

Original Article

Development of LLNA:DAE: a new local lymph node assay that includes the elicitation phase, discriminates borderline-positive chemicals, and is useful for cross-sensitization testing

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ABSTRACT — We developed a new local lymph node assay (LLNA) that includes the elicitation phase termed LLNA:DAE for discrimination of borderline-positive chemicals as classified by the LLNA modified by Daicel based on ATP content (LLNA:DA) and for cross-sensitization testing. Although the LLNA:DA method could help identify skin sensitizers, some skin irritants classified as non-sensitizers by the LLNA were classified as borderline positive. In addition, the evaluation for the cross-sensitization potential between chemicals was impossible. In the LLNA:DAE procedure, test group of mice received four applications of chemicals on the dorsum of the right ear for induction and one application on the dorsum of the left ear for elicitation. Control group of mice received one chemical application on the dorsum of the left ear. We evaluated the sensitizing potential by comparing the weights of the lymph nodes from the left ears between the two groups. The results of using the LLNA:DAE method to examine 24 chemicals, which contained borderline-positive chemicals, were consistent with those from the LLNA method, except for nickel chloride (NiCl₂). Two chemical pairs, 2,4-dinitrochlorobenzene (DNCB) with 2,4-dinitrofluorobenzene (DNFB) and hydroquinone (HQ) with p-benzoquinone (p-BQ), showed clear cross-sensitization with each other, while another chemical pair, DNFB with hexylcinnamic aldehyde (HCA) did not. Taken together, our results suggest that the LLNA:DAE method is useful for discriminating borderline-positive chemicals and for determining chemical cross-sensitization.

Key words: LLNA, LLNA:DA, LLNA:DAE, Sensitization, Induction, Elicitation, Lymph node weight, Cross-sensitization

INTRODUCTION

Many chemicals have the potential to cause allergic contact dermatitis, leading to serious health problems for workers in industrial areas. Furthermore, structurally similar chemicals may show cross-sensitization (Goon *et al.*, 2006). Thus, to protect humans from contact dermatitis, it is important to evaluate the skin sensitizing potential of chemicals. The guinea pig maximization test (GPMT; Magnusson and Kligman, 1969) as well as the Buehler assay (BA; Buehler, 1965) has been long used to

assess the sensitizing potential and the cross-sensitizing potential between chemicals. These methods involving the use of guinea pigs were adopted by the Organization for Economic Co-operation and Development (OECD) as test guideline (TG) number 406 (OECD, 1992). More recently, the local lymph node assay (LLNA) was developed and adopted by the OECD as TG 429 (OECD, 2002, 2010b; Kimber *et al.*, 1986, 1989; Basketter *et al.*, 1996; Dean *et al.*, 2001; Gerberick *et al.*, 2000; Haneke *et al.*, 2001; Sailstad *et al.*, 2001). In addition, the LLNA modified by Daicel on based on ATP content

(LLNA:DA) that we developed as a new non-radioisotope (RI) LLNA method was adopted by the OECD as TG 442A (Yamashita *et al.*, 2005; Idehara *et al.*, 2008; OECD, 2010a), and the LLNA:BrdU-ELISA was adopted by the OECD as TG 442B (OECD, 2010c) (Takeyoshi *et al.*, 2001, 2003). The LLNA:DA employs ATP measurement as an index of lymph node cell proliferation, with principles of evaluation similar to those of the LLNA. The reliability and relevance of the LLNA:DA were examined by a committee and have been validated by 17 laboratories belonging to that committee (Omori *et al.*, 2008). These LLNA methods have several advantages over the GPMT/BA, including generation of quantitative data, a reduction in test time, a reduction in the required number of animals, and an improvement in animal welfare (Haneke *et al.*, 2001; Basketter *et al.*, 1996). Thus, the LLNA became the method of first choice (Grindon *et al.*, 2008; Basketter *et al.*, 2009). However, these LLNA methods have given false-positive results for some skin irritants. In addition, especially for the LLNA:DA and LLNA:BrdU-ELISA methods, borderline-positive criteria were defined to avoid false-negative results when those methods were adopted. When the stimulation index was found to range from 1.8 to 2.5 by using the LLNA:DA, the result was classified as borderline positive (OECD, 2010a). Thus, a method was needed to distinguish the borderline-positive chemicals from the skin sensitizers and the non-skin sensitizers.

Recently, several cell-based *in vitro* test methods for skin sensitization have been proposed. A Japanese cosmetic company developed the human Cell Line Activation Test (h-CLAT) (Ashikaga *et al.*, 2006; Sakaguchi *et al.*, 2010; Takenouchi *et al.*, 2013). The endpoint of the h-CLAT is a skin sensitizer-induced change in the expression of a THP-1 cell surface specific marker.

Compared to the previous guinea pig tests, the LLNA, LLNA:DA, LLNA:BrdU-ELISA, and *in vitro* test methods evaluate earlier stages of the skin sensitization process. While the LLNA, LLNA:DA, and LLNA:BrdU-ELISA methods evaluate cell proliferation after migration of a sensitized dendritic skin cell to the draining lymph nodes, the cell-based *in vitro* methods evaluate even earlier stages of skin sensitization. However, because these newly adopted tests did not evaluate the elicitation response, they were unable to assess the potential for cross-sensitization between chemicals. The mouse ear-swelling test (MEST) was able to evaluate the elicitation response by measuring the swelling of the ear, and the MEST was included in the OECD's TG 406 (Gad *et al.*, 1986; Gad, 1994). The MEST evaluated ear thickness and the number of sensitized mice against the total number of mice tested for the

assessment of a potential chemical sensitization. Unfortunately, the sensitivity of the MEST was lower than that of the other OECD TG adopted methods described above (Dunn *et al.*, 1990; Hayes and Meade, 1999).

Thus, the two primary challenges regarding the use of the LLNA:DA method are that some skin irritants could be classified as either positive or borderline positive and that this assay could not evaluate the potential for cross-sensitization among chemicals, similar to the other LLNA methods. Therefore, we aimed to develop a new skin sensitization test method that eliminated these two concerns. Herein, we show the development of a new local lymph node assay that includes the elicitation phase termed the LLNA:DAE method. We validated the LLNA:DAE method by using 24 kinds of chemicals that included substances ranging from typical strong skin sensitizers such as DNCB to weak skin sensitizers such as methyl methacrylate (MM), skin irritants, and chemicals classified as borderline positive by the LLNA:DA. The main purpose of this validation was to evaluate the ability of the LLNA:DAE to discriminate those chemicals classified as borderline positive by the LLNA:DA. We also evaluated the potential of the LLNA:DAE method for use in cross-sensitization testing by using three chemical pairs.

