

Original Article

An improvement of LLNA:DA to assess the skin sensitization potential of chemicals

Hongwei Zhang, Ying Shi, Chao Wang, Kangfeng Zhao, Shaoping Zhang, Lan Wei,
Li Dong, Wen Gu, Yongjun Xu, Hongjie Ruan, Hong Zhi and Xiaoyan Yang

Department of Toxicology, National Institute of Environmental Health, Chinese Center for Disease Control and Prevention, China

(Received June 2, 2016; Accepted December 5, 2016)

ABSTRACT — We developed a modified local lymph node assay based on ATP (LLNA:DA), termed the Two-Stage LLNA:DA, to further reduce the animal numbers in the identification of sensitizers. In the Two-Stage LLNA:DA procedure, 13 chemicals ranging from non-sensitizers to extreme sensitizers were selected. The first stage used reduced LLNA:DA (rLLNA:DA) to screen out sensitive chemicals. The second stage used LLNA:DA based on OECD 442 (A) to classify those potential sensitizers screened out in the first stage. In the first stage, the SIs of the methyl methacrylate, salicylic acid, methyl salicylate, ethyl salicylate, isopropanol and propanediol were below 1.8 and need not to be tested in the second step. Others continued to be tested by LLNA:DA. In the second stage, sodium lauryl sulphate and xylene were classified as weak sensitizers. *a*-hexyl cinnamic aldehyde and eugenol were moderate sensitizers. Benzalkonium chloride and glyoxal were strong sensitizers, and phthalic anhydride was an extreme sensitizer. The 9/9, 11/12, 10/11, and 8/13 (positive or negative only) categories of the Two-Stage LLNA:DA were consistent with those from the other methods (LLNA, LLNA:DA, GPMT/BT and HMT/HPTA), suggesting that Two-Stage LLNA:DA have a high coincidence rate with reported data. In conclusion, The Two-Stage LLNA:DA is in line with the “3R” rules, and can be a modification of LLNA:DA but needs more study.

Key words: rLLNA: DA, LLNA: DA, Sensitization, Chemicals

INTRODUCTION

For many decades, human tests such as the human maximization test (HMT) and the human patch test (HPTA), and animal tests such as the guinea pig maximization test (GPMT), the Buehler test (BT), and the murine local lymph node assay (LLNA) have been the common tests used to assess the allergic contact dermatitis (ACD) of chemicals. Among these tests, LLNA has remained the preferred alternative to traditional animal skin sensitization models GPMT/BT (Kimber and Basketter, 1992). This scheme has technical advantages and responds to animal welfare concerns. However, the use of a radioactive endpoint has limited the adoption of the standard LLNA (OECD, 2002; Anderson *et al.*, 2011) assay. Consequently, several authors have studied alternative non-radioactive (RI) methods for LLNA. The murine local lymph node assay:DA (LLNA:DA) is a non-RI modified LLNA that involves the measurement

of the adenosine triphosphate (ATP) content to assess lymphocyte proliferation. It was adopted by the Organization for Economic Co-operation and development (OECD) in 2010 (Yamashita *et al.*, 2005; OECD, 2010a). However, from an animal welfare perspective, while LLNA and LLNA:DA reduce the overall animal numbers, there remains an aspiration to refine the existing animal approaches based on the “3R” rules (Replacement, Reduction and Refinement) as well as the REACH regulation (registration, evaluation, authorisation, and restriction of chemicals) (McGarry, 2007; SCCP, 2007). In 2006, Kimber proposed a cut-down assay of LLNA, named the reduced LLNA (rLLNA), in which only two dose groups (single high-dose group and a concurrent vehicle control group) of two mice each are used to screen chemicals (Kimber *et al.*, 2006). In 2009 the rLLNA was reviewed and validated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). It was included as an option in the revised OECD Test

Guideline 429 for the LLNA adopted in 2010 (OECD, 2010b). Because the rLLNA only uses one dose group, it may misclassify only the stimulation index (SI) of the high-dose group if it is below 1.8 and cannot provide dose-response information, but it can provide a “yes-no” result (Ezendam *et al.*, 2013). This can be used for hazard classification of skin sensitizing substances in cases that do not need dose-response information. Thus, there is also the opportunity to perform a rLLNA:DA to reduce the number of animals and to avoid the use of radiogen (ICCVAM, 2010a).

The rLLNA:DA decreases the number of sensitizers, and the LLNA:DA is one of the preferred non-RI methods for classification of sensitizers. The rLLNA:DA is less reliable, and the LLNA:DA conforms less to the “3R” rules. To facilitate the development of non-RI methods and to further decrease the demand for animals, we conducted a Two-Stage LLNA:DA combined rLLNA:DA and LLNA:DA. At the first stage, we used rLLNA:DA to screen 13 chemicals. We then used LLNA:DA to classify those potential sensitizers that had SI values equal or above 1.8. Finally, we compared the outcomes of Two-Stage LLNA:DA with reported data of LLNA:DA, LLNA, traditional animal tests (GPMT/BT) and human test (HMT/HPTA). We examined whether a combined method could be at least as good as singles in the hazard classification of skin sensitizing substances.

