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Effects of Strain Differences and Vehicles on Results of Local Lymph Node Assays

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Abstract: The Local Lymph Node Assay (LLNA) is now regarded as the worldwide standard. The analysis of accumulated LLNA data reveals that the animal strains and vehicles employed are likely to affect LLNA results. Here we show that an obvious strain difference in the local lymph node response was observed between DMSO-treated CBA/CaOlaHsd and CBA/CaHsdRcc mice. We also show that a vehicle difference in the response was observed when CBA/CaHsdRcc mice were exposed to 6 vehicles; 4:1 v/v acetone/olive oil (AOO), ethanol/water (70% EtOH), N,N-dimethylformamide (DMF), 2-butanone (BN), propylene glycol (PG), and dimethylsulfoxide (DMSO). The dpm/LN level was lowest in the 70% EtOH group and highest in the DMSO group. When alpha-hexylcinnamaldehyde (HCA) was used as a sensitizer for the LLNA, HCA was a weak sensitizer when AOO or DMSO was used as a vehicle, but a moderate sensitizer when the other 4 vehicles were used. This study showed that there are vehicle differences in the local lymph node response (dpm/LN level) in the LLNA and that the sensitization potency of HCA may be classified in different categories when using different vehicles. This suggests that careful consideration should be exercised in selecting a vehicle for the LLNA. A further comprehensive study will be needed to investigate why vehicle differences are observed in the LLNA.

Key words: animal strain, local lymph node assay, vehicle-effect

The local lymph node assay (LLNA) is the most commonly used among several alternatives of skin sensitization tests [1, 8, 10], including the human cell line activation test (h-CLAT), the peptide-binding test [7], and the quantitative structure-activity relationship (QSAR) system. Furthermore, a three-dimensional human skin model is now being actively developed as an *in vitro* model for the assessment of primary skin irritation [15], and put into trials for use as an *in vitro* model for the evaluation of skin sensitization. These newly developed alternative methods are expected to be implemented for practical use in the future. However, a considerable time will be required for these *in vitro* test methods to become reliable and accepted international standard test methods.

In 2001, Takeyoshi *et al*. developed the non-radioisotope (non-RI) LLNA [12, 13] in Japan. The novel test method was expected to become an alternative to the tra-

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ditional LLNA [14, 16]. However, the traditional LLNA using a radioisotope is still used in Japan, because the data of the traditional LLNA, which were obtained at laboratories outside Japan, are often used in chemical safety assessments in Japan. Currently, the European Centre for the Validation of Alternative Methods (ECVAM) and the Interagency Coordination Committee on the Validation of Alternative Methods (ICCVAM) are collaborating to harmonize the different methods of the LLNA [2, 6].

The LLNA method has been widely used throughout the world, however, it has several disadvantages; one of which is that false positive and false negative reactions may occur, and may not be detected by scientists or regulators [5]. Such false positive and/or false negative reactions may occur for several reasons, and there is the possibility that variations in experimental materials, such as animal strain or vehicles used, may cause differences in reactivity. If this is true, questions regarding the reliability of results (not only false positive or false negative results, but also true positive and true negative results) are raised. There are few studies focusing on the animal strains and vehicles used in the LLNA, and no comprehensive data have been reported. The present study was undertaken to elucidate the effects of strain and vehicle differences on LLNA results.

In this study, mouse strains and different vehicles which are commonly used in Europe and those described in the OECD Guideline 429 [3, 4] were used. Two strains of female mice aged 8 to 12 weeks at the time of treatment were used for the experiments. The mouse strains used were CBA/CaOlaHsd (Harlan Laboratories BV, Horst, Netherland), and CBA/CaHsdRcc (SPF) (RCC Ltd., Füllinsdorf, Switzerland). All animals were housed individually in Makrolon type-2 cages (Lignocel, Schill AG, Muttenz, Switzerland), and acclimatized for 5 days prior to sensibilization administration of test materials. The temperature and humidity of the animal rooms were controlled to maintain them within the target ranges of 22 ± 3°C and 30 to 70% relative humidity respectively. The air exchange rate was between 10 to 15 air changes per hour. The light/dark cycle was 12/12 h with light from 6:00 am to 6:00 pm daily. All animal procedures were based on the OECD guidelines for testing of chemicals, described in the updated guideline 429: Skin Sensitization; Local Lymph Node Assay (adopted on 24 April 2002) [11], and performed in an AAALAC-accredited facility, using protocols approved by the Animal Experimentation Ethics Committee of Harlan Laboratories.