MATERIALS AND METHODS

Animals

Female CBA/J mice (Charles River Japan Inc., Kanagawa, Japan; 7 to 11 weeks old) were used in the experiments, and each test group or control group contained five mice. The temperature and humidity inside the animal holding rooms were maintained at $23 \pm 2^\circ\text{C}$ and $55\% \pm 15\%$, respectively, with each room ventilated at 8-10 cycles per hour. All procedures were performed according to the guidelines of the institutional animal care and use committee of the Drug Safety Testing Center Co., Ltd.

Chemicals

The chemicals and test conditions used in this study are summarized in Table 1. The chemicals listed in the performance standard table, *Recommended Reference Substances for the LLNA PS*, of TG 429 were included in Table 1 except for 5-chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one (CMI/MI). We used CMI instead of CMI/MI. Suitable vehicles and concentrations for testing were determined based on previous reports (Gerberick *et al.*, 2005; Idehara *et al.*, 2008; OECD, 2010b). The sources of chemicals used in this study were as follows; p-phenylenediamine (ppD), MM and lactic

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Table 1. Tested chemicals and their sources, and test condition

Chemical name	CAS No	Source	Test conc.	Vehicle
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	AK Scientific	0.05%	AOO
2,4-Dinitrochlorobenzene	97-00-7	Wako	0.25%	AOO
2,4-Dinitrofluorobenzene	70-38-4	Nacalai	0.25%	AOO
p-Benzoquinone	106-51-4	Wako	1%	AOO
Hydroquinone	123-31-9	Wako	1%	AOO
p-Phenylenediamine	106-50-3	Sigma-Aldrich	2.5%	AOO
Cobalt chloride	7646-79-9	Wako	5%	DMSO
Isoeugenol	7646-79-9	Wako	10%	AOO
2-Mercaptobenzothiazole	149-30-4	Wako	25%	DMSO
Citral	5392-40-5	Wako	25%	AOO
Hexylcinnamic aldehyde	101-86-0	Wako	25%	AOO
Eugenol	97-53-0	Wako	50%	AOO
Cinnamic alcohol	104-54-1	Kanto	25%	AOO
Phenyl benzoate	93-99-2	Wako	10%	AOO
Imidazolidinyl urea	39236-46-09	ICN	40%	DMF
Methyl methacrylate	80-62-6	Sigma-Aldrich	100%	
Ethylene glycol dimethacrylate	97-90-5	Wako	50%	MEK
Nickel chloride	7718-54-9	Wako	10%	DMSO
Chlorobenzene	108-90-9	Wako	25%	AOO
Isopropanol	67-63-0	Wako	50%	AOO
Lactic acid	50-21-5	Sigma-Aldrich	25%	DMSO
Methyl salicylate	119-36-8	Wako	50%	AOO
Salicylic acid	69-72-7	Wako	50%	AOO
n-Hexane	110-54-3	Kanto	100%	
Sodium lauryl sulfate	151-21-3	Sigma	10%	DMF
Xylene	1330-20-7	Wako	100%	

Abbreviations; CAS No.: Chemical Abstracts Service Number, AK Scientific: AK Scientific, Inc., Wako: Wako Pure Chemical Industries, Ltd., Nacalai: Nacalai Tesque Inc., Sigma-Aldrich: Sigma-Aldrich Inc., Kanto: Kanto Chemical Co., Inc., Tokyo, Japan, ICN: ICN Biomedicals Inc., Sigma: Sigma Chemical Co., Test conc.: test concentration, AOO: acetone: olive oil (4:1, v/v), DMF: *N,N*-dimethylformamide, DMSO: dimethyl sulfoxide, MEK: methyl ethyl ketone. Test concentration and suitable vehicle were selected based on publish information.

acid (LA) were purchased from Sigma-Aldrich (Sigma-Aldrich Inc., St. Louis, MO, USA). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, except for CMI (AK Scientific, Inc., Union City, CA, USA), DNFB (Nacalai Tesque Inc., Kyoto, Japan), Imidazolidinyl urea (IU) (ICN Biomedicals Inc., Aurora, OH, USA), n-hexane (hexane) (Kanto Chemical Co., Inc., Tokyo, Japan), sodium lauryl sulfate (SLS) (Sigma Chemical Co., St. Louis, MO, USA) and olive oil (Yoshida Pharmaceutical Co. Ltd., Japan).

Assessment of equivalency of mouse auricular lymph nodes in both ears

Naïve mice were euthanized and both sides of their auricular lymph nodes were excised and were measured to evaluate the average lymph node weight of CBA/J mice ($n = 10$).

Two groups of mice ($n = 5$ each) were used to compare the lymphocyte proliferation levels in the lymph nodes of the right ear with that of the left ear induced by chemical application. The first group of mice was treated

with 25% HCA (25 μ l) in acetone: olive oil (AOO) (4:1, v/v) applied to the dorsum of the right ear while the second group of mice was similarly treated on the dorsum of the left ear. After 48 hr, the auricular lymph node of each ear was excised independently to measure the weight and ATP contents.

Evaluation of the influence of four applications of chemicals to the right ear on the lymph node weight of the left ear

On days 1, 2, 3, and 10, HCA (25%) in AOO, DNFB (0.25%) in AOO, sodium lauryl sulfate (SLS) (10%) in DMF, or xylene (25 μ l) was applied to the dorsum of right ear. Each group consisted of five mice. On day 12, each lymph node was excised. Lymph node weight and ATP content were measured to evaluate the influence of four separate applications of chemicals to the right ear on the lymph node weight of the left ear.

Optimization of the test schedule

The mice were divided into four groups, and each group was treated with HCA (25%) in 25 μ l of AOO using the following application schedule. The first group of mice was treated on days 1, 2, and 3 on the dorsum of the right ear (R3L0); the second group was treated on day 10 on the dorsum of the left ear (R0L1). The third group was treated on days 1, 2, and 3 on the dorsum of the right ear and treated on day 10 on the dorsum of the left ear (R3L1). The fourth group was treated on days 1, 2, and 3 on the dorsum of the right ear and treated on day 10 on the dorsum of both ears (R4L1). The fourth group was used to evaluate the influence of the four applications of skin sensitizer on the right ear to elicit a response on the left ear. On day 12, all groups of mice were euthanized. The draining auricular lymph nodes were excised and weighed, and the ATP content of each lymph node was measured. These procedures are shown in Fig. 1.

Evaluation of a quantitative relationship between the induction dose and the elicitation response

Various concentrations of DNCB (0.06%, 0.13%, and 0.25%) in 25 μ l of AOO were applied to the dorsum of the right ear for each test group of mice on days 1, 2, and 3, and DNCB (0.25%) in AOO was applied to the dorsum of both ears on day 10. The mice in the control group were treated topically on the dorsum of the left ear with DNCB (0.25%) in 25 μ l of AOO on day 10. On day 12, each lymph node was excised and weighed, and the ATP content was measured to evaluate the relationship between the induction dose and the elicitation response.