MATERIALS AND METHODS

Test Materials

Thirteen chemicals were obtained from the Sigma Chemical Co. (Saint Louis, MO, USA) and are summarized in Table 1. All of these were selected based on their sensitizing potential and published information about oth-

er sensitization tests especially the LLNA:DA. The vehicles used acetone/olive oil ph.Eur./DAB (4:1, v/v; AOO) and N,N-dimethylformamide (DMF) based on OECD guidelines. Three concentrations of each chemical were used in LLNA:DA and one maximum concentration in rLLNA:DA. The concentrations selected for each chemical were based on published information and preliminary experiments.

Animals

Female BALB/c mice were obtained from the Institute of Zoology at the Chinese Academy of Sciences (Beijing, China). All mice were 8- to 12-weeks old, and their weight was between 18 g and 22 g. Housing was specific pathogen free (SPF) with air temperature between 20°C and 24°C, a relative humidity of 60-80% and 12-hr light/dark cycles. Mouse chow obtained from Beijing Keaoxieli Feed Co., Ltd. (Beijing, China) and water were provided *ad libitum*.

The Two-Stage LLNA:DA Methods

The Two-Stage LLNA:DA was a combination of LLNA:DA and rLLNA. It has two stages: the first stage screens out the potential sensitizers. In this stage, 2 mice rather than 4 were used per group. The highest concentration was selected instead of three pre-chemicals. At the second stage, chemicals with SI ≥ 1.8 are measured at the same as the LLNA:DA based on OECD guideline 442 (A). These are classified according to the EC1.8 values. The 13 chemicals were tested in two batches. First, a-hexyl cinnamic aldehyde, benzalkonium chloride, eugenol, glyoxal, methyl methacrylate, phthalic anhydride, salicylic acid, sodium lauryl sulphate, xylene, control I (AOO) and control (DMF) were conducted. Then methyl salicylate, ethyl salicylate, isopropanol, propanediol and

Table 1. Test substance details.

	Substance	CAS number	Source	Reference
A	a-hexyl cinnamic aldehyde(HCA)	101-86-0	Sigma-Aldrich	Idehara <i>et al.</i> , 2008
B	benzalkonium chloride	8001-54-5	Sigma-Aldrich	Idehara <i>et al.</i> , 2008
C	eugenol	97-53-0	Sigma-Aldrich	Idehara <i>et al.</i> , 2008
D	glyoxal	107-22-2	Sigma-Aldrich	Haneke <i>et al.</i> , 2001
E	methyl methacrylate	80-62-6	Sigma-Aldrich	ICCVAM, 2010b
F	phthalic anhydride	85-44-9	Sigma-Aldrich	Idehara <i>et al.</i> , 2008
G	salicylic acid	69-72-7	Sigma-Aldrich	Idehara <i>et al.</i> , 2008
H	sodium lauryl sulphate	151-21-3	Sigma-Aldrich	Idehara <i>et al.</i> , 2008
I	xylene	1330-20-7	Sigma-Aldrich	Haneke <i>et al.</i> , 2001
J	methyl salicylate	119-36-8	Sigma-Aldrich	ICCVAM, 2010b
K	ethyl salicylate	118-61-6	Sigma-Aldrich	Basketter <i>et al.</i> , 1996
L	isopropanol	67-63-0	Sigma-Aldrich	Basketter <i>et al.</i> , 1996
M	propanediol	57-55-6	Sigma-Aldrich	Basketter <i>et al.</i> , 1996

control II were added.

In the first step of the Two-Stage LLNA:DA, groups of two female BALB/c mice were treated with 25 μ L of the highest concentration or vehicle on the dorsum of both ears. The concentration was based on literature and Per-Test. Pre-treatment with 1% sodium lauryl sulfate (SLS) solution was carried out one hour before each application. Treatment was performed on days 1, 2, 3, and 7.

On day 8, approximately 24 hr after the last application, the mice were sacrificed and the auricular lymph nodes on both sides were removed. Single cell suspensions were then prepared. The lymph nodes of each mouse were placed in glass homogenizers with 1 mL of phosphate-buffered saline (PBS, pH = 7.2). These were ground four to five times to separate the cells completely. The cell suspension was then filtered with a cell strainer (100 mesh) to remove membranes that were visible to the eye. After low-temperature centrifugation, the cells were suspended in 1 mL of PBS. A 20 μ L aliquot was collected with a micropipette and mixed with 1.98 mL of PBS to yield a 2 mL sample (diluted 100-fold). A second 2 mL sample was made so that the ATP content of each mouse could be measured twice.