The following 6 vehicles frequently used in the LLNA were selected as test chemicals: 4:1 v/v acetone and olive oil (AOO); 7:3 v/v ethanol/water (70% EtOH) (ethanol: VWR International GmbH, Darmstadt, Germany); N,N-dimethylformamide (DMF) (Merck KGaA, Darmstadt, Germany); 2-butanone (BN) (Merck KGaA); propylene glycol (PG) (Fluka Chemie AG, Buchs, Switzerland); and dimethylsulfoxide (DMSO) (Fluka Chemie AG).

As a common positive control of skin sensitizer, α-hexylcinnamaldehyde (HCA) (Fluka Chemie AG) was used at concentrations of 0, 5, 10, and 25%.

The test chemical was applied to the dorsum of both ears of each mouse at the same time each day for 3 consecutive days using a micropipette. Five days after the first topical application of the test chemical, 250 μl of ^{3}H-methylthymidine (^{3}HTdR) (GE Healthcare Bio-Science Limited, Buckinghamshire, England) was injected into the tail veins of the mice. Mice were euthanized 5 h after the intravenous injection of ^{3}HTdR. The draining auricular lymph nodes from each mouse were excised and pooled on an individual animal basis. Suspensions of lymph node cells (LNC) were prepared from the collected lymph nodes by filtration through a cell strainer (mesh size, 200 μm). To the LNC suspension, 3 ml of 5% trichloroacetic acid (Fluka Chemie AG) was added, and the mixture was then incubated for 18 h at 4°C. An aliquot of the suspension was transferred into a plastic scintillation vial (Perkin Elmer GmbH, Rodgau, Germany) containing scintillation liquid. The incorporation of ^{3}HTdR was measured as disintegrations per minute per lymph node (dpm/LN) for each mouse by a β-scintillation counter (Tri-Carb 2900TR, Perkin Elmer [LAS] GmbH, Rodgau, Germany) to detect the proliferation of immune cells induced by the test chemical.

The effect of strain difference on the incorporation of ^{3}HTdR (dpm/LN) was investigated in CBA/CaOlaHsd and CBA/CaHsdRcc (SPF) mice (Experiment 1). DMSO was topically applied to 5 mice of each strain, and the incorporation of ^{3}HTdR (dpm/LN) was determined. The incorporation of ^{3}HTdR (dpm/LN) was also measured in a non-treatment control group (NCG) (5 mice).

Table 1. Effects of strain differences on dpm/LN level

Strains	dpm/LN	
	Vehicle	Mean ± SD
CBA/CaOlaHsd	NCG	182 ± 78
CBA/CaHsdRcc(SPF)	NCG	189 ± 54
CBA/CaOlaHsd	DMSO	406 ± 133
CBA/CaHsdRcc(SPF)	DMSO	682 ± 151

DMSO was topically applied to CBA/CaOlaHsd and CBA/CaHsdRcc (SPF) mice, and the incorporation of ^{3}HTdR (dpm/LN) was determined. The incorporation of ^{3}HTdR (dpm/LN) was also measured in the no-treatment control group (NCG).

Table 2. Vehicle effect on dpm/LN level

Vehicle	dpm/LN
	Mean ± SD
NCG	189 ± 54
AOO	294 ± 46
EtOH 70%	132 ± 55
DMF	235 ± 108
BN	320 ± 124
PG	219 ± 40
DMSO	682 ± 15

AOO, EtOH 70%, DMF, BN, PG, and DMSO were topically applied to CBA/CaHsdRcc (SPF) mice (n=5 each), and the incorporation of ^{3}HTdR (dpm/LN) was determined. The incorporation of ^{3}HTdR (dpm/LN) was also measured in the NCG (n=5). The data for the NCG and the DMSO group were taken from experiment 1 (Table 1).