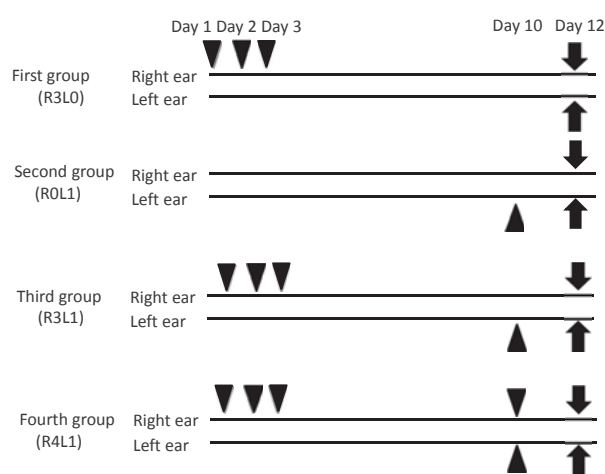


Fig. 1. Study design to optimize the LLNA:DAE method. The mice received an HCA (25%) treatment (triangles) to the dorsum of the indicated ear according to one of four schedules. Arrows indicate excision.

Evaluation of the potential for skin sensitization and cross-sensitization of chemicals

To evaluate the performance of the LLNA:DAE assay, 17 kinds of known skin sensitizers including those from CMI to ethylene glycol dimethacrylate (EG) as listed in Table 1, and 6 kinds of non-sensitizers including those from chlorobenzene (CB) to hexane as listed in Table 1 were used. NiCl_2 was shown to be a skin sensitizer in humans, but it was classified as negative in previous results obtained using the LLNA method. Borderline-positive chemicals as classified by LLNA:DA were included in these 24 chemicals. The test concentrations and the vehicles used for these chemicals are summarized in Table 1. The test groups of mice were treated topically on the dorsum of the right ear with test chemicals on days 1, 2, 3, and 10, and similarly treated on the dorsum of the left ear with test chemicals on day 10 (R4L1). Control mice were treated topically on the dorsum of the left ear with test chemicals on day 10 (R0L1). On day 12, each lymph node was excised and weighed. This procedure is shown in Fig. 2.

To evaluate the cross-sensitizing potential among chemicals, three chemical pairs were used: DNFB with HCA, DNCB with DNFB, and HQ with p-BQ. The test groups of mice were treated topically on the dorsum of the right ear with the induction chemical on days 1, 2, 3, and 10, and were treated topically on the dorsum of the left ear with a challenge chemical on day 10. On day 10, the control group was treated topically on the dorsum of

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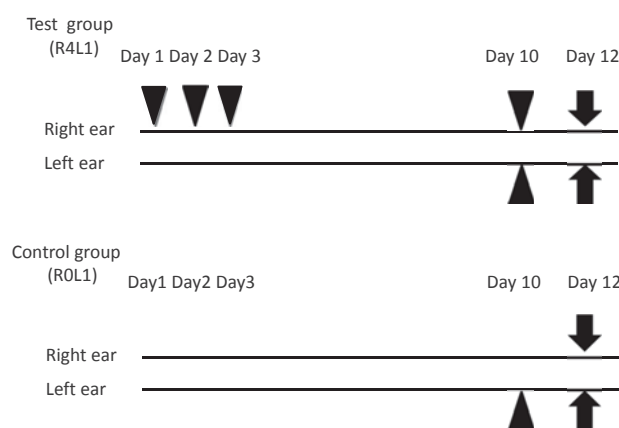


Fig. 2. Study design of the optimized LLNA:DAE method. The test and control mice received chemical treatments (triangles) to the dorsum of the indicated ear as illustrated. All animals were euthanized on day 12 and their lymph nodes were excised and weighed.

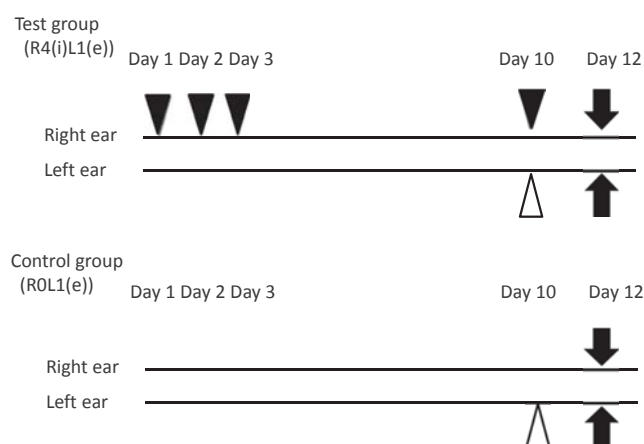


Fig. 3. Study design for evaluation of the cross-sensitization potential between chemicals. Black triangles indicate the treatment of the first type of chemical, and white triangles indicate the treatment of the second type of chemical. All animals were euthanized on day 12 and their lymph nodes were excised and weighed.

the left ear with only the challenge chemical. This procedure is shown in Fig. 3. The application dose was fixed at 25 μ l for all applications.

An elicitation response was defined as positive when the weight of the lymph nodes from the left ear of the test group was greater than that in the control group.

Measurement of lymph node weight and ATP content

On day 12, the mice were euthanized, and each auricular lymph node was excised and weighed. After weighing, the lymph node was placed between 2 glass slides, crushed, and spread. The cells were washed in 1.0 ml of phosphate-buffered saline (PBS) (Life Technologies Corp., Carlsbad, CA, USA) of pH 7.2 and diluted 100-fold with PBS. In all cases, 2 diluted solutions were prepared from each lymph node. ATP content in the diluted lymph node cell suspension was measured using an ATP kit (Check-Lite™ 250Plus, Kikkoman Corporation, Chiba, Japan). The diluted cell suspension (100 μ l) was added to an ATP releasing agent and mixed. After 20 sec, the luciferase reagent (100 μ l) was added and bioluminescence was measured. The ATP content as measured by bioluminescence was indicated in relative light units (Rlu). This process is described in more detail in previous reports (Yamashita *et al.*, 2005; Idehara *et al.*, 2008).

Statistical analysis

Student's *t*-test (Microsoft Excel, USA) was used to

compare lymph node weights and ATP content between two groups. The chemical was considered a skin sensitizer when the lower limit of 95% confidence interval was greater than 0. The 95% confidence interval was determined for the means in the difference between the average weights of the lymph nodes of the left ears in control versus the test groups.

RESULTS

The equivalency of right and left auricular lymph nodes

The average weights of the draining auricular lymph nodes from the right and left ears of naïve mice were 2.3 ± 0.3 mg and 2.2 ± 0.3 mg, respectively, while the average ATP content was $19,027 \pm 5,104$ Rlu and $17,314 \pm 4,982$ Rlu, respectively. The ATP content from the lymph nodes as determined using the Rlu measurement was consistent with the lymph node cell number. There was no statistically significant difference in weight or ATP content between the lymph nodes from the right and left ears in naïve mice. Thus, the draining auricular lymph nodes from the right and left ears of naïve mice are quantitatively equivalent in terms of ATP content and weight.

