

EVALUATION OF ALLERGENICITY OF CONSTITUENTS OF *MYOGA* USING THE MURINE LOCAL LYMPH NODE ASSAY

Q.J. WEI^{1,3}, C.N. WEI², K. HARADA³, K. MINAMOTO², Y. OKAMOTO⁴,
M. OTSUKA⁴ and A. UEDA²

¹The First Affiliated Hospital of Guangxi Medical University, Nanning, China;

²Department of Preventive and Environmental Medicine, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan; ³Department of Biomedical Laboratory Sciences, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan; ⁴Department of Bioorganic Medicinal Chemistry, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan

Received October 28, 2009 – Accepted April 26, 2010

Myoga (*Zingiber Myoga Roscoe*) is a perennial plant with a pungent smell from its flower buds. It is native to East Asia and has been reported to cause allergic contact dermatitis. The purpose of this study is to assess the allergenicity of *myoga* related to its major chemical components, α -pinene, β -pinene, limonene, limonene oxide and β -phellandrene, which are supposed to be the causative agents of contact dermatitis among *myoga* cultivators. We performed a toxicity study of the volatile constituents of *myoga* using the local lymph node assay (LLNA), in which limonene, limonene oxide and β -phellandrene had positive responses and the EC3 was 35.8%, 8.22%, and 0.54%, respectively. EC3 for both α -pinene and β -pinene was over 100%. Both chemicals failed to induce positive responses in the LLNA. While the maximization rating of limonene, limonene oxide and phellandrene were evaluated as moderate, extreme, and extreme respectively, α -pinene and β -pinene were evaluated as weak in the previously reported GPMT. The usage of LLNA was also confirmed by comparing with previously reported GPMT results to detect the allergenicity of *myoga* constituents. The actual risk of humans developing an allergy to *myoga* constituents depends on many factors. The concentration of the compounds, the frequency and duration of exposure and the condition of the skin are supposed to be important factors.

Myoga (*Zingiber Myoga Roscoe*) is a perennial plant with a pungent smell of its flower buds and is of East Asian origin (1), belonging to the ginger family (*Zingiberaceae*). The Japanese have long eaten the flower buds as a spice or pickle. The technology for cultivating *myoga* in greenhouses was established in the 1990s, enabling the flower buds to be supplied to markets all year round. *Myoga* greenhouses have increased in some areas in Japan,

while hand dermatitis suspected to be due to the allergenicity to *myoga* has been recognized among *myoga* greenhouse cultivators.

Minamoto et al. first reported a case study of contact dermatitis from *myoga* (2-3), before which contact dermatitis from *myoga* was unknown (4). According to our earlier investigation of *myoga* cultivators in Japan, 8 of 35 cultivators experienced contact dermatitis during the harvest season (2-3).

Key words: *Myoga* (*Zingiber myoga Roscoe*), local lymph node assay (LLNA), thymidine, allergic contact dermatitis, guinea-pig maximization test (GPMT), limonene, limonene oxide, β -phellandrene

Mailing address: Prof. Koichi Harada,
Kuhonji 4-24-1, Kumamoto-shi,
Kumamoto-prefecture,
862-0976 Japan
Tel/ Fax: ++81 96 373 5462
e-mail: haradako@kumamoto-u.ac.jp

0394-6320 (2010)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder. Unauthorized reproduction may result in financial and other penalties

Among the 8 workers who had experienced skin problems, the mean time from starting cultivation to the development of dermatitis was 5 (range 0-7) years (3). The affected region were hands, forearms, fingers, upper extremities and neck exposed to flower buds, leaves, and stems of *myoga*. Volatile constituents of fresh *myoga* flowers are supposed to be the causative materials of contact dermatitis. Volatile constituents, such as α -pinene, β -pinene, and limonene, were detected in *myoga* juice by gas chromatography (GC) in our previous study (5). We failed to identify limonene oxide by GC, but limonene oxide, an auto-oxidized constituent form of limonene, is also recognized as a constituent of *myoga*. β -phellandrene was reported as one of the major volatile compounds of *myoga* using GC and gas chromatography-mass spectrometry (GC-MS) (6).