The vehicle effect on incorporation of ^{3}HTdR (dpm/LN) was evaluated in CBA/CaHsdRcc (SPF) mice (Experiment 2). AOO, 70% EtOH, DMF, BN, PG, or DMSO was topically applied to the mice (n=5 for each vehicle), and the incorporation of ^{3}HTdR (dpm/LN) was determined. The incorporation of ^{3}HTdR (dpm/LN) was also measured in the NCG (n=5).

The vehicle effect on HCA response was also evaluated in CBA/CaOlaHsd mice (Experiment 3). HCA dissolved in the 6 different vehicles used in Experiment 2 was topically applied to the mice (n=4 for each vehicle) at concentrations of 0, 5, 10, and 25% (v/v). The stimulation index (SI) and EC3 value (the estimated concentration for the SI value of 3) were calculated from the observed incorporation of ^{3}HTdR (dpm/LN).

As shown in Table 1 (Experiment 1), the dpm/LN levels measured in the DMSO group were 406 in CBA/CaOlaHsd mice and 682 in CBA/CaHsdRcc (SPF) mice.

As shown in Table 2 (Experiment 2), except for the value observed in the NCG group, the dpm/LN level was the lowest in the 70% EtOH group, followed by the PG, DMF, AOO, BN, and DMSO groups. The difference between the highest dpm/LN (DMSO) and the lowest dpm/LN (70% EtOH) was 550 dpm/LN. The data of the DMSO group was taken from Experiment 1.

As shown in Table 3 (Experiment 3), the dpm/LN level at 5% HCA was the highest in the DMSO group, followed by the BN and DMF groups. At 10% HCA, the highest dpm/LN level was observed in the DMF group, followed by the DMSO, PG, and BN groups. At 25% HCA, the dpm/LN level was the highest in the DMSO group, followed by the PG, DMF, AOO, and 70% EtOH group, and the difference between the highest dpm/LN (DMSO) and the lowest dpm/LN (BN) was 2,395. The incorporation of ^{3}HTdR (dpm/LN) was dependent on the concentration of HCA in each vehicle.

An indicator of the allergic potential, the stimulation index (SI), was calculated (Table 4). At 5% HCA, the SI value was 3 or higher only in the BN group. The SI values were 3 or higher in all vehicle groups except AOO and DMSO, at 10% HCA and in all vehicles used at a concentration of 25% HCA.

The EC3 value (the estimated concentration for the SI value of 3) was also calculated (Table 4). The EC3 value was the lowest in the BN group (EC3=1.76) and the highest in the DMSO group (EC3=16.21). The difference between the highest and lowest value was 14.45.

The LLNA has been commonly used since the LLNA was adopted as part of the OECD 429 test guideline in 2002. Afterwards, although many studies were conducted according to the OECD test guidelines (TG429), different mouse strains and vehicles were used at each study site. In particular, the animal strains used in Europe are often different from those used in Japan. LLNA data obtained from laboratories in Europe are frequently used for chemical safety assessments in Japan. It is often unavoidable to use LLNA data using animal strains that are not available in Japan, or for which information

Table 3. Vehicle effect on HCA responses (dpm/LN)

HCA concentration (%)	dpm/LN					
	AOO	EtOH 70%	DMF	BN	PG	DMSO
0	334	211	437	296	364	1055
5	504	512	1096	1229	578	1614
10	755	960	2627	1705	1736	1952
25	2804	2764	2869	2589	3649	4984

HCA in 6 different vehicles, AOO, EtOH 70%, DMF, BN, PG, and DMSO, was topically applied to CBA/CaOlaHsd mice at concentrations of 0, 5, 10, and 25% (v/v) (n=4 each), and the incorporation of ^{3}HTdR (dpm/LN) was determined. The dpm/LN level was determined in lymph nodes pooled on an experimental group basis.