Two groups of mice were treated with a single exposure of 25% HCA to the right or left ear 48 hr prior to excision in each. The lymph node weight and ATP content of the right ear in the first group were 2.7 ± 0.3 mg and $21,473 \pm 6,186$ Rlu, and for the left ear in the second

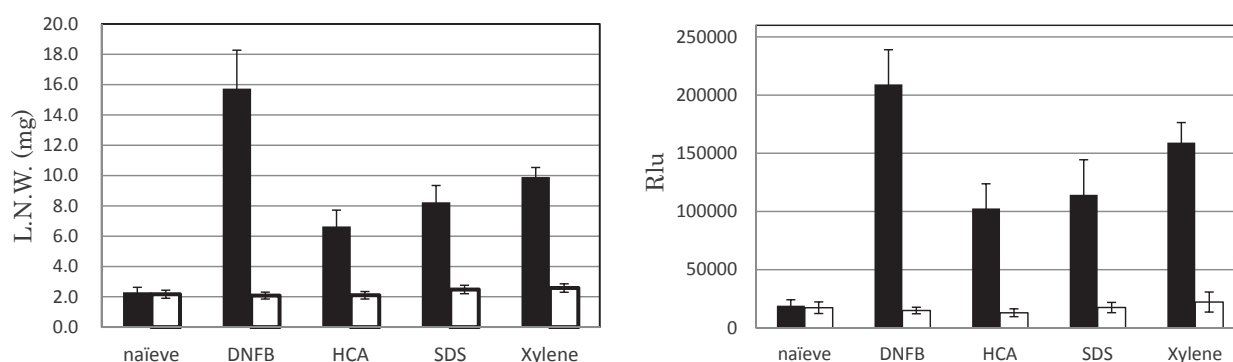


Fig. 4. Lymph node weight (L.N.W.) and ATP content (Rlu) in naïve mice and mice treated with four applications of DNFB (0.25%), HCA (25%), SDS (10%), or xylene (100%) only on the right ear. The black and white columns indicate the values for the right and left ears, respectively.

group were 3.1 ± 0.4 mg and $25,075 \pm 2,056$ Rlu, respectively. There was no statistically significant difference in the values obtained from the right side of the first group or left side of the second group, indicating similar levels of proliferation in the right and left auricular lymph nodes 48 hr after stimulation with 25% HCA.

Evaluation of the influence of four applications of chemicals to the right ear on the lymph node weight of the left ear

Fig. 4 shows the weight of the lymph node and ATP content following four applications of skin sensitizers or skin irritants to the dorsum of the right ear. The skin sensitizers DNFB and HCA induced proliferation of lymphocytes in the lymph node, and this was reflected by an approximately 6.8-fold and 2.9-fold increase in the weight of the lymph nodes from the right ear of the treated mice induced by DNFB and HCA, respectively, compared to that in the naïve mice. Similarly, the skin irritants xylene and SLS induced proliferation of lymphocytes, with the respective increases in the weights of the lymph nodes being approximately 4.3-fold and 3.6-fold. The pattern of results for the ATP content was similar to that for the lymph node weights. For both the skin sensitizers and the skin irritants, no significant lymphocyte proliferation was observed in the draining auricular lymph node of the left ear. Thus, four applications of chemicals to the dorsum of the right ear did not significantly affect the weight of the lymph nodes and ATP content of the left ear.

Optimization of test procedure

Fig. 5 shows the weight of the lymph nodes and ATP content for four groups of mice treated with 25% HCA after a different application procedure was used (for details see Materials and Methods *Optimization of the test schedule*). The weight of the lymph nodes from the right ear of first group (R3L0) was significantly increased compared to the weight of the left ear lymph nodes. The weight of the lymph nodes from the left ear of the second group (R0L1) was slightly increased compared to the weight of the lymph node from the right ear. The weight of the lymph node from the left ear of the third group (R3L1) was significantly increased as compared to the weight of the lymph node from the left ear of the second group of mice. These results suggest that sensitization to 25% HCA was established by at least day 10 and that the lymph node weight of the left ear could be used to evaluate the elicitation response. In addition, the weights of the lymph nodes from the right ears in both the first and third group were similar, suggesting that a single challenge chemical application to the left ear did not affect the lymph node of the opposite ear. The weight of the lymph node from the right ear of the fourth group (R4L1) significantly increased compared to that in the third group (R3L1) with no significant intergroup difference in the weight of the lymph nodes from the left ear. The results indicated that the elicitation response was dependent upon the first three applications on right ear; the fourth chemical application to the right ear did not affect the elicitation response of the left ear. The pattern of results for the ATP contents was similar to that for the lymph node weights.

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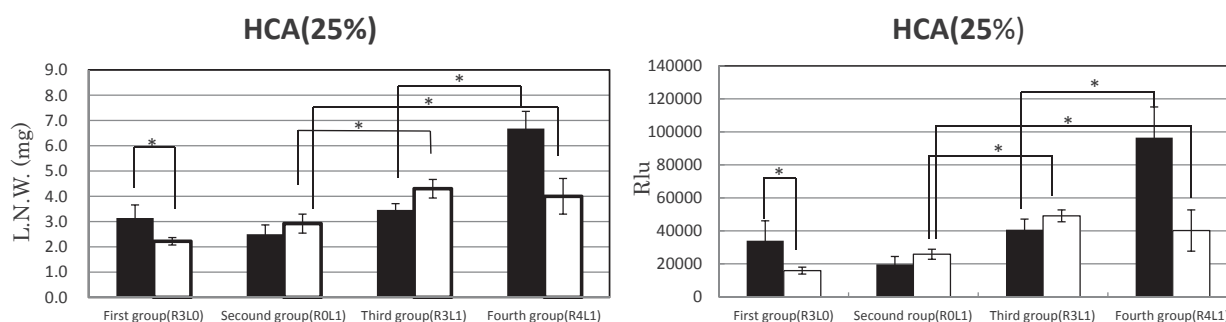


Fig. 5. Lymph node weight (L.N.W.) and ATP content (Rlu) in mice treated with HCA according to the treatment schedules shown in Fig. 1. The black and white columns indicate the values of the right and left ears, respectively. Values are expressed as the mean $\pm$ S.D. ($n = 5$). * represents $P < 0.05$ by the Student's t -test for the differences in lymph node weights between the groups.