We detected allergenicity using the guinea pig maximization test (GPMT) (7), and found that *myoga* juice, limonene, and limonene oxide were positive (5). We also detected the allergenicity of α -methylene- γ -butyrolactone (α -MBL), a constituent of *Alstroemeria* commonly known as Peruvian Lily or Lily of the Incas, which was shown to be the causative agent provoking delayed-type contact dermatitis using GPMT (8-9). The guinea pigs were initially exposed to the test substance by inter-dermal injection and/or epidermal application (induction exposure) in the GPMT. Following a rest period of 14 days (induction period), during which an immune response may develop, the animals were exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure in the test animals was compared with that demonstrated by control animals which underwent sham treatment during induction and received challenges exposure (OECD 406) (10).

The murine local lymph node assay (LLNA) (11-16) is a novel predictive method for identifying skin-sensitizing chemicals, in contrast to the guinea pig test such as the GPMT (7-9) and the occlusive patch test of Buehler (17). Activity is judged as a function of the induction, rather than the elicitation phase of sensitization. Specifically, LLNA measures the ability of topically applied chemicals to induce proliferative responses by draining proliferated lymph node cells in mice (15). The hypersensitivity reaction can be

recognized as T lymphocyte-dependent cellular immune responses. The defining event during the induction phase of skin sensitization is stimulation within draining lymph nodes of proliferative responses by allergen-reactive T lymphocytes (15).

The purpose of this study is to evaluate the allergenicity of α -pinene, β -pinene, limonene, limonene oxide, β -phellandrene and *myoga* juice as principal ingredients of *myoga* (6) using the LLNA. We tried to evaluate the effectiveness of the LLNA method using the previously reported GPMT results (5) adding the GPMT result of β -phellandrene in the present study.

MATERIALS AND METHODS

Chemicals

α -Pinene, β -pinene, limonene (as R-(+)-limonene), limonene oxide and hexylcinnamaldehyde (HCA) were obtained from Kanto Chemical Co. Inc. (Japan). Dimethylsulfoxide (DMSO) and acetone : olive oil (AOO v/v 4 : 1) was used as the vehicle for all test compounds. All reagents used were of the highest grade available.

β -phellandrene was synthesized as follows (18-19), because it was not available commercially (Fig. 1). β -Pinene (19.0 ml), water (25 ml), methanol (25 ml), sodium bisulfite (13.2 g), potassium nitrate catalyst (1.31 g) and $\alpha\alpha'$ -azobisisobutyronitrile (0.1 g) were added to a vessel and refluxed for 4 hours. After cooling, 30 g white crystalline solid was recovered from the reaction mixture. This solid was re-crystallized from 90% ethanol to yield 12.0 g re-crystallized solid. This solid was analyzed as sodium para-menth-1-ene-7-sulfonate with relatively high solubility in water. Ten grams (+)para-menth-1-ene-7-sulfonic acid sodium salt was added to a one liter distillation vessel with 20 g NaOH. The sodium sulfonate salt was heated to 260°C under reduced pressure (10 mmHg) for about 15 minutes. After the initial removal of water from the vessel by distillation, a yellow liquid was distilled from the vessel and collected in the distillation flask. The yellow oil (1.3 g), analyzed by GC, was mostly the desired (-)- β -phellandrene. [3H] methyl thymidine (3HTdR; specific activity 2 Ci mmol⁻¹) was purchased from Amersham International (UK).

Myoga juice(5)

Fresh flower buds of *myoga* were purchased at a local market. They were cut into suitable segments and were stirred for five minutes using a homogenizer (Polytron, Kinematica GmbH, Switzerland) on ice. The homogenized solution was filtered through a double layer

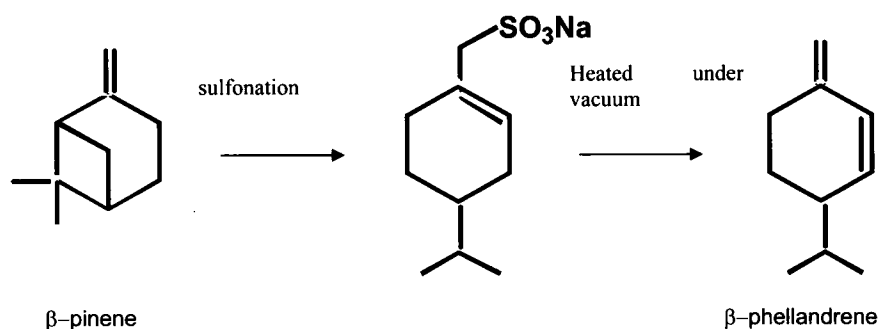


Fig. 1. Synthesis of β -phellandrene from β -pinene.

of gauze. The filtered solution was used as *myoga* juice(5) in the present study .