The diluted sample was added to each well (10 μ L/well) in a white 96-well plate that was treated with an ATP measurement kit (ApoSENSORTM ATP Luminescence Assay Kit, BioVision Co., Milpitas, CA, USA). These were added in triplicate wells, and 100 μ L of the nuclear releasing reagent was added per well. After five minutes, 5 μ L of the ATP monitoring enzyme was added and luminescence (Relative Light Units, RLU) was measured within 10 min by a Varioskan Flash (Thermo Scientific, Vantaa, Finland). The mean ATP content (RLU) was then determined and used in subsequent calculations.

In the second step, the chemicals picked out in step one were tested with LLNA:DA according to OECD guideline 442(A). The procedure is the same as the rLLNA:DA mentioned above, except that the second step used four mice per group and three concentrations per chemical. This procedure is aimed at classifying chemicals according to the EC1.8 value and assesses their sensitizing potential.

Data analyses

The mean values and standard deviations of the ATP content (RLU) for each experimental group was calculated, and the stimulation index (SI) was calculated according to the following equation: $SI = (\text{mean RLU of the test chemical group}) / (\text{mean RLU of the vehicle control group})$ (Idehara *et al.*, 2008). According to ICCVAM and OECD

test guideline 442(A) (ICCVAM, 2010b; OECD, 2010a), the cut-off point was set based on the SI value of the ATP content as follows. In the first step, $SI \geq 1.8$ required that the test continue; $SI < 1.8$ required that the test be terminated. In the second step, $SI \geq 1.8$ was positive; $SI < 1.8$ was negative.

For positive outcomes, the EC1.8 values were calculated by the following equation: $EC1.8 = c + [(1.8 - d) / (b - d)] \times (a - c)$ (Idehara *et al.*, 2008). Data points lying immediately above and below the SI value of 1.8 have coordinates (a,b) and (c,d) (Idehara *et al.*, 2008). The sensitization potency of a chemical was classified as follows: $EC1.8 < 0.1$, extreme; $EC1.8 \geq 0.1$ to < 1 , strong; $EC1.8 \geq 1$ to < 10 , moderate; $EC1.8 \geq 10$, weak (Kimber *et al.*, 2003; Gerberick *et al.*, 2005; Gamer *et al.*, 2008).

Using ANOVA to analyse the variance revealed significant differences in the parametric data. If the outcome was statistically valid, we used Dunnett's *t*-test to compare the experimental groups with the vehicle control groups. For non-parametric data, a Kruskal-Wallis test was used, followed by a Nemenyi test.

RESULTS

The outcomes of the 13 chemicals tested in the first step are listed in Table 2. Six chemicals methyl methacrylate, salicylic acid, methyl salicylate, ethyl salicylate, isopropanol and propanediol were negatives with SI values below 1.8. They do not need to be tested in the second step, which minimizes the number of animals needed. The others need to be tested for their SIs higher than 1.8.

The classification of 7 chemicals tested in step two are provided in Table 3. All of them have at least one group that induced a statistically significant increase in ATP versus each control group. The EC1.8 values for α -hexyl cinnamic aldehyde, benzalkonium chloride, eugenol, glyoxal, phthalic anhydride, sodium lauryl sulphate and xylene were 6.79, 0.28, 4.24, 0.28, 0.05, 11.04, and 46.8% respectively. Thus, in the Two-Stage LLNA:DA, upon using the classification standard, methyl methacrylate, salicylic acid, methyl salicylate, ethyl salicylate, isopropanol and propanediol were classified as non-sensitizers. Sodium lauryl sulphate and xylene were weak sensitizers, α -hexyl cinnamic aldehyde and eugenol were moderate sensitizers, benzalkonium chloride and glyoxal were strong sensitizers, and phthalic anhydride was an extreme sensitizer.

To assess the reproducibility of this test, we compared the mean ATP contents of both control groups and the same concentration groups of 7 chemicals between step one and step two (Fig. 1). Except for benzalkonium chlo-

Table 2. Test results obtained by evaluation of 13 chemicals in the rLLNA:DA.

Chemicals	Vehicle	Highest test concentration (%)	ATP content (RLU)		Continue to test (Y) or not (N)
			M $\pm$ S.D. ^a	SI	
Control I (AOO)			6734 $\pm$ 260	1	
Control II (AOO)			5580 $\pm$ 781	1	
Control (DMF)			7170 $\pm$ 366	1	
A a-hexyl cinnamic aldehyde	AOO ^b	25	28082 $\pm$ 735	4.17	Y
B benzalkonium chloride	AOO ^b	1	46805 $\pm$ 635	6.95	Y
C eugenol	AOO ^b	10	26534 $\pm$ 237	3.94	Y
D glyoxal	AOO ^b	2.5	30176 $\pm$ 1031	4.48	Y
E methyl methacrylate	AOO ^b	100	7610 $\pm$ 378	1.13	N
F phthalic anhydride	AOO ^b	0.5	47075 $\pm$ 958	5.51	Y
G salicylic acid	AOO ^b	100	11915 $\pm$ 1137	1.77	N
H sodium lauryl sulphate	DMF	20	21685 $\pm$ 1185	3.22	Y
I xylene	AOO ^b	100	17308 $\pm$ 1863	2.41	Y
J methyl salicylate	AOO ^c	50	9709 $\pm$ 1480	1.74	N
K ethyl salicylate	AOO ^c	10	9074 $\pm$ 1058	1.63	N
L isopropanol	AOO ^c	100	5971 $\pm$ 1324	1.07	N
M propanediol	AOO ^c	100	4297 $\pm$ 1394	0.77	N

^aValues are expressed as the mean $\pm$ S.D. (n = 2). ^bThe data are compared with control I (AOO). ^cThe data are compared with control II (AOO).

ride, the ATP contents of other 6 chemicals have no statistically significant differences between these two steps or between the control groups ($p > 0.05$).