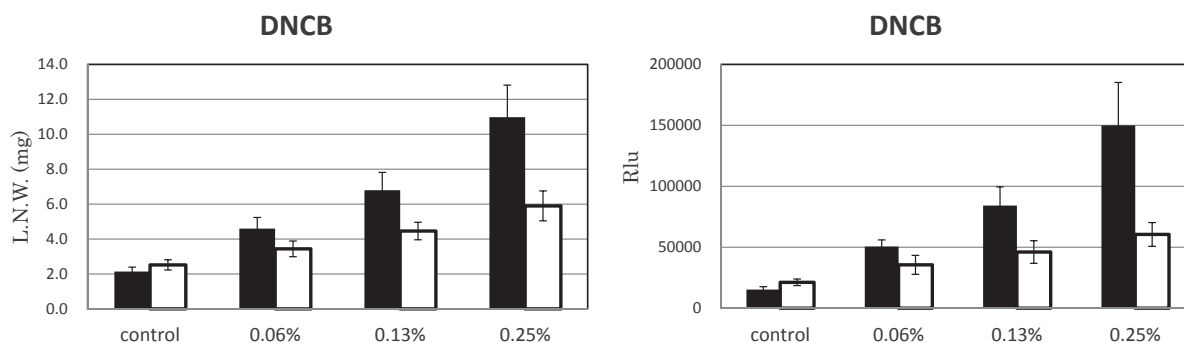


Fig. 6. Lymph node weigh (L.N.W.) and ATP content (Rlu) in DNCB-treated mice. Each test group of mice was treated with 0.06%, 0.13%, or 0.25% DNCB on the right ear on days 1, 2, 3 and was treated with 0.25% DNCB to both ears on day 10. Control group of mice was treated with 0.25% DNCB on the left ear on day10. The lymph node weight and ATP content were measured on day 12. The black and white columns indicate the values for the right and left ears, respectively. The labels of X-axis indicate the concentration of DNCB applied to the right ear.

Quantitative relationship between the induction dose and the elicitation of a response

Fig. 6 shows the weight and ATP content of the auricular lymph nodes from the mice that received various concentrations of DNCB during the induction phase. The weight of the lymph node from both ears was increased in a dose-dependent manner. The difference in the weights of the lymph nodes from the control group and from the three groups with an increasing dose of DNCB for the right ears was 2.5 mg, 4.7 mg, and 8.8 mg, respectively, and the corresponding weights for the left ears were 0.9 mg, 1.9 mg, and 3.4 mg, respectively. The weight of the lymph node from the left ear was increased by 1.5 ± 0.1 mg for every 0.1% concentration increase in the induction dose. And the ATP content of the lymph node

from the left ear increased by $19,577 \pm 4,099$ Rlu every 0.1% concentration increase in the induction dose. These results suggested that three applications of an induction dose of a chemical on right ear could establish a dose-dependent sensitization of the lymph nodes of the left ear.

Evaluation of the skin sensitizing potential of the chemicals

Table 2 and Fig. 7 show the findings for the skin-sensitization potential of the chemicals listed in Table 1 as evaluated using our LLNA:DAE method. In addition, in Table 2, we showed the skin sensitizing potential or potency category of chemicals reported in the literature (Garcia-Perez, 1978; Basketter *et al.*, 1999a; Haneke, *et al.*, 2001; Gerberick *et al.*, 2005; Idehara *et al.*, 2008;

Table 2. Results of 24 chemicals tested by LLNA:DAE method and comparison with other results

Chemical name	LLNA:DAE	LLNA:DA	LLNA potency category	Human
5-Chloro-2-methyl-4-isothiazolin-3-one	+	+ ^a	Extreme ^c	+ ^f
2,4-Dinitrochlorobenzene	+	+ ^a	Extreme ^d	+ ^e
2,4-Dinitrofluorobenzene	+	n.t.	Extreme ^d	+ ^g
p-Benzoquinone	+	+ ^b	Extreme ^d	+ ^h
Hydroquinone	+	n.t.	Strong ^d	+ ^h
p-Phenylenediamine	+	+ ^a	Strong ^d	+ ^e
Cobalt chloride	+	± ^b	Strong ^d	+ ^e
Isoeugenol	+	+ ^a	Moderate ^d	+ ^e
2-Mercaptobenzothiazole	+	± ^b	Moderate ^d	+ ^e
Citral	+	+ ^a	Weak ^d	+ ^e
Hexylcinnamic aldehyde	+	+ ^a	Weak ^d	+ ^e
Eugenol	+	+ ^a	Weak ^d	+ ^e
Cinnamic alcohol	+	+ ^a	Weak ^d	+ ^e
Phenyl benzoate	+	+ ^a	Weak ^d	+ ^e
Imidazolidinyl urea	+	+ ^a	Weak ^d	+ ^e
Methyl methacrylate	+	± ^b	Weak ^d	+ ^e
Ethylene glycol dimethacrylate	+	+ ^a	Weak ^d	+ ^e
Nickel chloride	+	- ^b	- ^e	+ ^e
Chlorobenzene	-	± ^b	- ^d	
Isopropanol	-	± ^b	- ^d	+ ^e
Lactic acid	-	- ^a	- ^d	
Methyl salicylate	-	- ^a	- ^d	- ^e
Salicylic acid	-	± ^b	- ^d	- ^e
n-Hexane	-	± ^b	- ^f	- ^f

Abbreviations: LLNA:DAE: local lymph node assay that includes the elicitation phase; LLNA:DA: LLNA modified by Daicel based on ATP content; LLNA: murine local lymph node assay; + = positive result; - = negative result; ± = borderline positive result; n.t. = not tested. Extreme: Extreme skin sensitizer; Strong: Strong skin sensitizer; Moderate: Moderate skin sensitizer; Weak: Weak skin sensitizer. The results of LLNA:DA had been taken from ^aIdehara *et al.* (2008) and ^bICCVAM publication (2010). The potency categories of LLNA had been taken from the ^cGerberik *et al.* (2005), ^dTakenouchi *et al.* (2013), ^eOECD TG429 (2010b) and ^fHaneke *et al.* (2001). The results of human had been taken from the ^eOECD TG429 (2010b), ^fHaneke *et al.* (2001), ^gA. Garcia-Perez (1978) and ^hBasketter *et al.* (1999a).

ICCVAM, 2010; OECD, 2010b; Takenouchi *et al.*, 2013). The following chemicals induced a statistically significant proliferation of lymphocytes in the lymph node of the left ear of the test group as compared with that of the control group: CMI, DNCB, DNFB, pPD, cobalt chloride (Co), NiCl₂, isoeugenol (IEU), 2-Mercaptobenzothiazole (2-MBT), citral, HCA, eugenol (EU), cinnamic alcohol (CA), phenyl benzoate (PBz), IU, MM, HQ, p-BQ, and ethylene glycol dimethacrylate (EG). Thus, these chemicals were identified as skin sensitizers. In case of CMI, CA, PBz, IU, and MM, the weights of the lymph nodes

from the right and left ears in the control groups were similar, indicating more clearly that the increase in weight of the lymph node from the left ear in the test groups was induced by an immune-specific response. On the other hand, chlorobenzene (CB), Isopropanol (IPA), LA, salicylic acid (SA), methyl salicylate (MS), and hexane were classified as negative by the LLNA method and similarly did not induce a statistically significant increase in the weight of the lymph node from the left ear in our test groups. In these chemicals, Co, 2-MBT, MM, CB, hexane, SA, and IPA gave borderline positive results when tested