Animals

Young adult (8-12 weeks old) CBA/J strain female mice for LLNA were obtained from Japan SLC, Inc. (Shizuoka Japan). Since susceptibility was not influenced by gender (11, 14) and females were easier to treat, only females were used in the present study. Guinea pigs, young adult 8-week-old Hartley strain female for GPMT were purchased from Kyudo Kumamoto Inc. (Kumamoto, Japan). The animals were kept at a constant temperature ($25 \pm 5^\circ\text{C}$), relative humidity (50–70%), and a 12/12-hour dark/light schedule. The animals received care in compliance with the Guide for the Care and Use of Animals, Laboratory Animal Research Center, Kumamoto University School of Medicine. All animals were fed a certified rodent diet (GB-1; Funabashi Co. Ltd, Japan) and water was available *ad libitum*.

LLNA

The LLNA was conducted as described previously (20-21). Groups of mice from Japan SLC Inc. (Shizuoka, Japan) ($n=4$ for each group) received topical administration of 25 μl of various concentrations of the test chemicals on the dorsum of both ears, or the same volume of the relevant vehicle alone, daily for 3 consecutive days. Five days after initiation of exposure, all mice were injected via the tail vein with 250 μl phosphate-buffered saline (PBS) containing 20 μCi [^3H] methyl thymidine. Five hours later, the mice were sacrificed and the draining auricular lymph nodes excised and pooled for each experimental group. Single cell suspensions of lymph node cell (LNC) were prepared by mechanical disaggregating through a cell strainer (BD Falcon, USA). Pooled LNCs were washed twice with PBS and precipitated in 5% trichloroacetic acid

(TCA) at 4°C overnight. Pellets were then re-suspended in 1 ml TCA and transferred to 10 ml scintillation fluid (ULTIMA GOLD XR; PerkinElmer Japan Co., Ltd). The incorporation of ^3H -TdR was measured as disintegrations per minute (dpm) per node possessing the potential to cause contact sensitization by β -scintillation counting using a liquid scintillation counter (Liquid Scintillation system LSC-3050; Aloka, Japan).

The effective concentration of a stimulation index of 3 (EC3) for each experimental group was calculated by linear interpolation (15, 20-24). In each case, a stimulation index (SI) relative to the concurrent vehicle-treated control was derived. An SI of 3 or greater indicated chemical sensitization. The estimated concentration of chemicals derived by linear interpolation as EC3 was required to induce a three-fold increase in proliferation compared with concurrent vehicle-treated controls.

GPMT for β -phellandrene

The threshold of primary irritation was evaluated female guinea-pigs by the patch test using FINN CHAMBERS (EPITEST Ltd. Oy; Tuusula, Finland). Aliquots of 20 μl of β -phellandrene-DMSO solution were applied to the abdominal skin of the female guinea-pigs for 24 hours. The erythema on the skin was observed to find the minimum criterion of the primary irritation (5).

According to the methods of Magnusson and Kligman (7), nine guinea-pigs were used for each sensitization group. The lowest concentration producing a mild irritation were used for induction, and the maximal non-irritating concentration was used for challenge. For induction, 0.1 ml of β -phellandrene-DMSO solution and Freud's adjuvant were hypodermically injected in the shaved 6 scapular regions. After 7 days, an occluded patch of 0.15 ml of the compound was placed on the injection site for 48 hours. After 14 days, all the guinea pigs were

exposed to a challenge dose on the shaved flank for 24 hours. Skin reactions were observed and scored according to a grading scale for the evaluation of challenge patch test reaction (5, 7).