Table 4 presents skin sensitizing data of 13 chemicals evaluated by Two-Stage LLNA:DA and other methods (LLNA:DA, LLNA, GMPT/BT, HMT/HPTA). The data evaluated by other methods were from the reported papers. Versus LLNA:DA and LLNA, the other positive/negative categories were the same except for methyl methacrylate, salicylic acid, methyl salicylate and isopropanol, which had both positive and negative categories in LLNA:DA and LLNA. In positive chemicals, classification were the same as LLNA:DA but higher than LLNA. Versus animal tests (GMPT/BT), only sodium lauryl sulphate was positive in the Two-Stage LLNA:DA but negative in animal testing. Versus human tests (HMT/HPTA), 7 species were similar except for isopropanol, which have both positive and negative categories in human testing.

DISCUSSION

Yamashita investigated the ATP content in LLNA using the ATP measurement kit in 2005 (Yamashita *et al.*, 2005). Kimber promoted rLLNA in 2006 (Kimber *et al.*, 2006) and Yamashita studied LLNA:DAE recently (Yamashita *et al.*, 2014, 2015). Studies about surrogate test systems for skin sensitization tests have never stopped. The ultimate aim is to pursue a simple but highly efficient method to accurately identify, classify and manage human skin sensitizers. The other goal is minimize

animal use and harm to follow the “3R” rules as best as possible. Accordingly, we conducted the Two-Stage LLNA:DA, which is a variant of the LLNA:DA. The clear advantage is the reduced use of animals. Here, there were 7 sensitizers and 6 non-sensitizers. In this case, we saved nearly 30% of the total number of animals versus the standard LLNA:DA in OECD 442(A).

The strain of mice is important. LLNA and both the two non-RI modified LLNA (LLNA:DA and LLNA BrdU-ELISA) recommended female CBA strain mice in the OECD guidelines (OECD, 2010a, 2010b, 2010c). But in China, CBA mice are limited to a few animal suppliers, and the price of this strain is 7 times than other normal strains such as BALB/c mice. This seriously limits the implementation and development of alternative methods of traditional animal skin sensitization in China. Fenxia H used BALB/c strain in LLNA BrdU-ELISA to test 32 LLNA sensitizers and 11 LLNA non-sensitizers, they found that using BALB/c mouse shows no statistical differences than the validation study with the LLNA:BrdU-ELISA using CBA/JN mouse (Fenxia *et al.*, 2015). Woolhiser MR assessed the response of chemical sensitizers in the LLNA across 6 strains of female mice, and concluded that “DBA/2, B6C3F1 and BALB/c mice are good choices for continued evaluation as additional mouse strains for use in the LLNA” (Woolhiser *et al.*, 2000). Other studies also showed that BALB/c mice may have small differences compared with CBA mice (Cockshott *et al.*, 2006; Guo *et al.*, 2013). Since the OECD guidelines state that “other strains and males may

Two-Stage LLNA:DA assess chemical sensitization

Table 3. Results of the second step of the Two-Stage LLNA:DA for 7 chemicals.

	Chemicals	Vehicle	Test concentration (%)	ATP content (RLU)		EC1.8 value	Classification
				M $\pm$ S.D. ^a	SI		
A	a-hexyl cinnamic aldehyde	AOO	0	7056 $\pm$ 369	1	6.79	moderate
			5	12004 $\pm$ 240	1.7		
			10	13980 $\pm$ 1069 ^b	1.98		
			25	23585 $\pm$ 721 ^b	3.34		
B	benzalkonium chloride	AOO	0	5041 $\pm$ 897	1	0.28	strong
			0.25	7231 $\pm$ 657	1.43		
			0.5	10934 $\pm$ 278 ^b	2.17		
			1	31198 $\pm$ 658 ^b	6.19		
C	eugenol	AOO	0	5986 $\pm$ 230	1	4.24	moderate
			2.5	9260 $\pm$ 1133 ^b	1.55		
			5	11405 $\pm$ 673 ^b	1.91		
			10	19943 $\pm$ 802 ^b	3.33		
D	glyoxal	AOO	0	7471 $\pm$ 134	1	0.28	strong
			0.5	20743 $\pm$ 118 ^b	2.78		
			1	31355 $\pm$ 607 ^b	4.2		
			2.5	32884 $\pm$ 701 ^b	4.33		
F	phthalic anhydride	AOO	0	6726 $\pm$ 539	1	0.05	extreme
			0.05	11198 $\pm$ 1056 ^b	1.66		
			0.1	23600 $\pm$ 801 ^b	3.51		
			0.5	51323 $\pm$ 1076 ^b	7.63		
H	sodium lauryl sulphate	DMF	0	6309 $\pm$ 303	1	11.04	weak
			5	9976 $\pm$ 514 ^b	1.58		
			10	10347 $\pm$ 1204 ^b	1.64		
			20	19987 $\pm$ 1314 ^b	3.17		
I	xylene	AOO	0	4758 $\pm$ 570	1	46.86	weak
			10	6339 $\pm$ 379	1.33		
			50	8749 $\pm$ 805 ^b	1.84		
			100	13029 $\pm$ 1429 ^b	2.74		

