF	5.537 ^e	Moderate	DMF	9.37 ^e	Moderate
11	CA	AOO	21	Weak	AOO	44.28	Weak	AOO	38.44	Weak
12	IU	DMF	24	Weak	DMF	32.02	Weak	DMF	28.58	Weak
13	MMA	AOO	90	Weak	AOO	—	Negative	AOO	—	Negative
14	CB	AOO	—	Negative	AOO	—	Negative	AOO	—	Negative
15	IP	AOO	—	Negative	AOO	—	Negative	AOO	—	Negative
16	LA	DMSO	—	Negative	DMF	—	Negative	DMF	—	Negative
17	MS	AOO	—	Negative	AOO	—	Negative	AOO	—	Negative
18	SA	AOO	—	Negative	DMF	—	Negative	DMF	—	Negative
Sensitivity					84.6% (11/13)			84.6% (11/13)		
Specificity					100.0% (5/5)			100.0% (5/5)		
Accuracy					88.9% (16/18)			88.9% (16/18)		

^a Data taken from Ahn et al., 2016.^b Phenyl benzoate was retested.^c DMF, *N,N*-dimethylformamide; AOO, acetone: olive oil (4:1, v/v); DMSO, dimethyl sulfoxide.^d ECETOC, 2003.^e Set y-axis = 1 because an EC2.7 value is below 0%.**Fig. 4.** Correlation of stimulation index values in LLNA: BrdU-FCM. SI of BALB/c versus SI of CBA/J when the test chemicals were exposed at a low, middle or high dose (r value = 0.7868 and $p < 0.0001$; $SI_{CBA/J} = 0.35SI_{BALB/c} + 1.82$). R value indicates Pearson's correlation co-efficient.

Acknowledgments

This research was supported by grants (15181MFDS457 and 16181MFDS386) from the Ministry of Food and Drug Safety of Korea in 2015 and 2016. We thank Ku Eun-kyung revising the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.yrtph.2016.10.021>.

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