RESULTS

Table I summarized the result of acute dermal irritation assay in the guinea-pig. β -phellandrene at concentration of 0.8% did not provoke any erythema on the abdominal skin of the guinea pigs. However β -phellandrene at the concentration of 4%, 12%, 20% and 100% caused erythema on the abdominal skin. Table II summarizes the onset of allergenicity observed at the challenge treatment sites on the guinea pigs' abdomens sensitized by 0.8% on their dorsal skin in the GPMT.

The results obtained are shown as the incorporation of $^3\text{HTdR}$ into the lymph nodes (dpm/node) in Table III. They are also displayed in the form of stimulation indices (SI). Where possible, the estimated concentration required to induce a three-fold increase in proliferation compared with concurrent vehicle-treated controls (EC3 value) was derived by linear interpolation for each compound, shown in Table III. Little variation was observed

in the levels of $^3\text{HTdR}$ incorporation in LNC derived from AOO vehicle-treated animals with levels extending over a relatively narrow range from 37dpm/node to 146dpm/node in the case of α -pinene to limonene, respectively. These data demonstrated a high degree of inter-experimental consistency between the LLNA conducted during these investigations and those reported previously (24). For limonene at a test concentration of 100%, the level of $^3\text{HTdR}$ incorporation was 1035dpm/node and the corresponding SI was 7.09. For limonene oxide at a test concentration of 25%, the level of $^3\text{HTdR}$ incorporation was 950dpm/node and the corresponding SI was 7.85. For β -phellandrene at a test concentration of 1%, the level of $^3\text{HTdR}$ incorporation was 353.5dpm/node and the corresponding SI was 4.71. α -Pinene, β -pinene and *myoga* juice failed at all test concentrations to induce a positive response in the LLNA with relatively low levels of $^3\text{HTdR}$ incorporation. The maximal SI for α -pinene, β -pinene and *myoga* juice were 2.59, 2.55 and 1.78 at the highest concentration in each case.

Table IV summarized the allergenicity rating of the test chemicals by the GPMT reported previously adding the result of β -phellandrene. Limonene, limonene oxide and β -phellandrene were evaluated

Table I. *The autodermal irritation assay by β -phellandrene in guinea pig.*

Material	Concentration	Guinea pig: 24 h
β -phellandrene	0.8%	0/9
	4%	6/9
	12%	9/9
	20%	9/9
	100%	9/9
Dimethyl sulfoxide	100%	0/9

Table II. *Allergenicity of β -phellandrene by guinea pig maximization test (GPMT).*

Induction material	Challenge material	Challenge result
4% β -phellandrene	0.8% β -phellandrene	7/9
4% β -phellandrene	Dimethyl sulfoxide	0/9
Dimethyl sulfoxide	0.8% β -phellandrene	0/9
Dimethyl sulfoxide	Dimethyl sulfoxide	0/9

Table III. Local lymph node assay responses to test chemicals.

Chemical	Vehicle	Exposure concentration (%)	dpm/node	SI**	EC3(%)
α -pinene	AOO*	0	37	1	
		1	38	1.03	
		25	61	1.65	
		100	96	2.59	
β -pinene	AOO	0	89	1	
		1	168	1.89	
		25	162	1.82	
		100	227	2.55	
R-(+)-limonene	AOO	0	146	1	
		1	234	1.60	
		25	337	2.31	
		100	1035	7.09	35.80
limonene oxide	AOO	0	121	1	
		5	251	2.07	
		25	950	7.85	
		50	2787	23.04	8.22
β -phellandrene	AOO	0	75	1	
		0.1	101	1.35	
		1	353.5	4.71	
		5	655	8.73	0.54
Myoga juice	AOO	0	45.7	1	
		5	47.7	1.04	
		25	56.3	1.23	
		50	81.7	1.78	
HCA	AOO	0	124	1	
		5	196	1.58	
		10	427.8	2.45	
		25	1373.9	11.08	10.86

*AOO: acetone : olive oil, 4:1

**SI: mean dpm/mouse (treatment group)/the mean dpm/mouse (vehicle-treated group)

SI>=3: test substance is regarded as a skin sensitizer

Table IV. Allergenicity rating by guinea pig maximization test (GPMT).