Table 4. Vehicle effect on HCA responses (stimulation index)

HAC concentration (%)	Stimulation Index（SI）					
	AOO	EtOH 70%	DMF	BN	PG	DMSO
0	1	1	1	1	1	1
5	1.5	2.4	2.5	4.1	1.6	1.5
10	2.3	4.6	6.0	5.8	4.8	1.8
25	8.4	13.1	6.6	8.7	10.0	4.7
EC3	11.72	6.36	5.71	1.76	7.35	16.21

HCA in 6 different vehicles, AOO, EtOH 70%, DMF, BN, PG, and DMSO, was topically applied to CBA/CaOlaHsd mice at concentrations of 0, 5, 10, and 25% (v/v) (n=4 each). From the observed incorporation of ^{3}HTdR (dpm/LN), the stimulation index (SI) and EC3 value (estimated concentration for the SI value of 3) were calculated.

is quite limited in Japan. Thus, the animal strains and the vehicles used in Europe, that differ from those in Japan, may be the source of differences from LLNA data derived in Japan, and this is an issue of extreme importance. This paper demonstrates that strain differences and vehicles affect the results of the LLNA in the mouse strains commonly used in Europe and described in the OECD guidelines.

Experiment 1 was conducted in 2 strains of CBA mice, CBA/CaOlaHsd and CBA/CaHsdRcc mice, to investigate whether the local lymph node response is similar between genealogically closely related strains. The experiment demonstrated that an obvious strain difference in the response (measured as incorporation of 3HTdR [dpm/LN]) was observed between DMSO-treated CBA/CaOlaHsd and CBA/CaHsdRcc mice compared with that between non-treatment groups (NCGs) of CBA/CaOlaHsd and CBA/CaHsdRcc mice. This suggests that the local lymph node response to DMSO is more sensitive in CBA/CaHsdRcc mice than in CBA/CaOlaHsd mice; however, a further study using other strains including genealogically divergent strains, in particular CBA/JNCrj and NMRI mice which are available in Japan, will be required to assess the extent of the strain difference observed between CBA/CaOlaHsd and CBA/CaHsdRcc mice.

Experiment 1 showed a local lymph node response only to DMSO. The reason why DMSO was used as a vehicle in Experiment 1 is that most test substances for the LLNA are insoluble in water, and in such cases DMSO is often used as a vehicle. We will continually evaluate the strain differences in the response to other vehicles recommended in the OECD TG429.

A vehicle that can adequately deliver a variety of test substances should be selected as the vehicle for the LLNA. The objectives of Experiment 2 were not to identify an appropriate vehicle, but rather to demonstrate that the local lymph node response is different among vehicles and to provide important fundamental knowledge allowing researchers to more accurately compare dpm/LN levels obtained with different vehicles in different laboratories or dpm/LN levels of similar pharma-

ceuticals or chemical substances in different vehicles. The difference in the local lymph node response to the vehicles is considered to indicate a vehicle effect in CBA/CaHsdRcc mice. However, additional studies using other strains will be required to further investigate vehicle differences in the LLNA.

As shown in Experiment 3, the difference between the highest EC3 (BN) and lowest EC3 (DMSO) was 14.45. Based on the 4-category classification (extreme [<0.1], strong [0.1≤ to <1], moderate [1≤ to <10], and weak [10≤ to <100]) [9], HCA was classified as a moderate sensitizer (EC3 <10) when BN, DMF, 70% EtOH, or PG was used as a vehicle, but a weak sensitizer (10< EC3) when AOO or DMSO was used. This indicates that the sensitization potency of HCA, even though based on the same classification methods, may be classified in different categories when using different vehicles. Further investigation with other sensitizers is needed to clarify whether other sensitizers fall in different categories when using different vehicles.

At first, we considered that the difference in the response to the vehicles was due to a difference in the solubility of HCA in the vehicles, but we soon had to accept that the vehicle difference in the response was difficult to explain only in terms of the solubility of HCA, because HCA is not soluble in water, glycerin, or PG. Based on the results from this study alone, it is also difficult to explain why the dpm/LN level at an HCA concentration of 10% was higher in the DMF group than in the DMSO group; whereas the dpm/LN levels at HCA concentrations of 5 and 25% were highest in the DMSO group (Table 3). In a forthcoming experiment, attention will also be paid to the appearance of the test solution. This is because when HCA is suspended in a test solution, undissolved HCA on the skin's surface may be gradually absorbed by the skin's sebum. For a more detailed discussion, further comprehensive studies will be required to investigate the possible relationship between the local lymph node response and the appearance of the test solution (suspension or solution) or between the response and the solubility of the test substance in a vehicle, as well as possible interactions between the vehicles and test substances.

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