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Group A

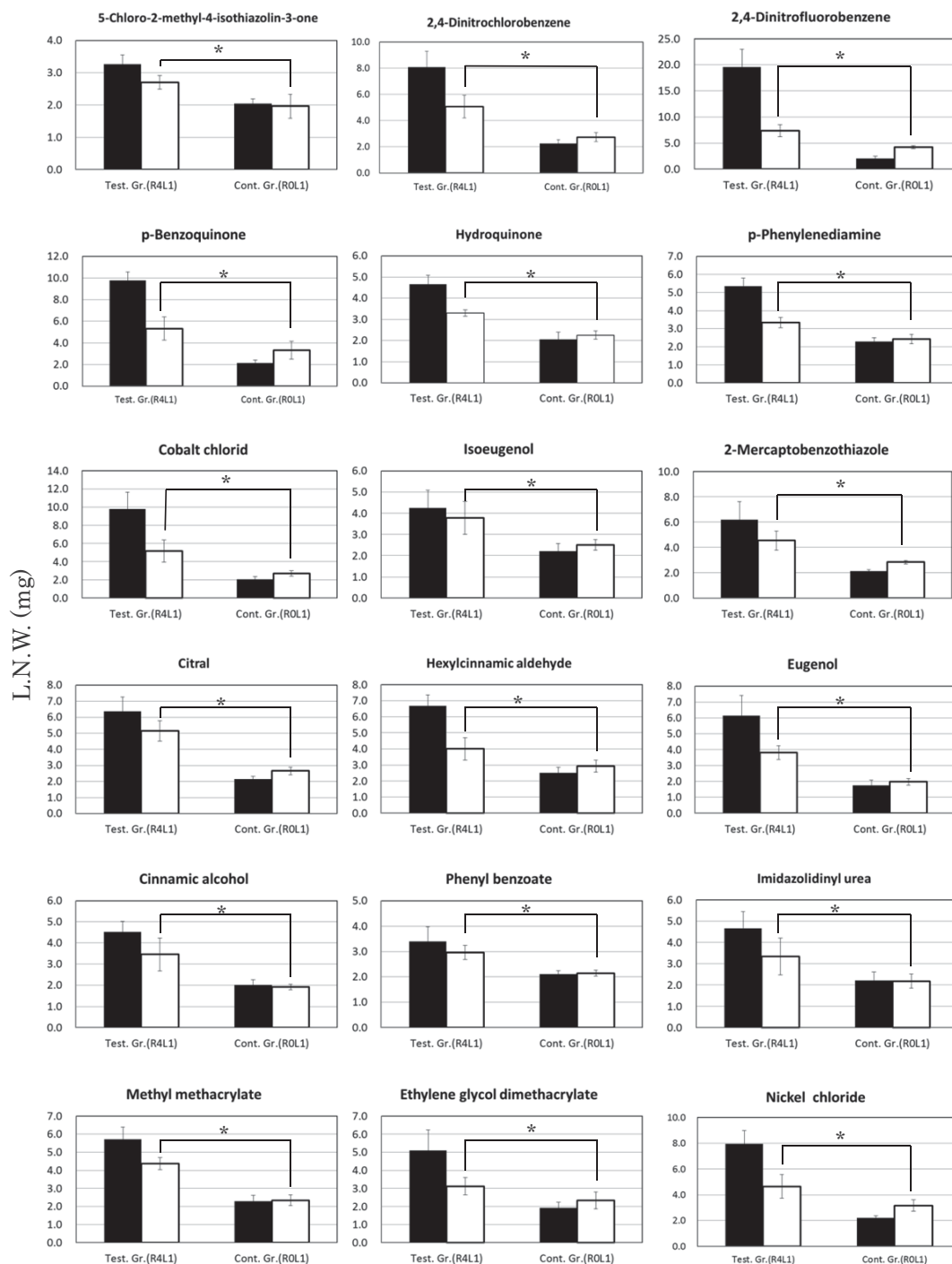


Fig. 7. The results of our LLNA:DAE test using 24 chemicals. The chemicals in Group A were classified as skin sensitizers while those in Group B were classified as non-sensitizers by using our LLNA:DAE method. The black and white columns indicate the values from the right and left ear, respectively. The Y-axis indicates the lymph node weight (L.N.W.). Values are expressed as the mean $\pm$ S.D. ($n = 5$). * represents a statistically significant difference in the average lymph node weights of the left ears between the control group and the test group indicated.

Group B

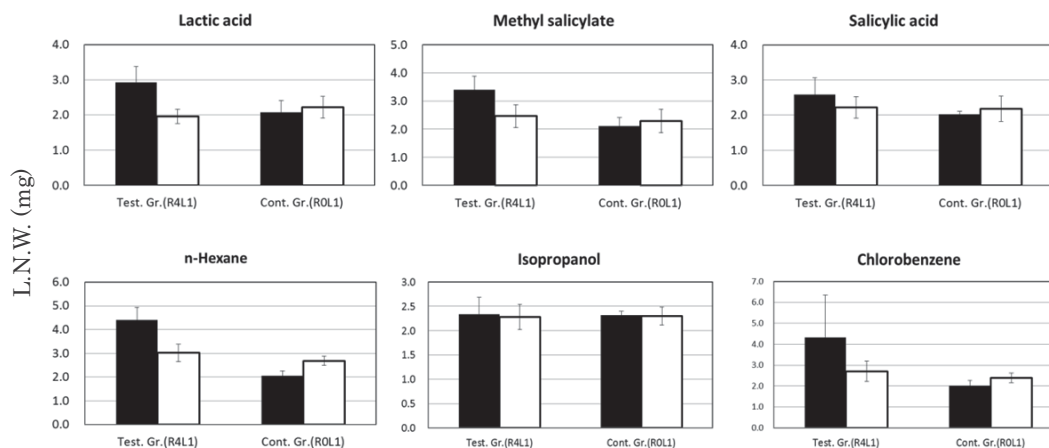


Fig. 7. (Continued).

by LLNA:DA method (ICCVAM, 2010). In fact, except for the results for NiCl_2 , the classification results for the 24 chemicals we examined with the LLNA:DAE method were consistent with the results of LLNA reported in the OECD TG 429 performance standard table and other previous reports (Haneke *et al.*, 2001; Gerberick *et al.*, 2005; OECD, 2010b; Takenouchi *et al.*, 2013).

These results suggest that the LLNA:DAE method can be used to distinguish true skin irritant from borderline-positive chemicals and to identify weak as well as strong skin sensitizers.