Antigens	GPMT ⁵⁾		
	% positive	Grade	Classification
α -pinene	0	I	Weak
β -pinene	0	I	Weak
limonene	33	III	Moderate
limonene oxide	100	V	Extreme
Myoga duice	17	II	Mild
β -phellandrene	78*	V*	Extreme*

*GPMT data for β -phellandrene was obtained by the additional experiment performed in the present study.

as moderate, extreme, and extreme respectively, in the GPMT in the maximization ratings. Both α -pinene and β -pinene were evaluated as weak in the GPMT. While *myoga* juice at 100% concentration had revealed mild rating in the previous study.

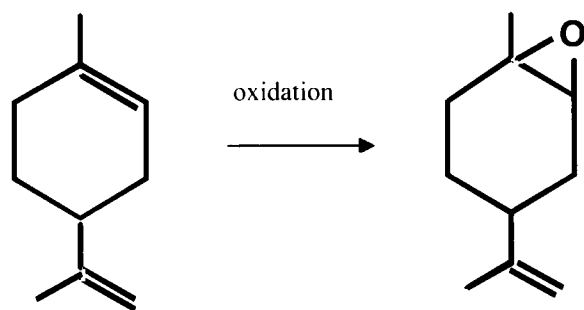


Fig. 2. Oxidation of limonene to limonene oxide.

DISCUSSION

The LLNA has the advantages of requiring a short test period and a low operation cost, and providing a quantitative value to evaluate the hallmarks of a skin sensitization response (25). Recently, the standard LLNA using radioisotopes has been recognized as a stand-alone sensitization test and has been incorporated into the official test guidelines published by the United States Environmental Protection Agency (US-EPA, 2003) (26) and the Organization for Economic Cooperation and Development (OECD, 2002) (21). Furthermore, OECD test guideline No. 429 (21) mentions that assessing the proliferation of lymph node cells must be performed with justification and appropriate scientific support, including full citations and description of the methodology. The standard LLNA uses the EC3 value as a parameter for evaluating the sensitization potency of chemicals. Regarding the use of radioisotopes, instead of H-thymidine, 5-bromo-2'-deoxyuridine incorporation into lymph node cells was measured immunohistochemically (27-29). Another sophisticated method was also proposed as non-radio isotopic modified LLNA in which adenosine triphosphate (ATP) content in the draining auricular lymphnode was measured

to evaluate the endpoint (30-31). We could also confirm that LLNA was a useful method to detect the allergenicity of *myoga* ingredients comparing it to the result from GPMT in the present study.

α -Pinene, β -pinene and 50% of *myoga* juice were negative in the LLNA. Both α -pinene and β -pinene failed to induce positive responses in the GPMT, but 100% of *myoga* juice had shown the mild reaction in the GPMT performed previously (5). As the concentration of *myoga* juice in the LLNA, 50% was thought to be sufficient to provoke allergenicity from the view point of the reaction observed in the previous study (5). The allergenicity on *myoga* juice at concentrations of 50%, however, increased gradually according to its concentration in the LLNA (Table III). It is concluded that *myoga* must have allergenicity, and some ingredient of *myoga* takes part in the reaction.

Although limonene showed a moderate reaction in the GPMT (5), limonene oxide, a secondary allergenic product, due to auto-oxidation (32) (Fig. 2), showed an extreme reaction in the GPMT. The EC3 values of limonene and limonene oxide changed similarly by 35.8% and 8.22%. Usage of the LLNA was recommended to detect the allergenicity of *myoga* constituents by the present results, comparing and evaluating the results obtained in the two tests.

Chemically synthesized β -phellandrene also induced a strong response with the EC3 value of 0.54% in the LLNA, and with the extreme in the GPMT in the present study. β -Phellandrene is a prominent volatile flavor component of *myoga* (6). The present study suggests that β -phellandrene is another allergen amongst the fragrant compounds of *myoga*, and this is the first paper describing its allergenicity. Since the LLNA, as is the case with all other toxicology tests, potentially gives some false positive results (33-35), there is a hypothesis that chemicals with unsaturated carbon-carbon double bonds may result in a higher number of false positive in the LLNA (36). Although β -phellandrene contains two carbon-carbon double bonds in its chemical formula (Fig. 1), the allergenicity of β -phellandrene is confirmed in the GPMT in the present study. It is thought that the positive reaction of β -phellandrene in the LLNA is a right reaction by observing the lesion in the guinea pigs in the GPMT, and not considered to be a false positive.