^a Values are expressed as the mean $\pm$ S.D. (n = 4). ^b represents a statistically significant difference in the ATP content between the control group and the test group ($P < 0.05$).

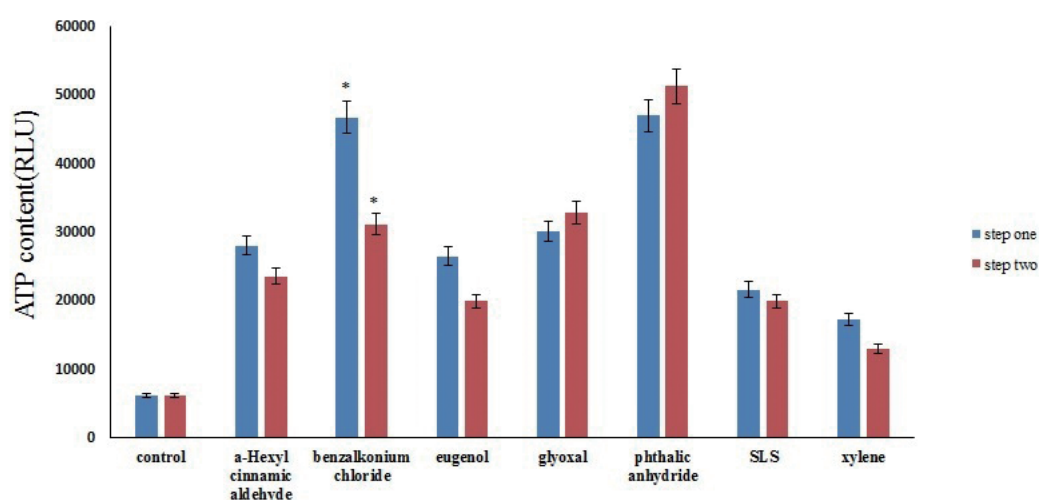


Fig. 1. Comparison of mean ATP contents in both control groups and seven chemical groups between step one and step two. *represents a statistically significant difference in the ATP content of benzalkonium chloride between step one and step two ($P < 0.05$).

Table 4. The category for 13 chemicals using LLNA:DA, LLNA, GMPT/BT, HMT/HPTA, and Two-Stage LLNA:DA.

Chemicals	LLNA:DA	LLNA	GMPT/BT	HMT/HPTA	Two-Stage LLNA:DA
A a-hexyl cinnamic aldehyde	+ abcd	+ ^b , ++ acd	p ^{ce}	n ^{abd}	++
B benzalkonium chloride	+++ ^d , +++ac	+acde	p ^{cde}	p ^{acde}	+++
C eugenol	++abcd	+bde	p ^{cdef}	p ^{bcdefg}	++
D glyoxal	/	++eh	p ^e	p ^e	+++
E methyl methacrylate	n/p ^b , + ^c	+bc	/	p ^{bi}	n
F phthalic anhydride	++++cd	+++cde	p ^{cdej}	p ^{cj}	++++
G salicylic acid	n ^{ackd} , n/p ^b	n ^{ackedly}	n ^{dek}	p ^{abdekn}	n
H sodium lauryl sulphate	++acd	+ ^g , ++chklm	n ^{dek}	n ^{dek}	+
I xylene	/	+ehl	/	n ^{en}	+
J methyl salicylate	n ^{bc}	n ^{co} , + ^a	n ^{fo}	n ^{abfo}	n
K ethyl salicylate	/	/	n ^f	n ^f	n
L isopropanol	n ^a , p ^{bn}	n ^{bm}	n ^f	p ^{bc} , n ^f	n
M propyleneglycol	/	n ^{am}	n ^f	n ^{afp}	n

\: not calculated because the substance was negative; /: not find relative data; n: negative; p: positive; +: weak; ++: moderate; +++: strong; ++++: extreme; GMPT: guinea pig maximization test; BT: Buehler test; HPTA: human patch test allergen; HMT: human maximization test; ^a data from Strickland *et al.*, 2012; ^b data from Yamashita *et al.*, 2014; ^c data from ICCVAM, 2010b; ^d data from Idehara *et al.*, 2008; ^e data from Haneke *et al.*, 2001; ^f data from Basketter *et al.*, 1996; ^g data from Basketter *et al.*, 2005; ^h data from Gerberick *et al.*, 2004; ⁱ data from Kligman, 1966; ^j data from van Och *et al.*, 2000; ^k data from ICCVAM, 2010a; ^l data from ECVAM, 2008; ^m data from Roberts *et al.*, 2007; ⁿ data from Basketter *et al.*, 2008; ^o data from Kimber *et al.*, 1995; ^p data from Takeyoshi *et al.*, 2005.

be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA response do not exist”, we chose female BALB/c strain mice instead of the OECD guidelines’ recommended strain during this study.