Evaluation of the potential for cross-sensitization between chemicals

The following three chemical pairs were tested to evaluate their potential for cross-sensitization, and the results are shown in Fig. 8: DNFB with HCA, DNCB with DNFB, and HQ with p-BQ. The first pair is not reported to have cross-sensitizing potential, while the latter two pairs are known to have a cross-sensitizing potential (Garcia-Perez, 1978; Basketter and Goodwin, 1988). When DNFB and HCA were tested using our LLNA:DAE method, the weight of the lymph node from the right ear that was sensitized by DNFB showed a significant increase, while that of the left ear did not increase by a challenge application of HCA. Similar results were obtained when HCA was used as the sensitizer and DNFB was used for the challenge application. Because DNFB and HCA are well-known skin sensitizers, this result suggests that the elicitation response observed using the

LLNA:DAE method was an immune-specific response. Thus, there was no significant difference in the weight of the lymph nodes from the left ear between the testing and control groups. In contrast, by using our LLNA:DAE method, we found that the mice sensitized by application of DNCB clearly showed an elicitation response against DNFB and the mice sensitized by DNFB clearly showed an elicitation response against DNCB. Similar results were also obtained by application of the HQ and p-BQ combination.

DISCUSSION

To date, no mouse skin-sensitization test can evaluate weak skin sensitizers like methyl methacrylate at the elicitation phase. In addition, there were no well-established methods based on the LLNA to evaluate the potential of cross-sensitization between chemicals.

The LLNA, LLNA:DA, and LLNA:BrdU-ELISA methods were used to evaluate the potential of chemicals to sensitize skin at the induction phase, and it was thought that cell proliferation in the lymph node was induced by immune-specific and/or nonspecific proliferation at the induction phase. These nonspecific proliferations of lymphocytes in the lymph node were thought to be one reason for the false-positive or borderline-positive results of the LLNA, LLNA:DA, and LLNA BrdU-ELISA methods (ICCVAM, 2010). To resolve this issue, we reported the results of preliminary experiments conducted in our laboratory (Idehara, 2013; Yamashita *et al.*, 2013). In the

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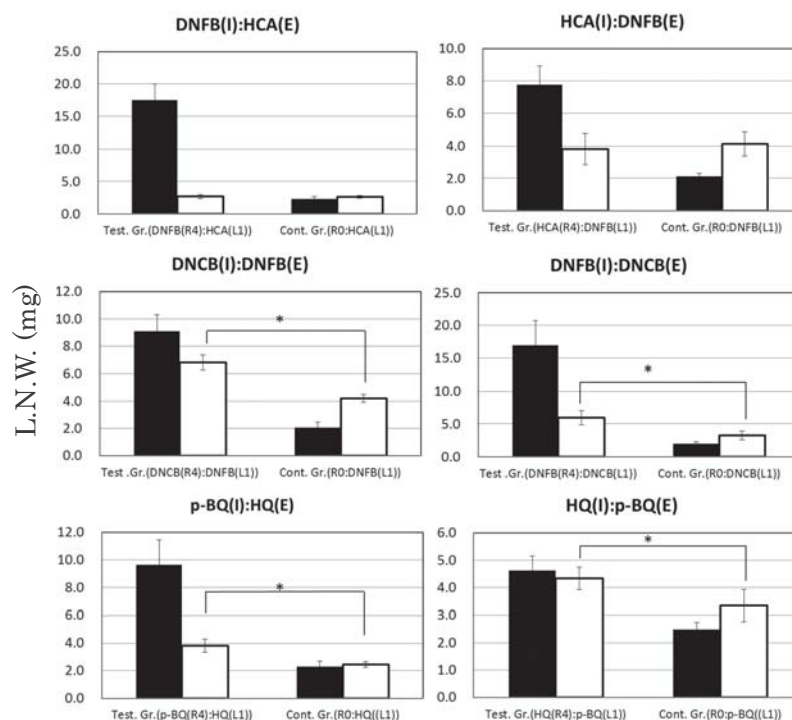


Fig. 8. The results of the cross-sensitization test for three chemical pairs. (I) indicates induction and (E) indicates elicitation. The labels on the X-axis indicate the treatments. For example, *DNCB(R4):DNFB(L1)* indicates that the mice received four treatments of DNCB to the right ear and one DNFB treatment to the left ear. The black and white columns indicate the values for the right and left ear, respectively. The Y-axis indicates the lymph node weight (L.N.W.). Values are expressed as the mean $\pm$ S.D. ($n = 5$). * represents a statistically significant difference in the average lymph node weights of the left ears between the control group and the test group indicated.

present study, we developed the LLNA:DAE method to distinguish immune-specific cell proliferation from non-specific cell proliferation of lymph nodes and we validated this method using 24 kinds of chemicals to evaluate the ability of this method to discriminate borderline-positive chemicals as classified by the LLNA:DA method. In addition, we showed the possibility of using this method to evaluate the cross-sensitization potential of chemicals.

To develop this new method, we first determined that the lymph nodes from the right and left ears of mice were suitable for use in evaluation of skin-sensitization tests by determining that the weight of the draining auricular lymph nodes from the right and left ears was not different nor was there any statistically significant difference in the cell proliferation response between the lymph nodes of the right and left ears during the 48-hr period after application of 25% HCA. We next used DNFB, HCA, xylene, and SLS to investigate the influence of four applications to the right ear on the lymph node weight or ATP content

of the left ear. Four applications of each of these chemicals alone to the right ear did not induce significant cell proliferation within the lymph node of the left ear. It was previously reported that the immune-specific lymphocyte response against an immunizing antigen in mice was only 0.08% of the lymphocytes (Tse *et al.*, 1980; Kanamori *et al.*, 2012). Our findings are consistent with these reports.

The test schedule was based on previous reports and on our experience during the development of the LLNA:DA method. It was known that skin sensitizers induce lymph node cell proliferation within three consecutive days of application on dorsum of the ear in the LLNA procedure. However, when using the MEST procedure, the challenge application was performed on day 10. Therefore, we used three consecutive days of application for the induction and an application on day 10 for the challenge. To optimize the test procedure, we investigated the response of lymph node cell proliferation following the chemical application using four groups of mice. The results indi-

cated that the elicitation response was dependent upon the first three applications on right ear; the fourth chemical application to the right ear did not affect the elicitation response of the left ear. However, the fourth chemical application to the right ear significantly increased the lymph node weight of right ear as compared with those right ears that did not receive the fourth application. Thus, in this study, we used the fourth application to right ear to clearly confirm the induction (Fig. 5).