In conclusion, limonene, limonene oxide and β -phellandrene are constituents of *myoga* which revealed their allergenicity by the LLNA. The usefulness of the LLNA to detect the allergenicity of *myoga* constituents was also confirmed when considering the differences between the LLNA in the present study and the previously reported GPMT. The actual risk of humans developing an allergy to *myoga* constituents depends on many factors. The concentration of the compounds, the frequency and duration of exposure and condition of the skin are all important factors.

REFERENCES

- Makino T. *Myoga*. In Makino's illustrated flora in colour. Tokyo, Hokuryukan, Tokyo, 1982, p.768. (in Japanese)
- Minamoto K. A case report of contact dermatitis from *myoga*. The 30th Annual Meeting of Japanese Society for Contact Dermatitis. Yokohama. 2005.
- Minamoto K, Harada K, Wei QJ, Wei CN, Ohmori S, Ueda A. Occupational allergic contact dermatitis from *myoga* (*Zingiber Mioga rosc.*) in greenhouse cultivators. *Int J Immunopathol* 2007; 20(S):31-34.
- Lovell CR. *Plants and the Skin*. Black Scientific Publications. Oxford. 1993.
- Wei QJ, Harada K, Ohmori S, Minamoto K, Wei CN, Ueda A. Toxicity study of the volatile constituents of *myoga* utilizing acute dermal irritation assay and the guinea-pig maximization test. *J Occup Health* 2006; 48:480-6.
- Kurobayashi Y, Sakakibara H, Yanai T, Yajima I, Hayashi K. Volatile flavor components of *myoga* (*Zingiber myoga*). *Agr Biol Chem* 1991; 55:1655-7.
- Mugnusson B, Kligman AM. The identification of contact allergens by animal assay. The guinea pig maximization test. *J Invest Dermatol* 1969; 52:268-76.
- Harada K, Ohmori S, Wei CN, Arimatsu Y, Ueda A. Experimental study of contact dermatitis due to *alstroemeria* in guinea pigs. *Contact Dermatitis* 1998; 39:82.
- Harada K, Ohmori S, Wei CN, Y. Arimatsu, Ueda A. Quantification of α -Methylene- γ -butyrolactone extracted from different parts of *Alstroemeria Wilhelmina* and evaluation of its antigenicity using the guinea-pig maximization test. *Environ Health Prev Med* 2002; 6:229-34.
- OECD guideline for testing of chemicals 406. *Skin Sensitisation* 1992.
- Basketter DA, Scholes W. Comparison of the local lymph node assay with the guinea-pig maximization test for the detection of a range of contact allergens. *Food Chem Toxicol* 1992; 30:65-9.
- Basketter DA, Gerberrick GF, Kimber I, Loveless SE. The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. *Food Chem Toxicol* 1996; 34:985-97.
- Basketter DA, Blaikie L, Dearman RJ, et al. Use on the local lymph node assay for the estimation of relative contact allergenic potency. *Contact Dermatitis* 2000; 42:344-48.
- Kimber I, Basketter DA. The murine local lymph node assay: A commentary on collaborative studies and new directions. *Food Chem Toxicol* 1992; 30: 165-69.
- Kimber I, Basketter DA, Berthold K, et al. Skin Sensitization Testing in Potency and Risk Assessment. *Toxicol Sci* 2001; 59:198-208.
- Betts CJ, Dearman RJ, Heyling JR, Kimber I, Basketter DA. Skin sensitization potency of methyl methacrylate in the local lymph node assay: comparisons with guinea-pig data and human experience. *Contact Dermatitis* 2006; 55:140-47.
- Buehler EV. Occlusive patch method for skin sensitization in guinea pigs: The Buehler method. *Food Chem Toxicol* 1994; 32:97-101.
- Traynor SG, Kane BJ, Betkouski MF, Hirschy LM. Facile Syntheses of optically active terpene sulfonic acids. Application to the resolution of ($\pm$)-phenylglycine. *J Org Chem* 1979; 44:1557-9.
- Hirschy LM, Kane BJ, Traynor SG. Preparation of β -phellandrene. United States Patent 4,136,126. 1979.
- Recommended Performance Standards. Murine Local Lymph Node Assay, in the URL of "http://iccvam.niehs.gov/docs/immunotox_docs/llna-ps/LLNAPerfStd.pdf"
- OECD Test guideline 429, 2002; OECD guideline for the testing of chemicals, Skin Sensitization: Local Lymph Node Assay. "<http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/OECD429.pdf>"
- Kimber I, Dearman RJ, Basketter DA, Ryan CA,