The first experiment was rLLNA:DA. It had rigorous criteria: substances were negatives only if the SI values were less than 1.8. Six cases accepted as LLNA negatives (methyl methacrylate, salicylic acid, methyl salicylate, ethyl salicylate, isopropanol and propanediol) were successfully screened out by the rLLNA:DA. This outcome supports rLLNA being added in OECD TG 429, which allows only two mice/group when each mouse is processed individually. In fact, Ezendam *et al.* (2013) showed that the accuracy, sensitivity and false negative of rLLNA were poor, but the specificity was 100% (1076/1076). This indicates that one dose per chemical and two mice per group are sufficient to screen out negatives. The second indication is that rLLNA:DA has high specificity and a great capacity to screen out negative substances.

Furthermore, to evaluate the reproducibility of this test, we compared the same concentration groups (including control groups) in two steps (Fig. 1). There were statistically significant differences between the same concentration of benzalkonium chloride in the first step and the second step. We considered that these might attributed to the individual differences between the animals or variation of experiment conditions such as operator, reagent supplies,

temperature and so on. Importantly though, the mean ATP contents of control groups in step one and step two were 6157 RLU and 6192 RLU respectively, and there were no statistically significant differences; secondly, except benzalkonium chloride, 6/7 chemical groups have no statistically significant differences between step one and step two. These analysis indicated that there were no large laboratory variations—the tests have satisfactory reproducibility.

To validate the Two-Stage LLNA:DA, we compared the skin-sensitizing potential of 13 chemicals in the novel assay with published data for LLNA:DA, LLNA, GMPT/BT and HMT/HPTA. Versus LLNA:DA, LLNA, GMPT/BT and HMT/HPTA, the coincidence rates were 9/9, 11/12, 10/11, and 8/13 (positive or negative only) respectively. It is important to note the discordances versus LLNA, GMPT/BT and HMT/HPTA. Versus LLNA, the discordance was methyl methacrylate, which had both positive and negative categories in LLNA (Yamashita *et al.*, 2014; ICCVAM, 2010b). Versus GMPT/BT, the discordance was sodium lauryl sulphate, which is a well-know false positive and can produce a borderline positive result in LLNA:DA (Yamashita *et al.*, 2014). Versus HMT/HPTA, there were 5 discordances. Except for methyl methacrylate and sodium lauryl sulphate mentioned above, a-hexyl cinnamic aldehyde and xylene are well-known and accepted LLNA false positives, salicylic acid is a well-known false negative. (van Och *et al.*,

Two-Stage LLNA:DA assess chemical sensitization

2000; ECVAM, 2008; Idehara *et al.*, 2008; ICCVAM, 2010a; OECD, 2010a, 2010b).

For those positives, the greatest issue here is 5 discordances between the Two-Stage LLNA:DA and LLNA. All of them were classed higher in LLNA:DA than in LLNA. This suggests that the use of ATP content as evaluation indicator was more sensitive than radioactive methods. Other groups support this conclusion (Yamashita *et al.*, 2005; Idehara *et al.*, 2008; Omori *et al.*, 2008).

Upon comparing the Two-Stage LLNA:DA and LLNA with human testing (HMT/HPTA), both have the same 8 coincidences. This suggests that the Two-Stage LLNA:DA has similar accuracy as LLNA. In this study, the data was limited (only 13 chemicals), but the classification and identification of 13 cases had a high coincidence rate with reported data. This is the main aim of the Two-Stage LLNA:DA.

In conclusion, the Two-Stage LLNA:DA is a combination of rLLNA:DA and LLNA:DA with the advantages of both accuracy and specificity. This approach also reduces the number of animals used—especially when it is used to evaluate mostly negative compounds. However, this does not imply that Two-Stage LLNA:DA can replace full LLNA:DA, and there are some limitations in this study: (1) The lack of direct evidence to prove there is no statistically significant differences between the performance of the LLNA:DA using BALB/c strain mice and that of using CBA strain mice; and (2) the 13 chemicals were insufficient to prove the feasibility and superiority of a method. The results need to be confirmed with additional future testing.