We next examined the relationship between the induction concentration and the elicitation response using DNCB. The proliferation of lymphocytes in the lymph nodes of the right ear increased in a dose-dependent manner as did the elicitation response observed in the lymph nodes of the left ear (Fig. 6). It was confirmed that the elicitation response of the left ear was dependent upon the first three applications to right ear and remained unaffected by the fourth application on right ear (Fig. 5). These results suggested that the dose-dependent sensitization of the lymph nodes of the left ear was induced by three chemical applications to right ear during the induction phase. The lymph node weight increase observed with every 0.1% concentration increase in the induction dose applied to the left ear was almost same in the three test groups. Nakamura *et al.* (1994) reported a quantitative relationship between induction and elicitation using a modified GPMT method. They adopted three indexes to evaluate the sensitizing potential: induction dose, challenge dose, and skin response in the elicitation phase. Their data showed a clear relationship among these three indexes. For example with 2-MBT, the combination of a high concentration of the induction dose and the challenge dose caused a greater skin response than that observed following the combination of lower doses. These authors recommended an experimental protocol and criteria for evaluating the sensitizing potential of chemicals. Similarly, in the LLNA:DAE method, the induction dose and the challenge dose can be selected, and the elicitation response can be evaluated as the difference in the lymph node weights of the left ears between the test groups and the control group. Thus, the LLNA:DAE method offers the same possibility as their method for evaluating a quantitative relationship between induction and elicitation.

In this study, CMI, DNCB, DNFB, pPD, Co, NiCl₂, IEU, 2-MBT, citral, HCA, EU, CA, PBz, IU, MM, HQ, p-BQ, and EG were classified as skin sensitizers, while CB, IPA, LA, SA, MS, and hexane were classified as non-sensitizers of the skin. Except for the results for NiCl₂, these results are consistent with those obtained by using the LLNA method. Even though NiCl₂ is known to cause skin sensitization in humans, the skin sensitization

results for it in the LLNA method were negative (OECD, 2010b). Of the 24 chemicals tested, Co, 2-MBT, MM, CB, hexane, SA, MM, and IPA gave the borderline-positive results when tested by the LLNA:DA method. The chemicals classified into borderline positive criteria consisted of skin sensitizers and skin irritants as classified by the LLNA method (ICCVAM, 2010; OECD, 2010b). The results described here suggest that the LLNA:DAE method is useful for discriminating true skin irritants from borderline-positive chemicals and that it is potentially useful as a new test for skin sensitization.

We did not use the values of the stimulation index (SI) for classification in this study. Rather we employed statistical analyses for classification of the skin sensitizing potential of chemicals using the lymph node weights. The data we obtained for the ATP content were not used for the classification. We decided these change from LLNA:DA method based on the following reasoning. In the LLNA:DA method, the classification of the skin-sensitizing potential were based on the SI of the ATP content, and the cut-off value for the SI was set to distinguish skin sensitizers from non-skin sensitizers. Some skin irritants are known to induced nonspecific proliferation of lymph nodes, suggesting that it may be necessary to set a cut-off value to distinguish skin sensitizers from skin irritants as well (ICCVAM, 2010). However, in the LLNA:DAE procedure, nonspecific proliferation could be ignored by subtracting the lymph node weight of the left ear in the control group from that in the test group because the nonspecific lymph node cell proliferation from the skin irritation would be induced equally in both the control and test groups. Therefore, we did not set a cut-off value for the SI to distinguish nonspecific lymph node cell proliferation caused by true skin irritants. Thus, we hypothesized that any difference in the average weights of the left ears between the test and control groups was caused by an immune-specific response from the skin sensitizing potential of the chemicals. Based on this hypothesis, we employed statistical analyses to evaluate the difference in the average lymph node weights of the left ears between the test and control groups. Accordingly, we found that the results of the classification of 24 kinds of chemicals were consistent with the results obtained by using the LLNA method, with the exception of NiCl₂. Of the 24 chemicals tested, weak skin sensitizers such as IU and MM showed a clear elicitation response. These results indicated that the difference in the lymph node weights of the left ears between test and control groups could be used to identify skin sensitizers. The procedure for measuring lymph node weights was easier than that for measuring ATP levels, likely reducing measurement errors, and the lymph node

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data were deemed suitable for statistical analysis.

NiCl₂ induced lymph node proliferation when it was tested by the LLNA method. In a previous report, the SI was found to be 2.4 when 5% NiCl₂ was dissolved in DMSO (Basketter *et al.*, 1999b); the classification of the LLNA results was based on an SI with a cut-off value of 3. Thus, NiCl₂ was classified as negative. However, in LLNA:DAE method, the classification was determined by the difference in the average weight of lymph nodes of the left ears between test and control groups. By this difference, we think that we were able to identify the skin sensitizing potential of NiCl₂.

The test concentrations used in this study were based on previous reports demonstrating the borderline-positive results in the LLNA:DA method or the significant lymph node cell proliferation in the LLNA method. When LLNA:DAE is used to discriminate skin irritants from borderline-positive chemicals as classified by LLNA:DA method, the test concentration should be selected based the results of LLNA:DA. For use of the LLNA:DAE method as stand-alone test in the future, further investigation will be required to establish an appropriate selection of test concentrations.

For the procedure described here, the right ear of the control group was not treated with vehicle during the induction phase because we aimed to estimate the lymphocyte proliferation in the left ear induced by single application of samples, to use the weight of the lymph node of right ear as the historical record of naïve lymph node weight, and to reduce the numbers and suffering of the animals. To avoid the influence of vehicle, we used the standard vehicle used in the LLNA:DA method. Therefore, whenever a new vehicle is to be used, another control group would be necessary to assess the sensitizing potential of the vehicle alone by this procedure.

We also studied the possibility of adapting the LLNA:DAE method to evaluate the potential for cross-sensitization between chemicals. Currently, the GPMT method is used for this (Basketter *et al.*, 1988). In our study, three chemical pairs were tested: DNFB with HCA, DNCB with DNFB, and HQ with p-BQ. The results showed that the LLNA:DAE method could evaluate the cross-sensitization potential between chemicals. In addition, the data obtained from the LLNA:DAE method were more quantitative than those obtained by using the GPMT method because the degree of the induction response could be estimated by weighing the lymph node from the right ear of the test groups and the degree of the elicitation response could be estimated by comparing the weight of the lymph node from the left ears of the test and control group. In this study, we chose the con-

centrations for cross-sensitization test based on the concentration used in the LLNA:DAE method that induced a clear positive response. The concentrations of chemicals should be used at least that which had induced a clear elicitation response using the LLNA:DAE method. However, the same or a higher concentration should be used for the challenge chemical because the immune response was expected to be weaker when two different chemicals were applied for the cross-sensitization test than when the challenge chemical was the same as that used to elicit the initial response.

The LLNA:DAE method could distinguish the skin sensitizing from non-sensitizing chemicals among borderline-positive chemicals by comparing the weights of the lymph nodes at the elicitation phase. Furthermore, the LLNA:DAE method showed potential for use in evaluating the likelihood for chemicals to cross-sensitize, which was impossible to determine by using the LLNA, LLNA:DA, LLNA:BrdU-ELISA, and *in vitro* methods. To assess the potential for cross-sensitization, it is important to determine the causative agent of the occupational skin disease.

In conclusion, the LLNA:DAE method may have the potential to distinguish true skin irritant from borderline positive skin-sensitizing chemicals and to evaluate the cross-sensitizing potential between chemicals.

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