- Gerbrick GF. The local lymph node assay: past, present and future. *Contact Dermatitis* 2002; 47:315-28.
23. Basketter DA, Lea LJ, Dickens A, Briggs D, Pate L, Dearman RJ, Kimber I. A comparison of statistical approaches to the derivation of EC3 value from local lymph node assay dose responses. *J Appl Toxicol* 1999; 19:261-6.
24. Warbrick EV, Dearman RJ, Lea LJ, Basketter DA, Kimber I. Local lymph node assay responses to paraphenylenediamine: intra- and inter-laboratory evaluations. *J Appl Toxicol* 1999; 19:255-60.
25. Gerberick GF, Ryan CA, Derman RJ, Kimber I. Local lymph node assay (LLNA) for detection of sensitization capacity of chemicals. *Methods* 2007; 41:54-60.
26. Lewis P, Matsumura F. Application of the local lymph node assay in dermal sensitization testing. SAP Report No. 2001 – 13A: 2001; 7-14.
27. Yamano T, Shimidzu M, Noda T. Allergenicity evaluation of p-chloro-m-cresol and p-chloro-m-xylol by non-radioactive murine local lymph node assay and multiple-dose guinea pig maximization test. *Toxicology* 2003; 190:259-66.
28. Yamano T, Shimizu M, Noda T. Quantitative comparison of the results obtained by the multiple-dose guinea pig maximization test and the non-radioactive murine local lymph-node assay for various biocides. *Toxicology* 2005; 211:165-75.
29. Yamano T, Shimizu M. Skin sensitization potency and cross-reactivity of p-phenylenediamine and its derivatives evaluated by non-radioactive murine local lymph node assay and guinea-pig maximization test. *Contact Dermatitis* 2009; 60: 193-8.
30. Yamashita K, Idehara K, Fukuda N, Yamaguchi G, Kawada N. Development of a modified local lymph node assay using ATP measurement as an endpoint, as an endpoint. *AATEX* 2005; 11:136-44.
31. Idehara K, Yamagishi G, Yamashita K, Ito M. Characterization and evaluation of a modified local lymph node assay using ATP content as a non-radio isotopic endpoint. *J Pharmacolog Toxicol Meth* 2008; 58:1-10.
32. Matura M, Goossens A, Bordalo O, Garcia-Bravo B, Magnusson K, Wrangsjö K, Karlberg AT. Patch testing with oxidized R-(+)-limonene and its hydroperoxide fraction. *Contact Dermatitis* 2003; 49:15-21.
33. Basketter DA, McFadden JF, Gerberick F, Cockshott A, Kimber I. Nothing is perfect, not even the local lymph node assay: a commentary and the implications for REACH. *Contact Dermatitis* 2009; 60:65-9.
34. Basketter DA, Gerberick GF, Kimber I. The local lymph node assay: current position in the regulatory classification of skin sensitizing chemicals. *Cutan Ocul Toxicol* 2007; 26:293-301.
35. Hans-Werner V, Jürgen AH. The local lymph node assay being too sensitive? *Arch Toxicol* 2005; 79: 721-8.
36. Kreiling R, Hollnagel HM, Hareng L, et al. Comparison of the skin sensitizing potential of unsaturated compounds as assessed by the murine local lymph node assay (LLNA) and the guinea pig maximization test (GPMT). *Food Chem Toxicol* 2008; 46:1896-904.