ACKNOWLEDGMENTS

This work was supported by China Research Special Fund of the Ministry of Health Public Service Sectors Funded Projects: Cosmetic Standards.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

- Anderson, S.E., Siegel, P.D. and Meade, B.J. (2011): The LLNA: A Brief Review of Recent Advances and Limitations. *J. Allergy (Cairo)*, **2011**, 424203.
- Basketter, D.A., Gerberick, G.F., Kimber, I. and Loveless, S.E. (1996): The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. *Food Chem. Toxicol.*, **34**, 985-997.
- Basketter, D.A., Clapp, C., Jefferies, D., Safford, B., Ryan, C.A., Gerberick, F., Dearman, R.J. and Kimber, I. (2005): Predictive identification of human skin sensitization thresholds. *Contact Dermatitis*, **53**, 260-267.
- Basketter, D.A., Cockshott, A., Corsini, E., Gerberick, G.F., Idehara, K., Kimber, I., Van Loveren, H., Matheson, J., Mehling, A., Omori, T., Rovida, C., Sozu, T., Takeyoshi, M. and Casati, S. (2008): An evaluation of performance standards and non-radioactive endpoints for the local lymph node assay. The report and recommendations of ECVAM Workshop 65. *Altern. Lab. Anim.*, **36**, 243-257.
- Cockshott, A., Evans, P., Ryan, C.A., Gerberick, G.F., Betts, C.J., Dearman, R.J., Kimber, I. and Basketter, D.A. (2006): The local lymph node assay in practice: a current regulatory perspective. *Hum. Exp. Toxicol.*, **25**, 387-394.
- ECVAM (2008): ECVAM murine local Lymph Node Assay (LLNA) Performance Standards. European Centre for the Validation of Alternative methods, October 2008; available from: http://ecvam.jrc.ec.europa.eu/ft_doc/ECVAM%20LLNA%20Performance%20Standards-Sec.pdf (accessed 12 January 2011).
- Ezendam, J., Muller, A., Hakkert, B.C. and van Loveren, H. (2013): Evaluation of the performance of the reduced local lymph node assay for skin sensitization testing. *Regul. Toxicol. Pharmacol.*, **66**, 66-71.
- Gamer, A.O., Nies, E. and Vohr, H.W. (2008): Local lymph node assay (LLNA): comparison of different protocols by testing skin-sensitizing epoxy resin system components. *Regul. Toxicol. Pharmacol.*, **52**, 290-298.
- Gerberick, G.F., Ryan, C.A., Kern, P.S., Dearman, R.J., Kimber, I., Patlewicz, G.Y. and Basketter, D.A. (2004): A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. *Contact Dermatitis*, **50**, 274-288.
- Gerberick, G.F., Ryan, C.A., Kern, P.S., Schlatter, H., Dearman, R.J., Kimber, I., Patlewicz, G.Y. and Basketter, D.A. (2005): Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis*, **16**, 157-202.
- Guo, T.L., Germolec, D.R., Zhang Ling, X., Auttachoat, W., Smith, M.J. and White, K.L.Jr. (2013): Contact sensitizing potential of pyrogallol and 5-amino-o-cresol in female BALB/c Mice. *Toxicology*, **314**, 202-208.
- Haneke, K.E., Tice, R.R., Carson, B.L., Margolin, B.H. and Stokes, W.S. (2001): ICCVAM evaluation of the murine local lymph node assay. Data analyses completed by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods. *Regul. Toxicol. Pharmacol.*, **34**, 274-286.
- Fenxia, H., Caihong, X., Bin, L., Juan, C.H., Wei, C.H. and Man, Z.H. (2015): Application of BALB/c mouse in the local lymph node assay: BrdU-ELISA for the prediction of the skin sensitizing potential of chemicals. *J. Pharmacol. Toxicol. Methods*, **72**, 53-58.
- ICCVAM (2010a): Appendix B ICCVAM-Recommended Test Method Protocol: The Murine Local Lymph Node Assay: DA, a Non-radioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products; available from: http://ntp.niehs.nih.gov/iccvam/docs/immunotox_docs/llna-da/appb-protocol.pdf.
- ICCVAM (2010b): ICCVAM test method evaluation report on the murine local lymph node assay: DA A nonradioactive alternative test method to assess the allergic contact dermatitis potential of chemicals and products. available from: http://ntp.niehs.nih.gov/iccvam/docs/immunotox_docs/llna-da/tmer.pdf (accessed 12 January 2011).
- Idehara, K., Yamagishi, G., Yamashita, K. and Ito, M. (2008): Characterization and evaluation of a modified local lymph node assay

- using ATP content as a non-radio isotopic endpoint. *J. Pharmacol. Toxicol. Methods*, **58**, 1-10.
- Kimber, I. and Basketter, D.A. (1992): The murine local lymph node assay: a commentary on collaborative studies and new directions. *Food Chem. Toxicol.*, **30**, 165-169.
- Kimber, I., Basketter, D.A., Butler, M., Gamer, A., Garrigue, J.L., Gerberick, G.F., Newsome, C., Steiling, W. and Vohr, H.W. (2003): Classification of contact allergens according to potency: proposals. *Food Chem. Toxicol.*, **41**, 1799-1809.
- Kimber, I., Dearman, R.J., Betts, C.J., Gerberick, G.F., Ryan, C.A., Kern, P.S., Patlewicz, G.Y. and Basketter, D.A. (2006): The local lymph node assay and skin sensitization: a cut-down screen to reduce animal requirements? *Contact Dermatitis*, **54**, 181-185.
- Kligman, A.M. (1966): The identification of contact allergens by human assay. 3. The maximization test: a procedure for screening and rating contact sensitizers. *J. Invest. Dermatol.*, **47**, 393-409.
- Kimber, I., Hilton, J., Dearman, R.J., Gerberick, G.F., Ryan, C.A., Basketter, D.A., Scholes, E.W., Ladics, G.S., Loveless, S.E., House, R.V. and Guy, A. (1995): An international evaluation of the murine local lymph node assay and comparison of modified procedures. *Toxicology*, **103**, 63-73.
- McGarry, H.F. (2007): The murine local lymph node assay: regulatory and potency considerations under REACH. *Toxicology*, **238**, 71-89.
- OECD (2002): Test Guideline 429: The Local lymph Node Assay. Paris: Organisation for Economic Cooperation and development.
- OECD (2010a): Test Guideline No:442(A): Guideline for the Testing of Chemicals.Skin Sensitization :Local lymph Node Assay:DA. Paris: Organisation for Economic Cooperation and development.available from: <https://ntp.niehs.nih.gov/iccvm/suppdocs/feddcs/oecd/oecd-tg442a.pdf>
- OECD (2010b): Test Guideline 429: The Local lymph Node Assay. Paris: Organisation for Economic Cooperation and development.
- OECD (2010c): Test Guideline No:442(B): Guideline for the Testing of Chemicals.Skin Sensitization :Local lymph Node Assay BrdU-ELISA. Paris: Organisation for Economic Cooperation and development.
- Omori, T., Idehara, K., Kojima, H., Sozu, T., Arima, K., Goto, H., Hanada, T., Ikarashi, Y., Inoda, T., Kanazawa, Y., Kosaka, T., Maki, E., Morimoto, T., Shinoda, S., Shinoda, N., Takeyoshi, M., Tanaka, M., Uratani, M., Usami, M., Yamanaka, A., Yoneda, T., Yoshimura, I. and Yuasa, A. (2008): Interlaboratory validation of the modified murine local lymph node assay based on adenosine triphosphate measurement. *J. Pharmacol. Toxicol. Methods*, **58**, 11-26.
- Roberts, D.W., Patlewicz, G., Kern, P.S., Gerberick, F., Kimber, I., Dearman, R.J., Ryan, C.A., Basketter, D.A. and Aptula, A.O. (2007). Mechanistic applicability domain classification of a local lymph node assay dataset for skin sensitization. *Chem. Res. Toxicol.*, **20**,1019-1030.
- SCCP (2007): Memorandum on Actual Status of Alternative Methods on The Use of Experimental Animals in The Safety Assessment Cosmetic Ingredients in The European Union. SCCP/1111/07. (adopted by SCCP at 12th plenary on 19 June 2007) .
- Strickland, J., Stack, F., Burns, T., Allen, D., Casey, W. and Stokes, W. (2012): Evaluation of two nonradiolabeled murine local lymph node assays(LLNA) for potency categorization of substances causing allergic contact dermatitis in humans. Nonradiolabeled LLNA Potency NICEATM-ICCVAM SOT 2012 poster.
- Takeyoshi, M., Iida, K., Shiraishi, K. and Hoshuyama, S. (2005): Novel approach for classifying chemicals according to skin sensitizing potency by non-radioisotopic modification of the local lymph node assay. *J. Appl. Toxicol.*, **25**, 129-134.
- van Och, F.M., Slob, W., de Jong, W.H., Vandebruel, R.J. and van Loveren, H. (2000). A quantitative method for assessing the sensitizing potency of low molecular weight chemicals using a local lymph node assay: employment of a regression method that includes determination of the uncertainty margins. *Toxicology*, **146**, 49-59.
- Woolhiser, M.R., Munson A.E. and Meade B.J. (2000): Comparison of mouse strains using the local lymph node assay. *Toxicology*, **146**, 221-227.
- Yamashita, K., Idehara, K., Fukada, N., Yamagishi, G. and Kawada, N. (2005): Development of a modified local lymph node assay using ATP measurement as an endpoint. *AATEX*, **11**, 136-144.
- Yamashita, K., Shinoda, S., Hagiwara, S. and Itagaki, H. (2014): 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. *J. Toxicol. Sci.*, **39**, 147-161.
- Yamashita, K., Shinoda, S., Hagiwara, S. and Itagaki H. (2015): Further development of LLNA:DAE method as stand-alone skin-sensitization testing method and applied for evaluation of relative skin-sensitizing potency between chemicals. *J. Toxicol. Sci.*, **40**, 137-150.